THE EFFECT OF SURFACE MODIFICATION
ON LYMPHOCYTE FUNCTION

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DOCTOR OF PHILOSOPHY
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by
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Statement

The experiments recorded in this thesis were done by myself. Mr. R. Hill prepared and cut the histological sections.

A. Kemp
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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER 1</th>
<th>INTRODUCTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbohydrate at the Cell Surface</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Methods for Detection of Sialic Acid at the Cell Surface</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sialic Acid in the Glycocalyx of Mammalian Cells</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Possible Biological Roles of Cell Surface Sialic Acid</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Sialic Acid and Cell Surface Antigens</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Recovery of Surface Sialic Acid by Neuraminidase Treated Cells</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Effect of Modification of the Cell Surface on Lymphocyte Migration</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>The Effect of Lymphocyte Surface Modification on Allogeneic Interactions</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte Circulation and the Immune Response to Sheep Erythrocytes</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Aims of the Present Investigation</td>
<td>27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 2</th>
<th>METHODS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rats</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Operative Procedures</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Preparation of Cell Suspensions</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Antigen and Immunization</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Assays for Haemolytic Plaque-Forming Cells</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Haemolysin Titration</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Gamma Irradiation</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Induction and Maintenance of Immunological Tolerance</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Incubation of Lymphocytes with Neuraminidase</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Assay of Sialic Acid Release</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Labelling of Lymphocytes with $^{51}$Cr and Injection of the Cells</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Assay for Radioactivity</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Incubation of $^{51}$Cr Labelled Cells with Isoantisera</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Preparation and Maintenance of Lymphocytes on Rat Embryo Monolayers</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Cell Counts and Haemoglobin Estimations</td>
<td>40</td>
</tr>
</tbody>
</table>
CHAPTER 2  METHODS (cont.)

Lymphocyte Cytotoxicity and Agglutination Testing 40
Viability Tests 42
Histology and Cytology 42
Incubation of Tissue Sections with Neuraminidase 42

CHAPTER 3  SIALIC ACID RELEASE FROM THE SURFACE OF RAT LYMPHOCYTES 44

Variability of assay 45
Optimum Conditions for Incubation 45
The Rate of Release of Sialic Acid 46
Release of Sialic Acid from Lymphoid Cells 46
Repeat Incubation of Cells with Neuraminidase 47
Effect of Guinea Pig Serum on Neuraminidase Treated Lymphocytes 49
The Effect of Strain Specific Isoantisera on Neuraminidase Treated Lymphocytes 50
Discussion 51

CHAPTER 4  THE OCCURRENCE OF SIALYL-CONTAINING MUCOSUBSTANCES IN LYMPHOID TISSUE 54

Demonstration of Sialic Acid 55
Acid Mucosubstance Around the Postcapillary Venule 56
Acid Mucosubstance Associated with other Small Vessels 57
Acid Mucosubstance in the Lymph Node Medulla 58
Discussion 59

CHAPTER 5  THE MIGRATORY PROPERTIES OF THORACIC DUCT LYMPHOCYTES 63

Labelling of Lymphocytes with \(^{51}\)Cr 63
Distribution of Lymphocytes in Syngeneic Recipients 66
Recirculation of Lymphocytes in the Thoracic Duct Lymph 67
Migratory Properties of Transfused Lymphocytes that have Reappeared in the Thoracic Duct after Transfusion 68
CHAPTER 5  THE MIGRATORY PROPERTIES OF  
THORACIC DUCT LYMPHOCYTES (cont.)

Migratory Properties of Lymphocytes  
Localizing in the Spleen  
70

The Effect of the Labelling Procedure  
on Lymphocyte Migration  
73

Distribution of Lymphocytes in Lethally  
Irradiated Recipients  
75

Distribution of Parental Cells in F1  
Hybrid Recipients  
76

Discussion  
78

CHAPTER 6  THE MIGRATORY PROPERTIES OF  
THYMOCYTES  
90

The Distribution of Normal Thymocytes  
in Syngeneic Recipients  
90

The Migration of Thymocytes into the  
Thoracic Duct  
91

Retransfer of Labelled Thymus Cells  
91

Discussion  
92

CHAPTER 7  THE MAINTENANCE OF  
NEURAMINIDASE TREATED  
LYMPHOCYTES ON RAT EMBRYO  
MONOLAYERS  
95

Preliminary Experiments to Establish  
Optimal Monolayers  
95

Behaviour of (Lewis x DA)F1 Thoracic  
Duct Lymphocytes on Outbred Monolayers  
96

Behaviour of (Lewis x DA)F1 Thoracic  
Duct Lymphocytes on Syngeneic  
Monolayers  
97

Migration of Lymphocytes after  
Maintenance on Monolayers  
98

Discussion  
103

CHAPTER 8  THE IMMUNE RESPONSE TO SHEEP  
ERYTHROCYTES OF IRRADIATED  
RATS RESTORED WITH NORMAL OR  
NEURAMINIDASE TREATED  
LYMPHOCYTES  
107

Colonization of the Irradiated Host's  
Spleen by Injected Lymphocytes  
108

Immune Response of Irradiated Rats  
Injected with Lymphocytes Before  
Antigen  
110
CHAPTER 8 THE IMMUNE RESPONSE TO SHEEP ERYTHROCYTES OF IRRADIATED RATS RESTORED WITH NORMAL OR NEURAMINIDASE TREATED LYMPHOCYTES (cont.)

Immune Response of Irradiated Rats Injected with Lymphocytes at the Same Time as Antigen

Immune Response of Irradiated Rats Injected with Lymphocytes 24 and Antigen 48 Hours after Irradiation

Immune Response of Irradiated Rats Injected with Antigen 6 Hours and Lymphocytes 24 or 48 Hours after Irradiation

The Inhibition of the Effect of Neuraminidase on the Reduction of the PFC Response by an Excess of Free Sialic Acid in the Incubation Medium

The Ability of Neuraminidase Treated Lymphocytes to Abrogate Tolerance to Sheep Erythrocytes

Discussion

CHAPTER 9 THE CAPACITY OF THORACIC DUCT LYMPHOCYTES INCUBATED WITH NEURAMINIDASE TO INITIATE AND SUSTAIN GvH REACTIONS

The Acute, Systemic Graft-Versus-Host Reaction

The Chronic, Systemic Graft-Versus-Host Reaction

The Local Graft-Versus-Host Reaction

The Fate of Neuraminidase Treated Parental Cells After Transfer to F1 Hybrid

The Response of Rats with Chronic GvH Disease to Sheep Erythrocytes

Discussion

CHAPTER 10 GENERAL SUMMARY AND CONCLUSIONS

REFERENCES
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr</td>
<td>Chromium</td>
</tr>
<tr>
<td>GvH</td>
<td>Graft-versus-host</td>
</tr>
<tr>
<td>Ndase</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>PFC</td>
<td>Plaque-forming cells</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>TDL</td>
<td>Thoracic duct lymphocytes</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

This review is concerned with the function of carbohydrates containing materials situated on the cell surface, in particular with the amino sugar N-acetylneuraminic acid which is one of the group of sialic acids. The occurrence and possible role of sialic acid at the cell surface is reviewed with special reference to the lymphocyte. This is followed by a consideration of the effects of surface modification on the migratory and immunological capabilities of lymphocytes.

1. CARBOHYDRATE AT THE CELL SURFACE

There has been a gradual accumulation of evidence of an extracellular material associated with the lipoprotein layer of the plasma membrane. In a study on Schistosoma eggs, Chambers (1940) described a proteolipid surface, the integrity of which was essential to life, and outside this an "extramembranous coat". This "extramembranous coat" was subsequently shown to be stainable by the periodic acid-Schiff (PAS) histochemical method for the detection of carbohydrate (Ward and Schauta 1950). In 1939, Lendahl described a PAS positive coat in the zona pellucida surrounding mammalian eggs. Further support for the presence of carbohydrate containing material at the cell surface was obtained by Laws and Sturgis (1961) with the demonstration of an extracellular material, which could be stained by aniline blue, at the surface of hepatic cells. In 1966, Hameury, Neutra and Lendahl, in an extensive histochemical study of over 50 different cell types in the rat, concluded that almost all cells possessed a surface coat of glycoprotein containing material anterior to the plasma membrane. Such carbohydrate containing material surrounding cells was subsequently termed the "glycocalyx" (Hameury 1963).

The identification of sialic acid as a component of the glycocalyx of cells is largely a result of studies on erythrocytes. Similar methods were subsequently utilized to demonstrate sialic acid on the surface of a variety of cells. Mirls (1942) found that chicken erythrocytes, which
INTRODUCTION

This review is concerned with the function of carbohydrate containing materials situated at the cell surface, in particular with the amino sugar N-acetyl neuraminic acid which is one of the group of sialic acids. The occurrence and possible roles of sialic acid at the cell surface is reviewed with special reference to the lymphocyte. This is followed by a consideration of the effects of surface modification on the migratory and immunological capabilities of lymphocytes.

1. CARBOHYDRATE AT THE CELL SURFACE

There has been a gradual accumulation of evidence of an extracellular material associated with the lipoprotein layer of the plasma membrane. In a study on Echinoderm eggs, Chambers (1940) described a protoplasmic surface, the integrity of which was essential to life, and outside this an "extraneous coat". This "extraneous coat" was subsequently shown to be stainable by the periodic acid-Shiff (PAS) reaction, a histochemical method for the detection of carbohydrate (Manne and Slatterback 1950). In 1950, Leblond described a PAS positive coat in the zona pellucida surrounding mammalian ova. Further support for the presence of carbohydrate containing material at the cell periphery was obtained by Laws and Stickland (1961) with the demonstration of an extracellular material, which could be stained by aniline blue, at the surface of hepatic cells. In 1966, Rambourg, Neutra and Leblond, in an extensive histochemical study of over 50 different cell types in the rat, concluded that almost all cells possessed a surface coat of glycoprotein containing material exterior to the plasma membrane. Such carbohydrate containing material surrounding cells has been termed the "glycocalyx" (Bennett 1963).

The identification of sialic acid as a component of the glycocalyx of cells is largely a result of studies on erythrocytes. Similar methods were subsequently utilized to demonstrate sialic acid on the surface of a variety of cells. Hirst (1942) found that chicken erythrocytes, which
had been once agglutinated by influenza virus, subsequently separated and could not be agglutinated again by further fresh virus. On the basis of these findings he suggested the existence of a surface receptor on the erythrocytes which bound the influenza virus. In 1948 Burnet reported that the agglutination of erythrocytes by heat inactivated influenza virus could be inhibited by mucins of human and animal origin. This ability of the mucins to inhibit the agglutination of erythrocytes by influenza virus was destroyed by pretreatment of the mucins with living influenza virus or with an enzyme derived from vibrio cholera. Hirst (1948) suggested that the influenza virus receptors on chicken erythrocytes which could be destroyed by treatment with trypsin and periodate belonged to the mucoprotein class of compounds. Gottschalk and Lind (1949) examined the specificity of the receptor destroying enzyme derived from influenza virus or from vibrio cholera cultures, and later named this enzyme neuraminidase (Gottschalk 1957), because of its ability to liberate acylated neuraminic acids. The isolation and purification of this enzyme (Ada and French 1959, Ada et al. 1961) enabled further progress to be made.

The above series of observations was linked together when Klenk and Uhlenbruck (1958) found that receptor destroying enzyme could liberate acylated neuraminic acids from erythrocyte stromata of various mammalian species. Klenk (1958) suggested that the negative charge of the erythrocyte surface might be due to an acylated neuraminic acid. In 1961, Cook, Heard and Seaman, using purified neuraminidase preparations and the technique of cell electrophoresis showed that neuraminidase treatment of erythrocytes decreased their negative surface charge, and that this was due to removal of sialic acid with loss of its anionic carboxyl group.

Thus the early observations of Hirst and Burnet, combined with the development of means for releasing and measuring the influenza virus receptors led to the identification of sialic acid as being a component of
the red cell membrane. Similar methods were later used to demonstrate surface sialic acid on most mammalian cells.

2. METHODS FOR DETECTION OF SIALIC ACID AT THE CELL SURFACE

Cell Electrophoresis

The fact that cells will migrate when placed in an electric field indicates that the cell surface carries an electric charge. Such a migration is termed the electrophoretic mobility of the cell, and reflects the zeta potential of the slip plane. This zeta potential is a composite measurement of the true surface potential of the cell due to its charged groups and absorbed counter-ions, and the contribution from that part of the ionic double layer enclosed between the plane of the bound charges and the hydrodynamic slip plane. Sialic acid, which has a pKa value of 2.6, and is therefore negatively charged at physiological pH is a major contributor to the negative charge of the cell surface. Hence electrophoretic methods have been extensively used to study cell surface sialic acid. Other ionic groups which may contribute to the surface charge of some cells are the carboxyl groups of amino acids e.g. aspartic and glutamic acids (Cook and Eylar 1965), the phosphate groups of surface bound RNA (Weiss and Mayhew 1966) and the positively charged amino groups (Cook and Jacobsen 1968). The determination of cellular electrophoretic mobilities before and after treatment with neuraminidase, coupled with the demonstration of liberated free sialic acid in the suspending medium, is an unequivocal method of demonstrating sialic acid at the cell surface (Cook 1968).

Alterations in cell surface charge after neuraminidase treatment may be caused not only by removal of negatively charged sialyl residues, but also by revealing cationic groups (Cook and Jacobsen 1968). For example certain leukaemic cells of mice, which have more sialic acid on the cell surface than normal cells, have a lower anodic mobility (Cook and Jacobsen 1968) as a result of an increase in surface cationic groups. The electrophoretic
mobility of one line of ascites hepatoma cells did not alter with neuraminidase treatment despite the fact that release of sialic acid could be demonstrated (Kojima and Maekawa 1970). The contribution of a charged molecule in the membrane to the surface potential will be influenced by a number of unmeasured variables such as the effective radius of the charge bearing site, the presence of other charges, and the depth of charges within the membrane. (Wallach 1969). For this reason it is difficult to make a direct correlation between the amount of sialic acid shown to be released by neuraminidase treatment of cells and the subsequent alteration in their electrophoretic mobility.

Chemical Methods

The development of the sensitive thiobarbituric acid method for measuring free sialic acid (Warren 1959, Aminoff 1961), coupled with the availability of purified preparations of neuraminidase (Ada and French 1959, Ada et al. 1961) provided means for the release of sialic acid from cells and its quantitation. Treatment of cells with neuraminidase, followed by assay of the sialic acid released into the supernatant has been widely used to measure cell surface sialic acid.

The question arises as to whether the sialic acid that is released by treatment of whole cells with neuraminidase is derived exclusively from the cell surface. There is no doubt that some of it is released from the cell surface. As only those charged molecules at the electrokinetic shear plane, which has a depth of 10Å, influence the electrophoretic mobility, any changes in mobility observed after neuraminidase treatment are likely to be due to alterations at this level. Histochemical methods showing loss of surface staining for sialic acid after neuraminidase treatment (Gasic and Berwick 1963, Walborg et al. 1969), alterations in the attachment of viruses to surface sialic acid receptors (Marcus 1959, Marcus, Salb & Schwartz 1965, Burnet 1948), alterations in cell surface reactions such as complement fixation (Arquilla et al. 1964) and antigen-antibody reactions
(Grothaus et al. 1971) all attest to the fact that removal of sialic acid by neuraminidase has altered the cell surface.

It is also clear that further sialic acid can be liberated by acid hydrolysis of cells which have been treated with neuraminidase previously (Kraemer 1966a, Madoff et al. 1964, Woodruff and Gesner 1969). This suggests that the residual sialic acid is bound in such a way that the glycosidic linkage is not susceptible to hydrolysis by neuraminidase or, alternatively that the enzyme has not reached all of the bound sialic acid. More direct evidence that neuraminidase does not enter the cell and release sialic acid was obtained by Marcus et al. (1965). Utilizing the specific attachment of myxovirus to sialic acid on the nuclear membrane or the cell surface of HeLa cells, they showed that sialic acid receptors on the nuclear membrane were not removed by treating the whole cell with neuraminidase, but could be removed by so treating isolated nuclei.

Simon-Reuss et al. (1964), postulated that the neuraminidase could enter cells by pinocytosis and release sialic acid from internal membranes. Some experimental evidence for this was obtained by Nordling and Mayhew (1966). Nuclei prepared from chicken erythrocytes or Ehrlich ascites cells which had been treated with neuraminidase, were shown to possess a significantly lower electrophoretic mobility than nuclei isolated from untreated cells. Furthermore, when neuraminidase labelled with fluorescein iso-thiocyanate was used to treat cells, intracellular fluorescence of both chicken erythrocytes and tumor cells was observed. These authors concluded that neuraminidase was able to enter cells and release sialic acid from within the cell. Fluorescein labelled neuraminidase did not enter Walker 256 tumor cells, even after 5 hours incubation, whereas rat neutrophils took up some in the form of tiny cytoplasmic droplets (Cormack 1970). Whist it seems likely that the majority of sialic acid liberated by treatment of whole cells is derived from the surface, one cannot exclude the possibility that a portion is derived from within the cell.
Histochemical Methods

Histochemical methods used to detect sialyl residues depend on the affinity of the reagent at low pH for the acidic carboxyl groups, and can also indicate other acidic groups such as the sulphate group of acidic mucopolysaccharides. Removal of these staining properties by neuraminidase treatment is taken as evidence that staining observed before enzyme treatment is due to the presence of sialyl residues.

The reagent most often used to demonstrate bound sialic acid on the cell surface is colloidal iron. Its use was first described by Hale (1946) and modified by Mowry (1958). This reaction is based on the affinity of ferric iron radicles in acid solution for the carboxyl groups of sialic acid, and the subsequent conversion of these ferric groups to ferric ferrocyanide which is visible as a bright blue material under the light microscope. The method has been adapted for electron microscopy (Gasic and Berwick 1963, Curran et al. 1965) where both the electron dense ferric ferrocyanide crystals or the ferric iron particles themselves can be readily seen.

3. SIALIC ACID IN THE GLYCOCALYX OF MAMMALIAN CELLS

Mammalian cells in general

The methods discussed above have been used to demonstrate sialic acid on the surface of a variety of mammalian cells. Wallach and Eylar (1961) established that sialic acid was a component of the glycoprotein coat of mouse ascites tumor cells, by removing material stainable by the colloidal iron method with neuraminidase. Cook, Heard and Seaman (1962) obtained further evidence for the occurrence of sialic acid on the cell periphery by demonstrating an alteration in the electrophoretic mobility of Ehrlich ascites tumor cells after treatment of the cells with neuraminidase. Gasic and Gasic (1962 a and b), using the Hale method, identified carbohydrate containing material at the surface of ascites tumor cells by light microscopy and
removed this material with neuraminidase. This observation was extended to the ultrastructural level by Gasic and Berwick (1963), where the Prussian blue crystals were identified on the cell membrane of ascites tumor cells. In 1963, Defendi and Gasic showed that embryonic hamster cells which had been transformed in vitro by polyoma virus possessed a glycoplyx which could be modified by neuraminidase. Kraemer (1966a) measured the surface sialic acid in a wide range of tumor and tissue culture cell lines, using the thiobarbituric acid method. As many tumor cells possess relatively large amounts of glycoprotein containing material at the cell surface, it is not surprising that much of the early work on histochemical identification and release of sialic acid was performed on these cells.

However it was also possible to demonstrate similar material at the surface of non-malignant cells. Material staining by Hale's method, although not identified as containing sialic acid, could be shown to surround a wide variety of cells in the rat (Rambourg, Neutra and Leblond 1966). Isolated plasma membranes from normal and neoplastic liver cells were shown by chemical methods to contain sialic acid (Emmelot, et al. 1964). This sialic acid was later located on the outer aspect of the membrane by using a combination of colloidal iron staining, neuraminidase treatment and electron microscopy (Benedetti and Emmelot 1967). Sialic acid has been demonstrated on the surface of platelets (Madoff, Ebbe and Baldini 1964, Behnke 1968), in the trophoblast (Bradbury, Billington and Kirby 1965), zona pellucida of the mammalian ovum (Soupart and Noyes 1964) and at the base of the epithelial cells of the kidney glomerulus (Mohos and Skoza 1969).

The Lymphocyte

In 1962 Ruehenstroth-Bauer et al. described alterations in electrophoretic mobility in a variety of human peripheral blood leucocytes, including lymphocytes, after neuraminidase treatment. Rambourg et al. (1966) reported that a cell coat staining with Hale's stain surrounded lymphocytes in blood
vessels, connective tissues and lymph nodes of adult rats. They surmised that this indicated acidic residues on the cell surface, although they did not identify these residues as sialic acid. Cook and Jacobsen (1968) demonstrated sialic acid on the surface of lymphocytes from the lymph nodes of normal and leukaemic mice. They were able to show changes in the electrophoretic mobility of these cells after neuraminidase treatment and could quantitate the amount of sialic acid released from $10^8$ leukaemic cells, but not from an equal number of normal cells. Woodruff and Gesner (1969) treated rat thoracic duct lymphocytes with neuraminidase and were able to measure the amount of sialic acid released and show a corresponding change in electrophoretic mobility. The sialic acid released by treatment of Burkitt EB$_2$ lymphoma cells with neuraminidase was quantitated by Pardoe et al. (1970).

The amount of sialic acid released from rat thoracic duct lymphocytes in the experiments of Woodruff and Gesner (1969), may be compared with that released from other cell types. Rat thoracic duct lymphocytes possess 4 times as much surface sialic acid per cell as do sheep or calf erythrocytes (Eylar et al. 1962). By comparison with rat lymphocytes, there is 1.4 times as much sialic acid per cell released from mouse lymphoma cells (Cook and Jacobsen 1968), 24 times as much from Burkitt lymphoma cells (Pardoe et al. 1970) and 21 times as much from HeLa cells (Kraemer 1966a). A better idea of the density of surface sialyl groupings may be gained from comparison of the relative amounts of surface sialic acid per unit area. Such a calculation can be made from the data of Woodruff and Gesner (1969), coupled with an estimate of the cell volume of rat thoracic duct lymphocytes. Taking this density as unity, the relative amount for L5178Y cells, a tissue culture line derived from a mouse lymphoma is 1.4, for HeLa cells, 1.9, for C$_{13}$ cells, a hamster fibroblast tissue culture cell line, 2.8 and for P183 a polyoma transformed line derived from C$_{13}$, 4.1, using the results of Kraemer (1966a), for the amounts of surface sialic acid per unit area on the tissue culture cell lines.
4. POSSIBLE BIOLOGICAL ROLES OF CELL SURFACE SIALIC ACID

The general approach in efforts to determine the biological role of surface sialic acid has been to remove the sialyl residues and then examine the cells for changes in function. The inference is then drawn that, as a function has been altered by removal of sialic acid, then sialic acid is important in the physiological mediation of that function. While this is the best experimental approach available to further understanding of cell surface sialic acid, it has obvious limitations. As will be discussed below treatment of cells with neuraminidase has been shown to produce a variety of effects. The possibility remains that an alteration in a particular cell function under study after removal of sialic acid may be a secondary effect due to the influence of sialic acid removal on another cell function.

Membrane Transport

Alterations in the movement of materials across the cell membrane have been described following neuraminidase treatment. Glick and Githens (1965), using L1210 leukaemia cells maintained in the ascitic form showed that removal of sialic acid interfered with both inward and outward movement of K⁺ ions across the cell membrane. In a further study on the same cell line Glick et al. (1966) demonstrated that neuraminidase treatment affected the spontaneous release of some proteins from the cells. The rate of release of some intracellular proteins, was reduced after neuraminidase treatment. The release of non-amino sugars and nucleotides from the cells was not affected. They postulated that sialic acid may be necessary to bind a membrane protein required for transport of other proteins out of the cell. Emmelot and Bos (1965) found that neuraminidase treatment could alter the Mg⁺⁺-ATPase, Na⁺-K⁺-Mg⁺⁺-ATPase and 5' nucleotidase activities of isolated plasma membranes from rat liver. Brown and Michael (1969) found the uptake of a ¹⁴C labelled amino acid, alpha-aminoisobutyric acid, into HeLa cells was decreased after treatment of the cells with neuraminidase, although its release was not affected.
Eylar (1966) conceived that the carbohydrate portion of excreted cellular glycoproteins acted as a "passport" for the protein. This concept was based on the observation that, while most excreted proteins have a carbohydrate portion, most intracellular proteins do not. Furthermore, the carbohydrate portion of the protein does not appear to be part of the biologically active site of these molecules and is without demonstrable function. Eylar suggested that the carbohydrate portion of the protein, upon interaction with a membrane receptor promoted transport of the newly synthesized glycoprotein into the extracellular environment.

In summary, there are several lines of evidence that sialic acid could play a role in the transport of small molecules and proteins across the cell membrane, both as a membrane receptor or as part of the excreted protein.

Cell Contact Phenomena

The properties of the cell surface are important in cell locomotion, invasion of tissues and in cell to cell contact. Sialic acid being a major component contributing to the negative charge on the cell surface probably plays an important role in these activities. Of particular interest in lymphocyte physiology is the movement of lymphocytes through tissues and the cellular interactions which occur in the immune response.

Studies of the locomotion of mammalian fibroblasts and epithelial cells have shown that movement depends on undulations of the cell membrane. The undulations appear to be produced by transient changes in membrane charge, an increase in the negative charge on the membrane causing expansion (Ambrose and Forrester 1968). These changes may provide a stimulus for the initiation of processes which provide the motive force for the cell. Alteration of the surface charge of an actively motile cell such as the small lymphocyte may well effect its normal ability to recirculate around the body.
Interactions between lymphocytes of different classes, and between lymphocytes and macrophages in the immune response are widely postulated. It is not known what influence cell surface sialic acid may exert in these interactions, and inferences on the possible behaviour of lymphocytes are largely drawn from other experimental models. Berwick and Coman (1962) showed that neuraminidase treatment of squamous epithelial cells reduced their ability to stick to siliconized glass, but had no effect on intercellular adhesion. A similar dissociation in the effect of neuraminidase on the ability of hamster fibroblasts to adhere to each other and to a foreign surface has been shown by Ambrose (1967). Woodruff and Gesner (1969) noted that neuraminidase treated rat thoracic duct lymphocytes tended to form spontaneous aggregates when resuspended in medium containing foetal calf serum. The killer cell activity of a sensitized spleen cell population, a phenomena which depends on close cell to cell contact, was somewhat increased by pretreatment of the spleen cells with neuraminidase (Weiss and Cudney 1971). Such evidence suggests that removal of cell surface sialic acid may not compromise the ability of lymphocytes to make contact with other cell types.

Treatment of the recirculating lymphocyte population with neuraminidase has been shown to alter their migratory properties after transfusion (Woodruff and Gesner 1969). An alteration in the ability of these cells to propel themselves through the tissues of the body, or in the nature of the cell to cell contact occurring between lymphocytes and the endothelial cells of postcapillary venules are possible explanations for this phenomenon.

It is difficult to draw firm conclusions from the behaviour of cells which have been exposed to other enzymes prior to neuraminidase treatment. Kemp (1958) found that the aggregation of embryonic chick fibroblasts was decreased if they were treated with neuraminidase after dissociation by trypsin. This emphasizes the difficulties of working on cells from solid tissues. Trypsin itself is known to release sialoglycopeptides from the cell surface (Cook et al. 1960, Langley and
Ambrose 1964, 1967, Ohkuma and Ikemoto 1966). As a result the condition of the surface of trypsin treated cells prior to incubation with neuraminidase may not be normal.

**Phagocytosis**

The stickiness of the surface of cells and their ability to adhere to foreign surfaces appear to depend on the carbohydrate containing surface material, and these properties can be reduced by treatment with neuraminidase (Berwick and Coman 1962, Appfel and Peters 1969). Sialic acid can increase the stickiness of the cell surface by increasing the ability of surface glycoprotein to bind water. Glycoprotein material surrounding the cell has been implicated in various pinocytotic and phagocytic mechanisms. The adhesion of foreign particles to the plasma membrane is important in the process of phagocytosis (Mudd and Mudd 1933). The process of phagocytosis has been considered to have two stages, one being the adherence of foreign particles to the cell surface and the other being the ingestion of these particles (Rabinovitch, 1967).

Morphological evidence that foreign particles adhere to an extracellular material prior to ingestion was obtained in the amoeba (Brandt and Pappas 1960) and toad bladder epithelium (Chio 1962). Experimental manipulations of the cell surface to study its role in phagocytosis involve of necessity free rather than fixed phagocytic cells, but the conclusions obtained can probably be applied to the phagocytic process in general. The phagocytic capacity of human monocytes was altered by neuraminidase treatment (Weiss et al. 1966). After neuraminidase treatment, more monocytes made contact with negatively charged, inert particles and more phagocytosis occurred. Bona et al. (1968) demonstrated that pinocytosis of foreign gamma globulin by rabbit neutrophils was inhibited by pretreatment of the cells with neuraminidase. Both trypsin and neuraminidase decreased the phagocytosis of foreign red cells. They concluded that a surface glycoprotein material was important in the uptake of foreign materials. The adherence of opsonized bacteria to mouse peritoneal macrophages was increased by neuraminidase treatment of macrophages but almost eliminated
by trypsin and chymotrypsin treatment (Allen and Cook 1970). These authors considered that the removal of charged sialic acid residues may have been accompanied by a reorientation of membrane constituents, allowing greater adherence of bacteria to receptor sites. These sites were considered to be removed by trypsin or chymotrypsin. This again pointed to the importance of surface material, possibly a glycoprotein, in the adherence of foreign particles to the cell.

There are thus several lines of evidence indicating that glycoprotein at the cell surface plays a role in the attachment and ingestion of foreign particles by cells.

Complement Fixation

Cell surface sialic acid appears to affect the process of complement fixation and cell lysis occurring at the surface. Arquilla et al. (1964) observed that sheep red blood cells which had been treated with neuraminidase lysed spontaneously in the presence of complement, without the requirement for haemolytic antibody. The complement used had been absorbed with relatively large quantities of sheep red blood cell stroma to remove any xenoantibody. This led them to suggest that an essential step in immune haemolysis was removal of a sialyl containing substrate from the cell surface. The spontaneous lysis of the neuraminidase treated red cells could be inhibited by conjugation of proteins containing sialic acid to the red cell surface. In addition, these workers compared the immune haemolysis by anti-insulin antisera, of sheep red cells sensitized with insulin alone or with insulin and transferrin, a sialic acid-containing glycoprotein. They found that haemolysis of sheep red cells sensitized with insulin and transferrin, was less than that of cells sensitized with insulin alone. They considered this inhibition was due to competition by the sialic acid-containing transferrin, in a step of complement fixation involving enzymatic cleavage of a sialic acid-containing substrate from the cell surface. Similar findings were made by Ray et al. (1971) using lymphoid cells. Rabbit serum activated by cobra venom factor was used as a source
of complement. They found that this serum had a cytotoxic effect on neuraminidase treated, but not on normal lymphocytes. However the mechanism whereby neuraminidase treatment increases the sensitivity of cells to lysis by complement is uncertain.

5. SIALIC ACID AND CELL SURFACE ANTIGENS

Cell surface sialic acid may be involved in surface antigenic systems either by being a structural component of the antigenic site or by influencing the expression of the surface antigens.

As part of the antigenic specificity site

Sialic acid has been shown to be involved in the specificity of 3 cell surface antigenic systems, the M and N antigenic system of human red cells, the HD antigen of human red cells and the M antigen of duck red cells (Uhlenbruck 1969). The antigenic activity of all three systems can be destroyed by treatment with neuraminidase. Influenza virus and enzymes derived from vibrio cholera were shown to inactivate M and N agglutinogens on human red cells (Springer and Ansell 1958, Mäkelä and Cantell 1958), and later purified neuraminidase preparations were demonstrated to have the same effect (Bird and Wingham 1970).

Expression of surface antigens

The term expression is used because any alterations noted in cell surface antigens after neuraminidase treatment depend on the methods used for their detection, and therefore may be due to an actual quantitative difference in the antigen under study or due to a variation in one of the steps used for detection.

Alterations in the expression of cell surface antigens after neuraminidase treatment have been measured in 3 ways; by observation of the in vivo immune response to neuraminidase treated cells, by measuring the response of immunocompetent cells to neuraminidase treated cells in vitro and by use of specific antisera directed against cell surface antigens.
The first way in which attempts have been made to demonstrate alterations in the expression of cell surface antigens after neuraminidase treatment, has been by observing differences in the in vivo immune response of animals to untreated or neuraminidase treated cells. Such observations are obviously very indirect, as a number of factors such as cell survival and the distribution and processing of the cells by the host’s immune system may affect the final result. Neuraminidase treatment of syngeneic (Sanford 1967, Currie and Bagshawe 1969) or allogeneic (Currie 1967, Sanford 1967) tumor cells prior to transfer led to an increased survival of recipient animals. Rechallenge of the surviving recipients of neuraminidase treated tumor cells with a lethal dose of untreated cells (Currie 1967) or skin graft syngeneic with the tumor cell line (Sanford 1967) suggested that immunization of the host against the tumor cells had taken place. In similar types of experiments, neuraminidase treated trophoblast (Currie et al. 1968) or mouse spleen cells (Simmons, Rios and Ray 1971) were more immunogenic than untreated cell preparations as judged by their ability to sensitize recipients against skin grafts. Simmons and Rios (1971) found that some established tumors regressed after immunization of the host with neuraminidase treated cells derived from the same tumor.

One explanation proposed to explain such findings is that neuraminidase treatment has "uncovered" histocompatibility or tumor specific antigens on the cell surface and thereby increased the immunogenicity of the cells (Currie 1967, Currie and Bagshawe 1968, 1969).

In support of such a mechanism are the findings of Schlesinger and Amos (1971) that mouse spleen cells are able to absorb increased amounts of anti-8 antibody or a xenoantibody in guinea pig serum after neuraminidase treatment. Kassulke et al. (1971) also found that human leukemic leucocytes demonstrated increased amounts of A and H antigens as assessed by mixed agglutination and
antibody absorption techniques after neuraminidase treatment. In contrast to these findings, the absorption of anti-H₂ antibody by mouse lymphoid cells (Ray et al. 1970) or TA₃ tumor cells (Hauschka et al. 1971) was not increased by neuraminidase treatment, and alternative explanations of the findings after immunization of animals with neuraminidase treated cells have been proposed. Hauschka et al. (1971) considered that the increased survival of animals after immunization with neuraminidase treated, as compared to untreated, TA₃ tumor cells was due to the diminished viability of the tumor cells as a result of neuraminidase treatment. Cormack (1970) found an increased survival in rats injected intraperitoneally with neuraminidase treated rat sarcoma cells as compared with rats injected with untreated cells. He proposed that this increased survival was due to a decrease in the ability of the enzyme treated tumor cells to attach to the peritoneum. The apparently greater immunogenicity of mouse lymphoid (Simmons, Rios and Ray 1971) or tumor (Simmons and Rios 1971) cells was considered to be due to an increase in the efficiency of processing of the cells by the host immune system, rather than to the "uncovering" of previously masked surface antigens.

The conflicts over the interpretations of these types of experiments are not easily resolved. Many experiments are not directly analogous because of the differing conditions present. For example the type and dose of cells used, their mode of preparation prior to neuraminidase treatment, the route of immunization and the parameters used to evaluate the response vary from one experiment to another.

A second means of examining the surface antigenicity of cells after neuraminidase treatment has been to measure the response of immunocompetent cells to neuraminidase treated cells in vitro. The results are again conflicting. Watkins et al. (1971) quantitated the response of peripheral blood lymphocytes from rats with Novikoff hepatoma to mitomycin-C treated, control or neuraminidase treated tumor cells. Greater lymphocyte
transformation as indicated by tritiated thymidine uptake occurred if the lymphocytes were incubated with enzyme treated rather than untreated target tumor cells, a result which they interpreted to indicate an uncovering of tumor associated antigens. It has been shown that treatment of the stimulatory cells in human one way mixed lymphocyte culture markedly increased the tritiated thymidine uptake of the responding cells (Lundgren and Simmons 1971). These workers favoured an increase in immunogenicity of the HLA antigens due to increased cell-to-cell contact rather than "uncovering" of antigenic determinants as the probable explanation. However Weiss and Cudney (1971) demonstrated that pretreatment of P815 mastocytoma cells with neuraminidase did not increase their susceptibility to the killer cell activity of sensitized spleen cells. They concluded that the concept of a sialomucin coat protecting tumor cells from immunological surveillance did not have general validity.

A third and more direct way of studying the effect of neuraminidase treatment on cell surface antigens has been by means of antisera directed against these antigens. As discussed above, absorption studies indicated that neuraminidase treatment uncovered θ antigen on mouse spleen cells (Schlesinger and Amos 1971) and the A and H antigens on leukaemic human leucocytes, but there was no increase in the corresponding antigens on normal human leucocytes (Kassulke et al. 1971). Neuraminidase treatment did not alter the absorption of anti-H2 antibody by mouse lymphoid (Ray et al. 1970) or TA3 tumor (Hauschka et al. 1971) cells.

Lymphocytes are more susceptible to the cytotoxic effect of antisera directed against some surface antigens after neuraminidase treatment. This has been demonstrated for the θ antigen on mouse thymus cells (Schlesinger and Amos 1971), an antigen on mouse lymphoid cells detected by a xenoantibody in normal guinea pig serum (Schlesinger and Amos 1971) and the HLA antigens on the surface of human lymphocytes (Grothaus et al. 1971). This may be due to the uncovering of more antigenic sites, but it could
also be a result of the increased efficiency of fixation and lysis by complement of neuraminidase treated cells (Arquilla et al. 1964, Ray et al. 1971), or a combination of both.

In order to account for such findings, Amos (1970) in discussing the structure of HLA antigens proposed that they consisted of a structural protein with peptide or oligosaccharide side chains, the terminal grouping of these side chains being the portion that is detected serologically. Sialic acid could block either the polysaccharide antigens themselves or the antigenic portions of the adjacent peptide chains in proximity to the sialyl group.

6. RECOVERY OF SURFACE SIALIC ACID BY NEURAMINIDASE TREATED CELLS

The first experimental evidence that regeneration of surface sialic acid could take place after its removal by neuraminidase was obtained by Marcus (1959). The ability of Newcastle disease virus to attach to and kill HeLa cells via surface sialic acid receptors was abolished after neuraminidase treatment of the cells. After maintenance of the cells in culture, virus could again attach to and kill the cells. The estimated half time for recovery of the surface receptors was about 6 hours. The surface receptors of HeLa cells for Newcastle disease virus returned within 20 hours of neuraminidase treatment of the cells (Marcus and Hirsch 1963) and some receptors could be detected within 15 minutes of treatment. The Hale staining material around TA3 tumor cells was restored after a minimum of 1 hour in culture following neuraminidase treatment (Gasic and Gasic 1962b).

Kraemer (1966b) studied the regeneration of sialic acid on the surface of Chinese Hamster ovary cells. The level of cell surface sialic acid, as assayed by the thiobarbituric acid method, following its release by neuraminidase, returned to normal levels within 16 hours after neuraminidase treatment. This recovery took place regardless of whether the cells were in a suspension or a monolayer, and could be inhibited by puromycin but not
by a concentration of actinomycin D sufficient to arrest cell division. Some lymphoid cells are also able to regenerate surface sialic acid after its removal. Pardoe et al. (1970) examined the sialic acid at the surface of Burkitt lymphoma cells. Maintenance of these cells in culture for 96 hours after treatment with neuraminidase resulted in the complete recovery of surface sialic acid. This was measured by chemical assay and by the return of the capacity of the cells to be agglutinated by haemolymph from Limulus polyphemus, which contains an agglutinin specific for sialyl residues. The administration of sublethal irradiation to the lymphoma cells suppressed this recovery.

Experimental systems demonstrating differences in behaviour between untreated and neuraminidase treated lymphocytes have also indicated that recovery of surface sialic acid can take place. After 4 days in culture, the increased sensitivity of neuraminidase treated, human peripheral lymphocytes to cytotoxic antisera had to a large extent been lost (Grothaus et al. 1971). Weiss and Cudney (1971) found that neuraminidase treated, sensitized spleen cells possessed greater killer cell activity against mastocytoma cells than did untreated spleen cells. After 5 hours in culture, differences in killer cell activity between treated and control cells were reduced, and the neuraminidase treated spleen cells showed some increase in electrophoretic mobility at this time. Weiss and Cudney interpreted these findings as indicating partial recovery of sialyl groupings at the cell periphery.

There is no indication as to whether recovery of surface sialic acid occurs by the addition of sialyl residues onto the sialic acid deficient oligosaccharides, or whether an entire new polysaccharide complex with a terminal sialyl residue is synthesized.
7. EFFECT OF MODIFICATION OF THE CELL SURFACE ON LYMPHOCYTE MIGRATION

Thoracic duct lymphocytes are predominantly a cell population which recirculates from blood to lymph (Gowans 1959, Gowans and Knight 1964) via the postcapillary venules (Marchesi and Gowans 1964). Factors controlling the specificity of this recirculation are poorly understood but properties of the lymphocyte surface are likely to be important. This problem has been approached by determining the effect of experimental modification of the lymphocyte surface on the subsequent migratory patterns of these cells.

Viability of the lymphocytes is a prerequisite for recirculation as lymphocytes killed by heat or ultraviolet irradiation did not recirculate from blood to lymph (Gowans 1957). Treatment of thoracic duct lymphocytes with a mixture of crude glycosidases (Gesner and Ginsburg 1964), trypsin (Woodruff and Gesner 1968), neuraminidase (Woodruff and Gesner 1969) and anti-lymphocyte serum (Martin and Miller 1967) have all been reported to modify the migration of the cells. Gesner and Ginsburg (1964) observed that the distribution in recipient animals of rat thoracic duct lymphocytes treated with a mixture of glycosidases obtained from Clostridium perfringens was altered. After this treatment, lymphocyte migration to the spleen and into the thoracic duct lymph was decreased while migration to the liver was increased. This effect of the glycosidases could be inhibited by addition of the sugars L-fucose or N-acetyl-D-galactosamine to the incubation medium. Hence, they considered that such changes were due to removal of cell surface sugars. They suggested that surface sugars acted as recognition sites for the specific migration of lymphocytes. Such a concept seemed additionally attractive as it would be in accord with the observation that sugars commonly found at mammalian cell surfaces include L-fucose, D-mannose, D-galactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and sialic acid but not D-glucose. As D-glucose, unlike
the other sugars, is found free in the blood it would interfere with a specific recognition system structurally based on itself.

These observations on cell migration were extended by Woodruff and Gesner (1969), who found that treatment of rat thoracic duct lymphocytes with neuraminidase prior to transfer to syngeneic recipients reduced migration of the cells to spleen, lymph nodes and thoracic duct lymph but increased uptake of these cells in the liver. About 24 hours after the infusion of labelled, neuraminidase treated lymphocytes, they noted a decrease in radioactivity in the liver associated with an increase in the lymph nodes. They suggested that the modified lymphocytes, after a period of residence in the liver, were able to leave and migrate into lymph nodes. The alterations produced in cellular migration by neuraminidase could be inhibited by the addition of an excess of free sialic acid to the incubation medium.

In contrast to these findings, incubation with trypsin, instead of neuraminidase, prior to infusion decreased the selective accumulation of thoracic duct lymphocytes in the lymph nodes in the first few hours after transfusion. However the migration to the liver and spleen was little different from that of untreated cells (Woodruff and Gesner 1968). This finding raised the possibility that factors influencing the migration of lymphocytes to the lymph nodes were not the same as those which determine migration to the spleen. One known difference is the route by which the lymphocytes leave the blood stream. Lymphocytes migrated into the white pulp of the spleen between gaps in the endothelial cells of the marginal sinuses (Goldschneider and McGregor 1968a), whilst they entered lymph nodes by migrating through or between the high endothelial cells of the postcapillary venules (Marchesi and Gowans 1964). However Ford (1969) in a study of lymphocyte migration from the isolated, perfused rat spleen showed that lymphocytes migrating from the spleen into the perfusate possessed the same migratory and immunological properties as thoracic duct lymphocytes,
and he concluded that these lymphocytes from the spleen belonged to the same population as the thoracic duct lymphocytes.

The basis of these enzyme-produced migratory changes is open to question. The explanation appears to be more complex than interference with a recognition mechanism on the lymphocyte surface. One must distinguish between the decreased migration to the lymph nodes after neuraminidase treatment as a result of inability of the lymphocytes to enter the node or as due to unavailability following sequestration elsewhere in the circulation e.g. in the liver. In an attempt to answer this question, Woodruff and Gesner transfused neuraminidase treated lymphocytes into hepatectomized and enterectomized rats. In one experiment in which the animals survived for more than 30 minutes, there was a 35% reduction in the radioactivity accumulating in the lymph nodes when neuraminidase treated cells were substituted for control cells. This is a result which the authors suggest should be viewed with great caution. An alteration in cell metabolism produced by enzyme treatments has also been suggested as a possible cause of change in migratory pattern (Woodruff and Gesner 1968, 1969), and has not been excluded.

As in many areas of immunology, there is a tendency to attribute any changes in function produced by alteration of the lymphocyte cell surface to modification of a specific 'receptor' site. Although Woodruff and Gesner made the point that an alteration of a lymphocyte surface 'recognition' site was only one possible explanation for the observed effects of neuraminidase treatment, such an explanation has been accepted and quoted by other authors (Griscelli et al. 1969), Mills and Cooperband 1971, Vincent and Gunz 1970). This explanation may prove to be too simplistic. Even the argument that, because removal of surface sialic acid can be shown to alter the distribution of lymphocytes, this surface constituent plays an important role in determining the normal fate and distribution of lymphocytes around the body, is open to question. For example it is possible that other sugar residues exposed
by removal of the terminal sialic acid may positively influence lymphocyte behaviour, and be the cause of the observed changes, and that the sialic acid residues play no active role in lymphocyte migration.

Host factors can also influence the migratory properties of normal and modified lymphocytes. The high frequency of uptake of thoracic duct lymphocytes treated with anti-lymphocyte serum in the liver appears to be due to phagocytosis by the Kupffer cells (Martin and Miller 1967, Martin 1969). Antigenic stimulation can alter the migratory pattern of lymphocytes through lymph nodes (Hall and Morris 1965, Griscelli et al. 1969) and to the spleen (Zatz and Lance 1971). It has also been proposed that the migration of lymphocytes rich in IgG surface receptors may be influenced by interaction with surface immunoglobulins on the reticulocytes of the follicular areas (Parrott and de Sousa 1971) and that complement fixation to the cell surface may play a part in this process (Dukor et al. 1970).

There is some suggestion that the postcapillary venule may not play an active role in lymphocyte migration. Neonatal thymectomy of rats depleted the recirculating lymphocyte pool and changed the morphological appearance of the postcapillary venules so that the endothelial cells appeared flattened and the vessels themselves appeared dilated and empty. Labelled lymphocytes were found to migrate across the endothelium of these vessels in a manner similar to that observed across normal endothelium (Goldschneider and McGregor 1968a).

The overall migratory pattern seen may be a summation of the distribution of various subpopulations of lymphocytes. Attempts have been made to identify lymphocyte subpopulations by the transfer to secondary recipients of labelled cells localizing in various tissues, and comparing their localization with that of the original population (Lance and Taub 1969, Zatz and Lance 1970). It was found that labelled lymph node cells that migrated to the lymph nodes after primary transfer,
showed an increased tendency to relocalize in the lymph nodes of a secondary host (Lance and Taub 1969). Such a phenomenon reflects to some extent the selection by the nodes for the recirculating population of lymphocytes from the original cell inoculum. However there is another possible selective influence in this type of experiment, namely selection for normal migratory properties from cells whose migratory properties have been altered by collection and labelling procedures. To assess the extent of such an occurrence, retransfer experiments utilizing thoracic duct lymphocytes would be needed. Only then could the full significance of a demonstrated 'subpopulation' be assessed.

There is other evidence for difference in migratory properties of various cell populations. Large lymphoid cells appearing in the thoracic duct lymph migrate predominantly to the bowel and gut associated lymphoid tissue on retransfer (Gowans and Knight 1964, Hall and Smith 1970). Thymocytes localize in a manner quite different to that of thoracic duct lymphocytes. Thymus cells have a much greater tendency than thoracic duct lymphocytes to be held up in the lungs soon after retransfusion, while fewer thymus cells migrate to the lymph nodes and spleen (Goldschneider and McGregor 1968b, Berney and Gesner 1970). There also appears to be a sub-population of cells within the thymus that has migratory properties resembling those of peripheral lymphocytes (Lance and Taub 1969). Additionally thymocytes localizing in the lymph nodes after transfer appear to have the surface antigenic properties of peripheral, thymus derived cells rather than those of the original population (Raff 1971). It is not known whether these antigenic differences are related to the different migratory pattern of this subpopulation of thymus cells.

Another question as yet unanswered is the fate of lymphocytes in vivo after treatment with neuraminidase. It has been suggested that changes produced in the cells by trypsin or neuraminidase treatment are reversible, on
the basis of increasing migration to the lymph nodes and thoracic duct lymph after a period of 24 hours or so residence in vivo (Woodruff and Gesner 1968, 1969). This return to a normal migratory pattern has not been directly demonstrated. If a reversibility of surface changes does occur it could be mediated by the host or by the lymphocyte itself.

8 THE EFFECT OF LYMPHOCYTE SURFACE MODIFICATION ON ALLOGENEIC INTERACTIONS

Modifications of the lymphocyte surface could affect in vivo allogeneic interactions such as the graft-versus-host (GvH) reaction either by altering the localization and fate of the grafted cells within the host animal or by interfering with interaction between the grafted cells and those of the host.

The evidence available from in vitro experiments suggests that the ability of lymphocytes to interact with allogeneic cells may not be compromised by prior removal of cell surface sialic acid. Incubation of human lymphocytes with a crude extract from vibrio cholera (Lindahl-Kiessling and Peterson 1969), or a more purified neuraminidase preparation (Lundgren and Simmons 1971) did not affect their response to mitomycin-C treated allogeneic lymphocytes in mixed lymphocyte cultures. As the responses are measured after several days in culture it is not possible to be sure whether regeneration of a normal surface has taken place before the lymphocytes can respond. Further evidence on the interaction of neuraminidase treated lymphocytes with allogeneic cells was obtained by Weiss and Cudney (1971). They found that the killer cell activity of a sensitized spleen cell population against an allogeneic monolayer was somewhat increased by pretreatment of the spleen cells with neuraminidase.

There is little evidence available on the effect of surface modifications on the ability of lymphocytes to undertake GvH reactions in vivo. Neuraminidase treated rat thoracic duct lymphocytes appeared equivalent to untreated cells in their ability to produce a lethal
GvH reaction after transfer to lethally irradiated, bone marrow restored mice (Gesner and Woodruff 1969). The only criteria of the reaction recorded was the mortality at 14 days after the transfer of immunocompetent cells, and while showing that some neuraminidase treated lymphocytes can participate in GvH reactions there was little indication of the relative GvH activity of normal and enzyme treated cells.

It is possible that surface modification of lymphocytes by neuraminidase treatment could affect the GvH reaction by altering the distribution of the cells within the host animal. There are some indications that the site of localization of parental cells soon after transfer is important in determining the nature of the GvH syndrome which subsequently develops.

Biozzi et al. (1964) found that F₁ hybrid mice splenectomized before the injection of parental strain lymphocytes tended to be more resistant to the ensuing GvH reaction, and they proposed that this may be due to the fact that donor cells failed to gain a "foothold". Such a result suggests that the severity of the disease depends on the site in which lymphocytes settle after transfusion. Syngeneic immunocompetent cells can protect neonatal mice (Siskind et al. 1960) or rats (Silvers and Billingham 1969) against the GvH disease produced by allogeneic cells. For such protection, the two cell types must be administered simultaneously. A delay of as little as 4 hours in the administration of the protective cell inoculum greatly reduced its effect (Silvers and Billingham 1969), an indication that some initial events which determine the course of the disease had taken place very soon after cell transfer.

The importance of the initial site of parental cell localization in determining the course of the GvH syndrome, and the subsequent capabilities of the lymphocyte population itself, is indicated by the experiments of Streilein and Billingham (1970). F₁ hamsters developed epidermolytic features of acute GvH disease only when given parental cells intracutaneously but not when given
an equivalent number of parental cells by the intravenous, intraperitoneal or intramuscular routes. Furthermore, the epidermolytic syndrome could be transferred to secondary F₁ hosts by lymph node cells derived from the primary F₁ host, given by either the intravenous or intracutaneous route, provided that the original primary host had been injected by the intracutaneous route. A further example of the importance of the route of transfer of cells has been given by Billingham (1968). The severity of GVH disease produced in neonatal mice varied with the route of cell administration, with the intravenous, intraperitoneal and subcutaneous routes giving a decreasing severity of disease. This is presumably because of differences in the site of localization of the injected cells.

One of the most distinctive characteristics of GVH activity is that it appears to involve predominantly the host's lymphomyeloid tissues. Proliferative changes are present in those organs in which lymphoid cells are known to "selectively localize" namely the spleen and lymph nodes. Streilein and Billingham (1970) considered that the only immunologically specific event in the induction of GVH disease was interaction between the lymphoid cells of the host and those of the donor. The particular site in which this interaction took place determined the type of lesion that followed.

In view of the above observations on the induction of the GVH syndrome and factors affecting the severity of GVH disease, the effect of altering the localization of parental cells by treatment with neuraminidase prior to transfer to F₁ recipients, would be of interest.

9. LYMPHOCYTE CIRCULATION AND THE IMMUNE RESPONSE TO SHEEP ERYTHROCYTES

Modification of the lymphocyte surface could theoretically affect the in vivo immune response to an antigen in two ways; either by an alteration in the distribution and recirculation of the lymphocytes or by directly compromising the ability of the cells to respond to antigenic stimulus.
The recirculation of lymphocytes is an important factor in the evolution of an immune response. The cells of the recirculating lymphocyte pool in the rat can initiate and maintain an antibody response to sheep red blood cells. The magnitude of the haemolysin response produced by $2-3 \times 10^8$ of these lymphocytes on transfer to a sublethally irradiated rat is equivalent to that produced by an intact animal (McGregor et al. 1967). The migration of lymphocytes from the blood can restore the immune response in the rabbit spleen (Taliaferro and Taliaferro 1956) or sheep lymph node (Hall and Morris 1964), depleted of lymphoid cells by local irradiation. Good evidence that the lymphocytes migrating through the spleen are of importance in the mounting of an immune response in that organ has been obtained by Ford and Gowans (1967), using the isolated, perfused rat spleen preparation. The magnitude of the haemolysin response to sheep red blood cells produced by this preparation was dependent on the number of lymphocytes migrating through the spleen at the time of antigenic stimulation, although the cells in the course of migration represented less than 20% of the total lymphocyte pool of the spleen. The lymphocyte population migrating through the spleen appeared to be identical with that recirculating via the thoracic duct (Ford 1969). Approximately half of the total recirculating pool of lymphocytes passed through the spleen every 18 hours (Ford 1968a). It has been suggested that this recirculation of immunocompetent lymphocytes may provide a means whereby the maximum number of competent cells can be exposed to an antigen (Ford and Gowans 1967). Experiments of Ford (1968b) suggested that the recruitment of lymphocytes from the recirculating pool contributed substantially to the haemolysin response in the spleens of irradiated rats restored with thoracic duct lymphocytes. The major period of recruitment occurred within the first 24 hours after the injection of antigen. After this time the inductive effect of antigen diminished.
That lymphocyte migration is of such relevance in the inductive stages of the immune response implies that modification of this migration might be reflected in changes in the evolution of the response.

Another factor to be considered is that cell surface changes produced by neuraminidase may compromise the ability of lymphocytes to respond to antigenic stimulation. There is no direct evidence on this point. David et al. (1964) in a study of the migration inhibition response, which depends on the interaction of antigen with sensitized lymphoid cells, found that incubation of the sensitized cells with trypsin destroyed their ability to inhibit migration of macrophages after exposure to antigen. However this ability returned if the cells were maintained in vitro for 24 hours after trypsinization (David 1968). Neuraminidase treatment of lymphocytes did not reduce their response to allogeneic cells in vitro (Lindahl-Kiessling and Peterson 1969, Lundgren and Simmons 1971, Weiss and Cudney 1971), suggesting that neuraminidase treatment may not compromise the ability of lymphocytes to respond to antigen.

10. AIMS OF THE PRESENT INVESTIGATION

The consequences of removal of the cell surface sialic acid from the recirculating lymphocyte population obtained from the thoracic duct lymph have been studied. Particular attention has been paid to the fate of these cells in vivo and in vitro, and to their ability to undertake a graft-versus-host reaction, and to participate in the immune response to sheep erythrocytes.
1. **RATS**

Rats used in these experiments were obtained from several sources. Rats obtained from a non-inbred Albino colony are referred to in the text as 'albino'. Three inbred colonies of CA, Lewis and hooded strains of rats were also maintained. CA and Lewis strain rats were bred by brother-sister mating from pairs obtained from the Walter and Eliza Hall Institute, Melbourne, the colonies having originally been developed by Dr. E. E. Silvers. Hooded strain rats were obtained from the C.S.I.R.O. Division of Animal Physiology, Florey, New South Wales, Australia. They were originally obtained from James Laboratories, Inc.

When F76A x Lewis/Hybrid or F76A x Hooded/Hybrid rats were used from these colonies, both strains were used at a ratio of 1:1. In all experimental procedures no sex difference was observed in the case of thoracic duct cannulation for which male rats of 250 to 250 grams were employed.

2. **OPERATING PROCEDURE**

**Cannulation of the Thoracic Duct**

The thoracic duct was cannulated in the abdomen under ether anaesthesia using 'Seldas' (1948) modification of the method of Rolleston, Cato and Sandiford (1947). After cannulation, rats were confined in restraining cages where they remained anaesthetised, during the period of lymph collection. During this period, rats received intermittent infusions of saline phosphate buffered saline containing 1 unit of heparin/ml. Intravenous fluids were administered at the approximate rate of 40 ml/24 hours. Cannulated rats were provided with 0.9% solution of sodium chloride for drinking in the immediate post-operative period and with tap water thereafter. Hay cubes were provided in a hopper attached to the restraining cage. Lymph from the thoracic duct cannula...
METHODS

1. RATS

Rats used in these experiments were obtained from several sources. Rats obtained from a non-inbred albino colony are referred to in the text as 'outbred'. Three inbred colonies of DA, Lewis and Hooded strains of rats were also maintained. DA and Lewis strain rats were bred by brother-sister mating from pairs obtained from the Walter and Eliza Hall Institute, Melbourne, the colonies having originally been developed by Dr. W.K. Silvers. Hooded strain rats were obtained from the C.S.I.R.O. Division of Animal Physiology, Prospect, New South Wales, Australia. They were originally obtained from Glaxo Laboratories, U.K.

When (DA x Lewis)F₁ hybrid or (DA x Hooded)F₁ hybrid rats were bred from these colonies, both strains were used as a source of either parent. Use in any experimental procedure was not restricted to rats of one sex except in the case of thoracic duct cannulation for which male rats of 200 to 250 grams were employed.

2. OPERATIVE PROCEDURES

Cannulation of the Thoracic duct

The thoracic duct was cannulated in the abdomen under ether anaesthesia using Gowans' (1959) modification of the method of Bollman, Cain and Grindlay (1948). After cannulation, rats were confined in restraining cages where they remained, unanaesthetized, during the period of lymph collection. During this period, rats received intermittent infusions of Dulbecco's phosphate buffered saline containing 2 units of heparin/ml via a femoral vein cannula. Intravenous fluid was administered at the approximate rate of 20 ml/24 hours. Cannulated rats were provided with 0.45% solution of sodium chloride for drinking in the immediate post-operative period and with tap water thereafter. Rat cubes were provided in a hopper attached to the restraining cage. Lymph from the thoracic duct cannula
was collected in sterile flasks at room temperature for periods of up to 16 hours. Each flask initially contained 5 ml of medium 199 plus 100 units of heparin.

**Assay of local GvH activity**

Recipients were anaesthetized with ether. With the rat immobilized on its back an incision approximately 1 cm long was made in the right or left iliac fossa. The overlying bowel was pushed aside with moist packs, and the site for injection identified by locating the iliolumbar artery and vein which enter the retroperitoneal fat together. 0.1 ml of the cell suspension in Hanks' saline was injected using a 1 ml tuberculin syringe fitted with a 25 gauge needle. The needle was inserted into the adipose tissue 0.8-1 cm inferior to the site of entry of the iliolumbar artery and vein into the retroperitoneal fat. The bowel was replaced over the site of injection of the cells and the incision in the abdominal wall repaired with black silk sutures. Recipients were killed 7 days after the injection, and the largest diameter of the inflammatory lesion in the adipose tissue was measured as an index of size. The lateral iliac nodes were identified situated adjacent to the iliolumbar vessels, removed, cleaned and weighed to an accuracy of 0.1 mg.

The foot pad assay for GvH activity was performed as described by Ford, Burr and Simonsen (1970). 0.1 ml of a suspension of lymphocytes in Hanks' saline was injected subcutaneously into the foot pad using a tuberculin syringe and 25 gauge needle. Popliteal nodes were removed and weighed with an accuracy of 0.1 mg 7 days later.

**Skin Grafting**

(Lewis x DA)F₁ hybrid rats which had received $10^8$ DA strain neuraminidase treated lymphocytes at the age of 4-5 weeks were grafted with Lewis and DA skin grafts 6-9 weeks later. Full thickness Lewis and DA skin grafts of approximately 3.5 x 2.5 cm in size were transferred, one to each flank of the host.
3. PREPARATION OF CELL SUSPENSIONS

Thoracic duct lymphocytes

Thoracic duct lymphocytes were collected as described above. Where lysis of red cells was required (in radioactive labelling experiments and in determination of surface sialic acid), after centrifugation from the lymph the cell pellet was suspended in 2 ml of 0.9 N saline for 20-30 seconds to lyse red cells. The saline was then made isotonic by addition of 1/2 N saline.

Spleen cells

Rats were killed with ether and the spleen removed by transection of the splenic pedicle.

After removal the spleen was placed in ice-cold Hanks' saline. Spleen cell suspensions were prepared by disrupting the organ by means of a mesh of stainless steel. The suspension was prepared and maintained in ice-cold Hanks' saline.

Thymus cells

Rats were killed with ether, the thorax opened and the thymus removed by blunt dissection. The organ was then gently teased apart in ice-cold Hanks' saline using fine forceps. Suspensions prepared in this manner were filtered through gauze.

4. ANTIGEN AND IMMUNIZATION

Sheep erythrocytes used as antigen were prepared from sheep blood collected and stored in Alsever's solution. Before use in an experiment, erythrocytes were washed three times in a 0.9% solution of sodium chloride and resuspended in this solution. For the purpose of immunization or the induction of tolerance, suspensions of intact sheep erythrocytes were used. The immunizing or challenging dose of sheep erythrocytes used in these experiments was 1 ml of a 1% suspension given via the intravenous route.
5. ASSAYS FOR HAEMOLYTIC PLAQUE-FORMING CELLS

Cunningham and Szenberg's (1968) modification of the plaque assay of Jerne, Nordin and Henry (1963) was used.

6. HAEMOLYSIN TITRATION

Blood was collected from the tail veins of anaesthetized rats, allowed to stand until clot retraction had occurred, and then centrifuged. The separated sera were stored frozen. Immediately before use, sera were inactivated by heating to 56°C for 30 minutes. Titrations for haemolysin were always undertaken against a control serum of known haemolysin titre (Commonwealth Serum Laboratories, Melbourne). A micro-titration apparatus (Takatsy, Budapest) was used for haemolysin determinations. Serial two-fold dilutions of serum with calcium-magnesium (Ca-Mg) saline solution (Fazekas de St. Groth, Graham and Jack 1958) were prepared in perspex titration plates, the first dilution being 1:2. Sheep erythrocytes were washed three times in 0.9% sodium chloride solution and once in Ca-Mg saline solution before being adjusted to a 1% suspension in the latter. Lyophilized guinea pig serum (Commonwealth Serum Laboratories, Melbourne) was reconstituted with distilled water and diluted 1:8 with Ca-Mg saline immediately before use. 0.025 ml of the erythrocyte suspension and 0.025 ml of diluted guinea pig serum were added to the 0.05 ml of diluted serum in each cup. The plates were incubated at 37°C for 30 minutes and then maintained at 4°C to permit erythrocytes to settle before reading. The highest dilution of serum showing complete haemolysis was read as the end point, titres being recorded as the log₂ of the reciprocal of this serum dilution.

7. GAMMA IRRADIATION

Irradiation was performed at the Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Black Mountain, A.C.T. A 100
curie cobalt 60 source was used. Rats were irradiated in rotating metal drums placed 20 cm from the source. The doses administered in the experiments to be reported were either 455, 730 or 910 rad.

Preliminary experiments established that 730 rad was sufficient to completely prevent a haemolysin response by irradiated rats injected with sheep erythrocytes.

Injection of sublethally irradiated (455 rad) rats was carried out within the first 24 hours after irradiation.

8. **INDUCTION AND MAINTENANCE OF IMMUNOLOGICAL TOLERANCE**

Immunological tolerance of sheep erythrocytes was induced in rats by the injection of a 50% suspension of washed sheep erythrocytes on the day of birth. 0.125 ml of this suspension was given intravenously followed by a similar dose intraperitoneally. Further injections of 0.25 ml of a 50% suspension of sheep erythrocytes were given intraperitoneally twice weekly. A rat was classified as 'tolerant' if no haemolysin could be detected at the lowest serum dilution (1:4) employed in titration. When a tolerant rat was to be subjected to additional procedures, injections of the 50% suspension of sheep erythrocytes were discontinued in the week preceding the experiment. Serum haemolysin titre was determined several days after the last injection.

9. **INCUBATION OF LYMPHOCYTES WITH NEURAMINIDASE**

Neuraminidase, derived from vibrio cholera and prepared to contain 500 units/ml was obtained from Hoechst Pharmaceutical Co., Melbourne, Australia and was manufactured by Behringwerke Ag., Marburg-Lahn, West Germany. The enzyme was supplied dissolved in sodium acetate buffer solution at 0.05 mol/litre at pH 5.5 with an addition of 9 mg of sodium chloride and 1 mg of calcium chloride per ml. One unit of neuraminidase was defined as the amount of the enzyme required to release 1 µg of N-acetyl neuraminic acid from human α-acid glycoprotein in 15 minutes at 37°C.
Lymphocytes were washed once with phosphate buffered saline pH 7.4, and resuspended in Dulbecco's phosphate buffered saline pH 7.4 containing 50 units of neuraminidase (0.1 ml of enzyme solution) per ml, at a concentration of $5 \times 10^8$ cells per ml. Incubation in a water bath at $37^\circ C$ was carried out for 30 minutes with occasional agitation. At the completion of the incubation, ice cold Hanks' saline was added, the cells were centrifuged and resuspended in Hanks' saline, counted and injected.

When an excess of sialic acid was required in the incubation medium, sialic acid was made up in Hanks' saline, containing phenol red as an indicator, and adjusted with $1.4\%$ NaHCO$_3$ to a pH of 7.2. The final concentration was 20 mg/ml. 0.1 ml (2 mg) of this solution was added per 25 units of enzyme when an excess of sialic acid was required.

10. ASSAY OF SIALIC ACID RELEASE

Thoracic duct lymphocytes or thymus cells were suspended in phosphate buffered saline pH 7.4 and counted. $2.5 \times 10^8$ thoracic duct lymphocytes or $5 \times 10^8$ thymocytes were centrifuged, the supernatant removed and the cells resuspended in 0.45 ml of Dulbecco's phosphate buffered saline pH 7.4. 25 units (0.05 ml) of neuraminidase were added and the cells incubated at $37^\circ C$ for 30 minutes. After centrifugation, a 0.2 ml sample of the supernatant was assayed for sialic acid by the thiobarbituric acid method of Warren (1959). The amount of sialic acid released from the lymphocytes was calculated by multiplying the amount present in the 0.2 ml sample by 2.5 to obtain the amount present in the total volume.

Optical densities were measured at both 532 and 549 nm to correct for absorbancy at 549 nm not due to sialic acid. N-acetyl neuraminic acid was used as a standard.

When normal lymphocytes were incubated for 30 minutes in buffer without any enzyme, a certain amount of material was released which gave a colour reaction.
with the Warren assay. This is probably 2 deoxyribose which forms a chromophore with an absorption spectrum similar to N-acetyl neuraminic acid (Warren 1959). Allowance is made for this by taking readings at 532 nm and 549 nm. However 5 samples of 5 x 10^8 thymus cells incubated in buffer alone gave a calculated mean release of 0.002 µm of sialic acid which was probably due to the interference by the chromophore. In an effort to increase the sensitivity of the Warren assay, the volumes of reagents used were decreased as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Original Volume</th>
<th>Decreased Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodate</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Arsenite</td>
<td>1 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Thiobarbituric acid</td>
<td>2.5 ml</td>
<td>1.5 ml</td>
</tr>
</tbody>
</table>

The chromophore was extracted with 1.5 ml of cyclohexanone instead of 4.3 ml as in the original assay, and read at the same optical densities. Standard curves for the Warren method and micro method are shown in Figure 2.1.

11. LABELLING OF LYMPHOCYTES WITH ^51 Cr AND INJECTION OF THE CELLS

Radioactive chromium (\(^51\)Cr) was obtained from "The Radiochemical Centre", Amersham, England as a sterile solution of sodium chromate in isotonic saline. The solutions contained 14-22 µg sodium chromate per ml and the activity varied from 2-5 millicuries per ml.

A suspension of lymphocytes (2 x 10^8/ml) was made in Dulbeco's phosphate buffered saline containing 5% foetal calf serum. 50 µcuries of \(^51\)Cr were added per 2 x 10^8 cells. The cells were incubated for 1 hour with occasional agitation in an atmosphere of 5% CO₂ and air at 37°C. After incubation the cells were isolated by centrifugation, and then washed once in phosphate buffered saline containing 5% rat serum. The cell pellet was then resuspended in phosphate buffered saline and equally divided. The suspensions were centrifuged, one cell pellet was treated with 25 units of neuraminidase in 0.45 ml of phosphate buffered
FIGURE 2.1

STANDARD CURVES RELATING SIALIC ACID CONCENTRATION TO OPTICAL DENSITY

Warren method

'Micro' modification of the Warren method
saline per $2.5 \times 10^6$ cells, the other with $0.5$ ml of phosphate buffered saline alone. After incubation in a water bath at $37^\circ C$ for 15 minutes, the cell suspensions were washed twice in medium 199 containing 5% normal rat serum and resuspended in the same medium for injection, at a concentration of $50 - 100 \times 10^6$ cells/ml. 0.1 ml portions of the final suspension were taken for assay of radioactivity, cell concentration and viability.

12. **ASSAY FOR RADIOACTIVITY**

Samples were counted for 100 seconds in a Packard model 300 L tri-carb scintillation spectrometer.

**In organs**

Animals were killed by ether anaesthesia. The superficial and deep cervical nodes, thymic, thoracic and mesenteric nodes were dissected and pooled. Other organs were collected as stated. The liver was cut into pieces of sufficient size to be placed in the counting tube. Correction was made for loss of counting efficiency due to the height of the sample in the counting tube in the case of the liver and small bowel.

**In thoracic duct lymph**

The lymphocytes were separated from the lymph by centrifugation, and resuspended in 1 ml of phosphate buffered saline and the whole sample assayed for radioactivity. Where this sample was reinjected, the cell suspension was drawn up into a tuberculin syringe and injected intravenously via the tail vein. After injection the syringe was rinsed with phosphate buffered saline into the original container which was then re-assayed for residual radioactivity. This figure was then subtracted from the original count to determine the amount of radioactivity injected. A similar procedure was followed after labelling lymphocytes which had been maintained in *vitro* on rat embryo monolayers.
13. **INCUBATION OF \(^{51}\)Cr LABELLED CELLS WITH ISOANTISERA**

Rat isoantisera were prepared by injecting members of one strain of rat with lymphoid cells from another strain. Each prospective antiserum donor received an initial intraperitoneal injection of approximately \(1.5 \times 10^8\) spleen cells suspended in complete Freund's adjuvant. Two subsequent intraperitoneal injections of \(3-4 \times 10^8\) spleen cells were given at 10 day intervals. At the time of the second intraperitoneal spleen cell injection, \(10^8\) thoracic duct lymphocytes were given intravenously followed by a further intravenous dose of \(10^8\) thoracic duct lymphocytes 10 days later. This was followed by a further \(3-6 \times 10^8\) spleen cells injected intraperitoneally after a further 10 days. Antiserum was prepared from blood taken 10 days after the last injection.

To \(0.2\) ml of the cell suspension of thoracic duct lymphocytes, at an approximate concentration of \(50 \times 10^6\) cells/ml in medium 199, was added \(0.2\) ml of freshly reconstituted lyophilized guinea pig serum (Commonwealth Serum Laboratories, Melbourne) as a source of complement and \(0.2\) ml of the undiluted specific isoantiserum or \(0.2\) ml of normal rat serum as a control. After incubation at \(37°C\) for 30 minutes, \(1.5\) ml of phosphate buffered saline was added and the cell suspension centrifuged. \(1.5\) ml of the supernatant was removed and assayed for radioactivity. The total radioactivity released was calculated from the knowledge of the radioactivity present in the original cell sample and the final volume of cell suspension.

14. **PREPARATION AND MAINTENANCE OF LYMPHOCYTES ON RAT EMBRYO MONOLAYERS**

**Preparation of monolayer**

Rat embryo monolayers were prepared by a modification of the method of Ginsburg and Sachs (1965).

Embryos were removed under sterile conditions from the uterus, at the 17 day stage in gestation. Usually 6 - 8 embryos were obtained from 1 rat. After removal
of the abdominal and thoracic viscera, the embryos were minced, using fine scissors, in a petri dish in Hanks' saline adjusted to pH 7.4 with 1.4% NaHCO$_3$ and containing 20 units of heparin per ml. The minced suspension was washed twice in Hanks' saline plus heparin, then 5-10 ml of 0.5% trypsin in Puck's saline at pH 7.4 was added per embryo. This suspension was incubated at $37^\circ$C for 25 minutes with continual stirring. The suspension was then filtered through 12 layers of sterile gauze, centrifuged and resuspended in medium 199 containing 10% foetal calf serum, and the cell concentration was determined. The appropriate cell number (usually $10^6$ cells) in 4 ml of medium 199 plus 10% foetal calf serum was transferred to 30 ml tissue culture flasks (Falcon Plastics California, U.S.A.). The monolayers were maintained at $37^\circ$C.

It was found that $10^6$ embryo cells took about 7 days to establish a suitable monolayer. The growth medium (medium 199 containing 10% foetal calf serum) was replaced every 4 to 5 days. Further monolayer cultures could be made by passaging primary cultures. 2-4 ml of 0.5% trypsin in Puck's saline were added to each monolayer after removal of the growth medium, and incubated for 10 minutes at $37^\circ$C, by which time monolayer cells were detached from the flask. The cells were then washed once in cold growth medium counted and plated to new flasks.

**Preparation of Thoracic Duct Lymphocytes**

Thoracic duct lymphocytes were prepared for plating onto monolayers by removal from the lymph by centrifugation, a single washing in phosphate buffered saline and resuspension in medium 199 containing 10% foetal calf serum and 200 µg of carbenecillin ("Pyopen" Beecham Laboratories) per ml. Carbenecillin was found to be necessary in order to prevent the growth of contaminating pseudomonas pyocynea organisms. 4 ml of the suspension of lymphocytes containing 50-60 x $10^6$ cells were plated onto each monolayer.
Labelling and injection of lymphocytes from monolayers

After agitation, medium and lymphocytes were removed from the monolayers by aspiration. The lymphocyte suspension was centrifuged and resuspended so that the cells obtained from 2 monolayers were contained in 0.5 ml of incubation medium. 12.5 μ curie of $^{51}$Cr was added and the cells were incubated for one hour at 37°C in an atmosphere of 5% CO$_2$ and air. The cell suspension was then washed twice in phosphate buffered saline containing 5% rat serum and halved. One half was incubated in a volume of 0.5 ml of phosphate buffered saline containing 25 units of neuraminidase, while the other half was incubated in phosphate buffered saline alone, for 15 minutes at 37°C. The cells were then washed once in medium 199 and 5% rat serum. The whole suspension was assayed for radioactivity prior to injection in a final volume of 1 ml. Lymphocytes which had been labelled with $^{51}$Cr prior to plating on the monolayer were prepared for injection as above with omission of the labelling procedure.

Lymphocytes for injection without prior labelling with $^{51}$Cr were removed from the monolayer, washed once with phosphate buffered saline, resuspended in Hanks' saline, counted and injected.

15. CELL COUNTS AND HAEMOGLOBIN ESTIMATIONS

Cell counts were performed using a Coulter counter model B (Coulter Electronics, Hialeah, Florida). Where suspensions were contaminated with red blood cells, these were lysed by the addition of 1% saponin, 30 seconds prior to counting, to a final concentration of 0.01% saponin. Haemoglobin estimations were done by means of a grey wedge M.R.C. Photometer (Keeler, London).

16. LYMPHOCYTE CYTOTOXICITY AND AGGLUTINATION TESTING

Antisera were prepared as described previously (Section 12). Cytotoxicity tests were performed using trypan blue uptake by a modification of the method of Gorer and O'Gorman (1956). 0.05 ml of freshly reconstituted lyophilized guinea pig serum (C.S.L.
Melbourne) as a source of complement, 0.05 ml of the isoantisera and 0.1 ml of cell suspension at $10^8$ cells/ml in Hanks' saline were incubated in perspex titration plates at $37^\circ$C for 30 minutes. After incubation 0.1 ml of the cell suspension was added to 0.4 ml of 0.2% trypan blue. The percentage of cells taking up trypan blue was calculated from a count of 200 cells made in a haemocytometer 5-10 minutes after the addition of trypan blue.

For absorption of guinea pig serum freshly reconstituted lyophilized guinea pig serum (C.S.L., Melbourne) was diluted 1:2 with Ca-Mg saline. 1.5 ml of the diluted serum was absorbed with $4 \times 10^8$ untreated or neuraminidase treated DA thymus cells, at $4^\circ$C for 60 minutes. The cells were removed by centrifugation. Cytotoxicity tests were performed as above using 0.1 ml of cell suspension in Hanks' saline and 0.05 ml of the 1:2 dilution of guinea pig serum.

Complement fixation tests were performed in round bottom microtitre perspex titration plates. Dilutions of complement from 1:10 to 1:100 were made in Ca-Mg saline. Unit volumes at these dilutions were incubated with 2 volumes of Ca-Mg saline and one volume of 8% sheep red cells sensitized with 8 HD$_{50}$ of rabbit anti-sheep haemolysin. After settling of the red cells, the dilution of complement giving 50% haemolysis was taken as the end point.

Lymphocyte agglutination titrations were performed by preparing serial two-fold dilutions of antisera with Ca-Mg saline in perspex titration plates, the first dilution being 1:1. Similar dilutions of normal rat serum were prepared as a control. To 0.05 ml of the antiserum was added 0.1 ml of lymphocyte cell suspension at $10^8$ cells/ml in Hanks' saline. After incubation at $37^\circ$C for 2 hours, a drop of cell suspension from each well was placed on a clean glass slide, which was examined 5-10 minutes later.

The slides were examined under a microscope at 45X magnification and the degree of agglutination recorded.
as o, + or ++. The agglutination was assessed with an eyepiece graticule which divided the field into 36 squares, on an arbitrary basis as follows:

++ obvious clumping of cells with many large clumps of 10 cells or more.

+ more than 5 small clumps of 4-10 cells per square in field.

o less than 5 small clumps per square.

17. VIABILITY TESTS

Viability of cell suspensions was assessed by means of trypan blue uptake. 0.1 ml of cell suspension was added to 0.4 ml of a solution of 0.2% trypan blue in isotonic saline. The number of cells taking up trypan blue were counted in a haemocytometer 5-10 minutes after exposure of the cells to trypan blue.

18. HISTOLOGY AND CYTOLOGY

Smears of lymphocyte suspensions were prepared in a cytocentrifuge. 0.1 ml of cell suspension containing approximately $10^5$ cells suspended in foetal calf serum was spun onto a glass slide using a cytocentrifuge for 15 minutes at 180 g until dry. Blood films were prepared by spreading a drop of blood from the tail vein and drying in air. Smears were stained using McNeal's tetrachrome stain.

Tissues were fixed in formal saline or Carnoy fluid, and stained with haematoxylin and eosin, methyl green pyronin or Masson's trichrome stains.

19. INCUBATION OF TISSUE SECTIONS WITH NEURAMINIDASE

Paraffin was removed from tissue sections by passage through acetone and alcohol to water. 0.1 to 0.2 ml of active or heat-inactivated (by maintenance at $100^\circ C$ for 30 minutes) neuraminidase solution, at a concentration of 50 units per ml in phosphate buffered saline at either pH 5.6 or 7.4, was pipetted onto the sections. The slides, covered with enzyme solution, were placed in a petri dish on glass rods and incubated.
in a moist atmosphere at 37°C for periods of 2-16 hours. After incubation the slides were washed in distilled water prior to staining with Mowry's (1958) modification of Hale's colloidal iron stain for acidic mucosubstances. As additional controls, completely untreated sections were included, and in one section the colloidal iron step was omitted in order to identify ferric iron normally present in the tissues.

The sections were counterstained by Scarba red. Photography of these specimens was performed using Kodak pantomatic X film and an Ilford 202 orange filter.
CHAPTER 3

SIALIC ACID RELEASE FROM THE SURFACE OF RAT LYMPHOCYTES

The release of sialic acid from the cell surface has been measured in a wide variety of tumor and tissue culture cell lines, [Heilborn and Yllar 1961, Kramer 1965a] erythrocytes from a number of different species, [Yllar et al. 1963], and human plasma [Badoff et al. 1964] using the Dithioniterbituric assay of Warner (1955). Gock and Jacobson (1969) were able to measure the release of sialic acid by neuraminidase from 10^8 lymph node cells from leukemic mice using the Warner assay. However, the release of sialic acid from an equivalent number of lymph node cells from normal animals could not be detected by the use of this assay. Woodruff and Griffin (1969) were able to measure the amount of sialic acid released from 5 x 10^5 rat thymus T-1 lymphocytes.

Neuraminidase treatment has been shown to alter the expression of surface antigens on a range of cells, including T-2 lymphocytes, [Schlesinger and Amos 1971], A and B antigens on human peripheral blood lymphocytes, [Grotthus et al. 1971], A and B antigens on human leukemic lymphocytes [Keevins et al. 1971], an antigen on human lymphocytes detected by normal rabbit serum [Grotthus et al. 1971] and an antigen on mouse thymus cells detectable by a serum antibody in guinea pig serum [Schlesinger and Amos 1971].

The following experiments were carried out in order to establish suitable conditions for incubation of rat lymphocytes with neuraminidase, to quantitate the sialic acid released during incubation and to look for any change in surface antigenic expression.
The release of sialic acid from the cell surface has been measured in a wide variety of tumor and tissue culture cell lines, (Wallach and Eylar 1961, Kraemar 1966a) erythrocytes from a number of different species, (Eylar et al.1962) and human platelets (Madoff et al. 1964) using the thiobarbituric assay of Warren (1959). Cook and Jacobsen (1968) were able to measure the release of sialic acid by neuraminidase from $10^8$ lymph node cells from leukaemic mice using the Warren assay. However the release of sialic acid from an equivalent number of lymph node cells from normal animals could not be detected by the use of this assay. Woodruff and Gesner (1969) were able to measure the amount of sialic acid released from $5 \times 10^8$ rat thoracic duct lymphocytes.

Neuraminidase treatment has been shown to alter the expression of surface antigens on a range of cells including the theta antigen on mouse thymus cells, (Schlesinger and Amos 1971), HLA antigens on human peripheral blood lymphocytes, (Grothaus et al. 1971), A and H antigens on human leukaemic lymphocytes (Kassulke et al. 1971), an antigen on human lymphocytes detected by normal rabbit serum (Grothaus et al. 1971) and an antigen on mouse thymus cells detectable by a xenoantibody in guinea pig serum (Schlesinger and Amos 1971).

The following experiments were carried out in order to establish suitable conditions for incubation of rat lymphocytes with neuraminidase, to quantitate the sialic acid released during incubation and to look for any changes in surface antigenic expression.
RESULTS

1 VARIABILITY OF ASSAY

Sialic acid released from lymphocytes was assayed by the method of Warren (1959). To establish the variation expected in an assay on a given sample of cells, 11 samples, each consisting of $5 \times 10^8$ thymus cells were incubated in phosphate buffered saline containing 25 units of neuraminidase for 30 minutes at pH 7.4 and the amount of sialic acid (NANA) released was assayed. The results ranged from 0.015 to 0.017 µmole of NANA released and gave a mean of $0.016 \pm 0.0002$ (S. error).

2 OPTIMUM CONDITIONS FOR INCUBATION

Neuraminidase derived from vibrio cholera has a pH optimum of 5.6 (Ada et al. 1961). In order to determine suitable conditions for incubation of lymphocytes, the effect of pH, duration of incubation, and amount of enzyme on the release of sialic acid was examined. $5 \times 10^8$ thymus cells in phosphate buffered saline at pH of either 5.6 or 7.4 were incubated with 25 or 50 units of neuraminidase for either 30 or 60 minutes at $37^\circ C$. The amount of sialic acid released was determined using the Warren method and the results are shown in table 3.1. The values are all equivalent, any differences being within the limits of experimental variation. It can be seen that similar amounts of sialic acid were released at pH 5.6 and 7.4 and that increasing the time of incubation from 30 to 60 minutes did not result in the release of more sialic acid. Fifty units of neuraminidase released the same amount of sialic acid as 25 units. From these results a standard incubation procedure was adopted in the subsequent experiments to be described in this thesis. $5 \times 10^8$ thymus cells or $2.5 \times 10^8$ thoracic duct lymphocytes were incubated with 25 units of neuraminidase at pH 7.4 for 30 minutes at $37^\circ C$. 

### Table 3.1

**Release of Sialic Acid from Thymus Cells**

<table>
<thead>
<tr>
<th>pH</th>
<th>Time of incubation (minutes)</th>
<th>Units of neuraminidase</th>
<th>NANA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6</td>
<td>30</td>
<td>50</td>
<td>0.018</td>
</tr>
<tr>
<td>7.4</td>
<td>30</td>
<td>50</td>
<td>0.017</td>
</tr>
<tr>
<td>5.6</td>
<td>30</td>
<td>25</td>
<td>0.016</td>
</tr>
<tr>
<td>7.4</td>
<td>30</td>
<td>25</td>
<td>0.016</td>
</tr>
<tr>
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<td>60</td>
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</tr>
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<td>60</td>
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<td>0.016</td>
</tr>
<tr>
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<td>0.017</td>
</tr>
<tr>
<td>7.4</td>
<td>60</td>
<td>25</td>
<td>0.017</td>
</tr>
</tbody>
</table>

5 x 10^8 thymus cells were incubated with neuraminidase at 37°C under the conditions shown. Free sialic acid released into the medium was assayed by the method of Warren.
3 THE RATE OF RELEASE OF SIALIC ACID

The rate of release of sialic acid from thymus cells is shown in figure 3.1. It can be seen that the release of sialic acid from the cells was complete within 15 minutes and that most of the sialic acid had been released within the first five minutes of incubation.

4 RELEASE OF SIALIC ACID FROM LYMPHOID CELLS

The amount of sialic acid released by neuraminidase from 2 different types of lymphoid cells, namely thymocytes from 5-9 week old rats and thoracic duct lymphocytes from 10-14 week old rats is shown in table 3.2.

**TABLE 3.2**

THE RELEASE OF SIALIC ACID FROM LYMPHOID CELLS

<table>
<thead>
<tr>
<th>Lymphoid Cells</th>
<th>Strain of rat</th>
<th>Number of experiments</th>
<th>NANA (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>Outbred</td>
<td>8</td>
<td>0.008±0.0002</td>
</tr>
<tr>
<td>Thymus</td>
<td>DA</td>
<td>3</td>
<td>0.007±0.0005</td>
</tr>
<tr>
<td>TDL</td>
<td>Outbred</td>
<td>4</td>
<td>0.016±0.0007</td>
</tr>
<tr>
<td>TDL</td>
<td>DA</td>
<td>7</td>
<td>0.014±0.0009</td>
</tr>
</tbody>
</table>

*Sialic acid release expressed as micro mole per 2.5 x 10^8 cells. (mean ± standard error)

2.5 x 10^8 thoracic duct lymphocytes or 5 x 10^8 thymocytes were treated with 25 units of neuraminidase. The sialic acid (NANA) released into the supernatant was assayed by the Warren method.

The amount of sialic acid released from rat thoracic duct lymphocytes by neuraminidase is similar to the figure of 0.034±0.003 µ moles/500 x 10^6 cells obtained by Woodruff and Gesner (1969). This investigation shows that thymocytes only have half as much surface sialic acid that can be released by neuraminidase as do thoracic duct lymphocytes.
FIGURE 3.1

SI ALIC ACID RELEASE FROM THYMUS CELLS

The sialic acid released from $5 \times 10^8$ thymus cells, incubated with 25 units of neuraminidase in phosphate buffered saline at 37°C, was determined by the Warren assay at the times shown.
5 REPEAT INCUBATION OF CELLS WITH NEURAMINIDASE

In order to examine the possibility of some early reacquisition of surface sialyl groups, an experiment was performed in which neuraminidase treated thymus cells or thoracic duct lymphocytes were incubated in medium 199 containing 50% normal rat serum for 1 hour. These cells were then retreated with a further 25 units of neuraminidase. As controls the sialic acid release from normal lymphocytes before and after maintenance in vitro was included. The results are shown in table 3.3.

**TABLE 3.3**

**SIALIC ACID RELEASE FROM LYMPHOCYTES MAINTAINED FOR 1 HOUR IN VITRO**

<table>
<thead>
<tr>
<th>Lymphocytes†</th>
<th>NANA (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unincubated TDL</td>
<td>0.011</td>
</tr>
<tr>
<td>Untreated TDL incubated for 1 hour</td>
<td>0.010</td>
</tr>
<tr>
<td>Neuraminidase treated TDL incubated for 1 hour</td>
<td>0.001</td>
</tr>
<tr>
<td>Unincubated thymocytes</td>
<td>0.015</td>
</tr>
<tr>
<td>Untreated thymocytes incubated for 1 hour</td>
<td>0.014</td>
</tr>
<tr>
<td>Neuraminidase treated thymocytes incubated for 1 hour</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*Sialic acid release is expressed as micro mole.

+2.5 × 10⁸ thoracic duct lymphocytes or 5 × 10⁸ thymocytes were treated with neuraminidase and the release of sialic acid was determined (unincubated lymphocytes). These enzyme treated lymphocytes and previously untreated lymphocytes were maintained for 1 hour in medium 199 + 50% rat serum and then treated with neuraminidase.

There was no further significant release of sialic acid from the neuraminidase treated lymphocytes after maintenance in vitro. The small amount apparently released on retreatment of the cells probably represents a contamination by deoxyribose chromophores, as it is of a similar order to the apparent release of sialic acid when normal cells were incubated without neuraminidase.
To examine this point further, thoracic duct lymphocytes were treated with neuraminidase then retreated with the enzyme either immediately or after maintenance for 1 hour in medium 199 containing 50% rat serum. In each case the sialic acid released was assayed using the micro modification of the Warren method (methods). The results are shown in table 3.4.

**TABLE 3.4**

RETREATMENT OF THORACIC DUCT LYMPHOCYTES IMMEDIATELY OR AFTER 1 HOUR MAINTENANCE IN VITRO

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>NANA (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>First treatment</td>
<td>0.012</td>
</tr>
<tr>
<td>Retreated immediately</td>
<td>0.001</td>
</tr>
<tr>
<td>Retreated after 1 hour</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Sialic acid release is expressed as micro mole per 2.5 x 10⁸ cells.

Further sialic acid was not released after maintenance for 1 hour in vitro then was released after immediate retreatment. As discussed above the apparent release of 0.001 µM of sialic acid on retreatment is probably due to contamination by deoxyribose chromophores.

These experiments do not provide any evidence for the reacquisition of cell surface sialic acid within an hour after removal by neuraminidase. This indicates that at the time when neuraminidase treated lymphocytes are utilized in experiments to be described in the following chapters, there has been no regeneration of their surface sialyl groups.
EFFECT OF GUINEA PIG SERUM ON NEURAMINIDASE TREATED LYMPHOCYTES

Fresh guinea pig serum has been shown to have a cytotoxic effect on thymocytes from mice (Reif 1963, Schlesinger 1965) and rats (Wakefield and Batchelor 1966). This activity is thought to be due to a naturally occurring antibody (Schlesinger 1965). Neuraminidase treatment of mouse thymocytes produced a four fold increase in this cytotoxic effect of guinea pig serum (Schlesinger and Amos 1971).

A comparison was made of the cytotoxic effect of guinea pig serum on normal and neuraminidase treated DA rat lymphocytes. The lymphocytes were incubated with a 1:2 dilution of guinea pig serum in calcium-magnesium saline then their viability was determined by trypan blue uptake (methods). Table 3.5 shows that there was no detectable cytotoxic activity of the guinea pig serum against control or neuraminidase treated thoracic duct lymphocytes.

### TABLE 3.5

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Trypan blue uptake%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline alone</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>3</td>
</tr>
</tbody>
</table>

*Mean of two separate experiments*

In contrast, the guinea pig serum at the same dilution did not show cytotoxic activity against normal DA thymus cells but was markedly cytotoxic for neuraminidase treated thymocytes. This cytotoxic activity could be reduced by prior adsorption of the serum with normal thymus cells and completely eliminated by prior adsorption with neuraminidase treated thymus cells (Fig. 3.2). The activity was also removed by
FIGURE 3.2

THE EFFECT OF GUINEA PIG SERUM ON NEURAMINIDASE TREATED THYMOCYTES

Control untreated or neuraminidase treated DA thymus cells were incubated with normal guinea pig serum (compt.) or guinea pig serum which had been absorbed with either normal (abs. control cells) or neuraminidase treated (abs. ndase cells) thymus cells. Thymus cells were also incubated in saline alone (saline).

The viability of the cells after incubation was assessed by trypan blue uptake, and is expressed as a mean and range of 2 separate experiments.
heating the guinea pig serum at 56°C for 30 minutes. Complement fixation tests showed that complementary activity was still present in the guinea pig serum after adsorption. The complement fixation titre of unabsorbed guinea pig serum and of that adsorbed with normal thymus cells was 40, whilst it was 30 for the serum absorbed with neuraminidase treated thymus cells. It appears that the cytotoxic activity of guinea pig serum against neuraminidase treated thymus cells is due to a naturally occurring xenoantibody.

7 THE EFFECT OF STRAIN SPECIFIC ISOANTISERA ON NEURAMINIDASE TREATED LYMPHOCYTES

In experiments to be described in the following chapters neuraminidase treated, DA strain thoracic duct lymphocytes were used to initiate graft-versus-host reactions, in studies on cell migration and in the restoration of the immune response to irradiated animals. In such experiments it is of importance to know whether neuraminidase treatment has altered the presence or expression of the DA histocompatibility antigens on the cell surface. To examine this possibility, the expression of DA histocompatibility antigens on normal and neuraminidase treated thoracic duct lymphocytes was studied.

Control or neuraminidase treated thoracic duct lymphocytes were incubated with undiluted normal Lewis or Lewis anti-DA serum and normal guinea pig serum as a source of complement (methods). After incubation the viability of the cells was determined by trypan blue uptake (Table 3.6.)
TABLE 3.6
THE EFFECT OF LEWIS ANTI-DA SERUM ON CONTROL AND NEURAMINIDASE TREATED DA THORACIC DUCT LYMPHOCYTES

<table>
<thead>
<tr>
<th>DA Lymphocytes</th>
<th>Trypan blue uptake %&lt;sup&gt;o&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Lewis serum&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>8</td>
</tr>
</tbody>
</table>

Mean of two separate experiments

Treatment of the thoracic duct lymphocytes with neuraminidase has not altered the cytotoxic effect of strain specific isoantisera on the cells.

It has been reported that neuraminidase treatment may increase the susceptibility of lymphocytes to the lytic effects of complement (Ray et al. 1971). Consequently to eliminate any confusion arising from the possibility that the process of complement fixation has been altered on neuraminidase treated lymphocytes agglutination tests, which are independent of complement fixation, were performed (methods). As neuraminidase treated lymphocytes may tend to agglutinate spontaneously, (Woodruff and Gesner 1969) parallel control experiments were performed using normal sera. The agglutination patterns obtained with normal Lewis or Lewis anti-DA serum and DA thoracic duct lymphocytes are shown in figure 3.3. The control and neuraminidase treated lymphocytes are agglutinated by the specific antiserum to a similar titre.

DISCUSSION

These experiments have established conditions to be used for incubation of lymphocytes with neuraminidase. Neuraminidase treatment has revealed basic surface differences between populations of lymphoid cells derived from the thymus and the thoracic duct lymph as regards the total amount of sialic acid releasable and the cytotoxic effect of normal guinea pig serum.
FIGURE 3.3

THE AGGLUTINATION OF NORMAL AND NEURAMINIDASE TREATED THORACIC DUCT LYMPHOCYTES

Control untreated or neuraminidase treated DA thoracic duct lymphocytes were incubated with serial two fold dilutions of either Lewis anti-DA serum or normal Lewis serum as a control. After incubation for 2 hours at 37°C the degree of agglutination was recorded.
The pH optimum of neuraminidase derived from vibrio cholera is 5.6 (Ada et al. 1961). In the present experiments, release of sialic acid from lymphocyte surfaces was as efficient at pH 7.4 as at pH 5.6. Neuraminidase also shows optimal activity in the presence of 0.001 M CaCl₂ (Ada et al. 1961). No CaCl₂ was added to the buffer in these experiments, as the preparation of neuraminidase used contained 1 mg of CaCl₂ per ml. Under the conditions of incubation used in these experiments, 0.05 ml of enzyme (25 units) solution was added to 0.45 ml of cell suspension. This gave a final concentration of CaCl₂ of 0.001 M. There was a very rapid release of sialic acid from lymphoid cells, the majority being released after 5 minutes incubation. Release of sialic acid by neuraminidase from the surface of tumor cells (Cormack 1970, Walborg et al. 1969) and from a hamster cell line (Kraemer 1966a) was shown to be complete by one hour. The rapid release of sialic acid obtained in the present experiments suggests that the sialic acid is removed from the surface rather than from the interior of the cell.

Approximately twice as much sialic acid is released by neuraminidase treatment of rat thoracic duct lymphocytes as compared with rat thymocytes. Other differences in the surface properties of thymus cells and peripheral lymphocytes have been described, such as the presence of the TL antigen and a greater quantity of the θ antigen on thymocytes in mice (Reif and Allen 1966, Aoki et al. 1969, Raff 1970) and the RTA antigen on thymocytes in rats (Colley et al. 1970). Demonstration of a difference in chemical composition of the surface region of the thymus cells adds another parameter to those differences previously demonstrated between thymus cells and peripheral lymphocytes.

A comparison of the effect of guinea pig serum on neuraminidase treated thymocytes or thoracic duct lymphocytes revealed another difference between these two cell populations. The cytotoxicity appears to be due to a naturally occurring xenoantibody similar to
that previously described by other workers (Reif 1963, Schlesinger 1965, Wakefield and Batchelor 1966). Whilst a detailed examination of the titres of this antibody was not carried out, the cytotoxic activity detected against neuraminidase treated thymus cells but not against neuraminidase treated thoracic duct lymphocytes indicates further differences in the cell surface of these two types of lymphocyte after neuraminidase treatment.

Cytotoxicity and agglutination studies of DA thoracic duct cells showed that neuraminidase treatment had not altered the ability of anti-DA serum to recognize DA cells. This finding indicates that sialic acid is not part of the antigenic site of the DA histocompatibility antigen.
CHAPTER 4

THE OCCURRENCE OF SIALYL-CONTAINING MUCOSUBSTANCES IN LYMPHOID TISSUE
THE OCCURRENCE OF SIALYL-CONTAINING MUCOSUBSTANCES IN LYMPHOID TISSUE

Cell surface glycoproteins, in particular sialoglycoproteins have been implicated as being of importance in many functions appropriate to the immune system such as phagocytosis (Weiss et al. 1966, Bona et al. 1968), adhesion of foreign particles to the cell surface (Allen and Cook 1970), complement fixation (Arquilla et al. 1964, Ray et al. 1971), and lymphocyte migration (Gesner and Ginsberg 1964, Woodruff and Gesner 1969). There are also suggestions from the literature that material of a carbohydrate nature is associated with the postcapillary venules (Smith and Henon 1959, Kruger 1968) which are one of the main sites of lymphocyte migration from the blood stream.

Most of the activities of the cells involved in immune responses entail contact between cells, as well as contact of cells with foreign material. It is likely that glycoproteins situated both at the surface of these cells and in their immediate environment play an important role in the cell contact phenomena occurring in an immune response.

In order to study the existence of such material in lymphoid tissue, a survey was made of spleen, lymph nodes, thymus and Peyer's patches using Mowry's modification (1958) of Hale's (1946) colloidal iron stain to define acid mucosubstances. This stain acts by the binding of the colloidal iron at low pH to the strongly acidic carboxyl and sulphate groups of the glycoproteins. This iron is then converted to ferric ferrocyanide (Prussian blue) which is visible as a bright blue material. The photographs to be presented were taken through an orange filter which renders the blue ferric ferrocyanide black in a monochrome print.

In the results described below particular attention is paid to the postcapillary venules and the lymph node medulla where there were findings of interest.
RESULTS

1 DEMONSTRATION OF SIALIC ACID

There is no histochemical stain specific for sialic acid alone and thus its identification requires comparison of staining properties of a tissue before and after treatment with neuraminidase.

In order to define suitable conditions for treatment, sections of various tissues were incubated with a solution containing 50 units of neuraminidase in phosphate buffered saline, at 37°C, at a pH of either 5.6 or 7.4. Incubations were carried out for 1, 2, 4, 6, 8, 12 and 16 hours. 3 controls were performed: (1) incubation with heat-inactivated neuraminidase solution, (ii) staining of an unincubated specimen, (iii) omission of the ferric iron step in the staining reaction in order to distinguish true acid mucosubstance from ferric iron already present in the tissue.

As sialic acid is known to be a component of the rat glomerulus (Mohos and Skoza 1969) this structure was used to demonstrate the activity of the neuraminidase. When tissue sections were incubated with heat-inactivated enzyme solution the intensity of the Hale stain was diminished in comparison with completely untreated sections. Some reduction in intensity of staining of mucosubstances in the kidney after incubation with heat-inactivated enzyme for 2 hours at pH 7.4 can be seen, (Fig. 4.1B) as compared to the untreated specimen (Fig. 4.1A). Treatment of the section with neuraminidase for 2 hours at pH 7.4 was sufficient to completely abolish the staining (Fig. 4.1D). This effect of the enzyme could be partially inhibited by an excess of free sialic acid in the medium (Fig. 4.1C). Thus, while sialyl groups can be demonstrated by comparison of sections incubated with active or heat-inactivated enzyme, the loss of staining intensity produced by incubation alone made photographic documentation of sialic acid removal more difficult.
FIGURE 4.1

The effect of neuraminidase on acid mucosubstance in the kidney.

A. Kidney cortex. The dark Hale positive material can be seen in the glomeruli. There is some lighter staining of the tubules.

B. Kidney cortex incubated with heat inactivated neuraminidase solution for 2 hours prior to staining. Some loss of intensity of the Hale stain has occurred, particularly in the lighter staining areas of the tubules.

C. Kidney cortex incubated with active neuraminidase solution (25 units), plus free sialic acid (2 mgm), for 2 hours prior to staining. Hale positive material is still present in the glomeruli.

D. Kidney cortex incubated with active neuraminidase solution (25 units) for 2 hours prior to staining. The Hale positive material has been completely removed from the glomeruli.

Hale stain Magnification x 130
The postcapillary venules of the Peyer's patches were found to be associated with a considerable amount of material staining with Hale's stain. Rats of 1 - 15 weeks of age were examined. At one week, there were few postcapillary venules identifiable and they were relatively small. Some vessels with a high endothelium associated with Hale positive material were seen at this time. By 6 weeks the vessels were well developed and staining showed Hale positive material concentrated around the base of the endothelial cells (Fig. 4.2A). Sometimes this material was split into laminae, and lymphocytes were situated between the laminae. Similar material was seen at the base of the endothelial cells of 10 week (Figs. 4.2B and C) and 15 week old rats. The staining of this material was reduced in intensity by incubation with heat-inactivated enzyme, and completely abolished by treatment with active enzyme. Similar material was identified surrounding the postcapillary venules of lymph nodes, though it frequently appeared to be less in amount than that seen in the Peyer's patches.

Postcapillary venules situated at the periphery of a Peyer's patch are sometimes asymmetrical, with a high endothelium on the lymphoid side of the venule and a low endothelium on the side of the venule away from the lymphocytes. It was noted that, in this situation, the mucosubstance was associated with the high endothelial cells (Fig. 4.2D).

Taken together with its occurrence on the lymphocyte surface, the accumulation of Hale positive material around the base of the endothelial cells would suggest that this material may be related to the lymphocyte migration which is known to occur at this site (Gowans and Knight 1964). It is possible that such material is deposited by lymphocytes on their migration through the postcapillary venules or that it may be produced by the endothelial cells in response to lymphocyte migration. In either case, it might be expected that there could be diminished
Acid mucosubstance around postcapillary venules

A. Postcapillary venules from a 6 week old rat

Hale positive material can be seen at the base of the endothelial cells of the postcapillary venules. In places the material appears to be split into laminae which surround the lymphocytes.

Magnification x 600

B. Postcapillary venules from a 10 week old rat

Hale positive material is concentrated at the base of the endothelial cells. In the top right hand corner some acid mucosubstance lining an intestinal crypt can be seen. This material was not removed by neuraminidase treatment.

Magnification x 330

C. Postcapillary venules from a 10 week old rat

Hale positive material is present at the base of the endothelial cells.

Magnification x 600

D. Postcapillary venule from a 10 week old rat

The left hand side of the vessel which is adjacent to the lymphoid tissue has a high endothelium, whilst the opposite side facing the intestinal mucosa has a lower endothelium. The Hale positive material is concentrated on the side of the vessel with a high endothelium, where lymphocytes can be seen passing through the vessel wall.

Magnification x 530

All sections were stained with Hale stain
amounts of this material associated with the postcapillary venules if the migration of lymphocytes through these vessels was diminished. In order to test this hypothesis, staining of Peyer's patches was performed after two procedures known to deplete the recirculating pool of lymphocytes, namely prolonged thoracic duct drainage and gamma irradiation. Figures 4.3A and B are low power photomicrographs of Peyer's patches from a normal rat (A) and from a rat lethally irradiated 7 days previously (B). The marked depletion of lymphocytes in the irradiated animal can be seen. Postcapillary venules become readily identifiable in the irradiated animal by contrast with the lymphocyte-free sub-endothelial region. Despite this depletion of lymphocytes, there is no diminution of Hale positive material around the base of the endothelial cells (Fig. 4.3B). This material can be easily seen even at low magnification. Figure 4.3C depicts postcapillary venules 5 days following lethal irradiation. Considerable amounts of Hale positive material were present at the base of the endothelial cells. Similarly this material was not diminished after 4 days thoracic duct drainage (Fig. 4.3D). In fact the accumulation of acid mucosubstance frequently appeared to be increased in irradiated rats or in those depleted of recirculating lymphocytes by thoracic duct fistulae.

3 ACID MUCOSUBSTANCE ASSOCIATED WITH OTHER SMALL VESSELS

The previous examination of postcapillary venules demonstrated material that was stained by Hale's stain around the base of the high endothelial cells of the postcapillary venules, and it was suggested that the occurrence of this material may be related to the migration of lymphocytes which occurs at this site. A variety of similarly sized small vessels of tissues in which a similar magnitude of lymphocyte migration does not occur were examined. The vessels depicted were in tissues which were fixed and stained at the same time as those used to demonstrate the normal postcapillary venules.
FIGURE 4.3

The presence of acid mucosubstance in tissues depleted of lymphocytes.

A. Peyer's patch from a normal adult rat

Postcapillary venules are present with some Hale positive material at the base of the endothelial cells, but are not obvious due to the large numbers of lymphoid cells present.

Magnification x 140

B. Peyer's patch from an adult rat 7 days after 910 rad gamma irradiation

Note the decreased cellularity particularly around the postcapillary venules which become prominent by contrast with the lymphocyte free subendothelial region. Hale positive material is present at the base of the endothelial cells. The Hale positive material which can be seen in the intestinal crypts and lining the serosal surface was not removed by neuraminidase treatment.

Magnification x 95

C. Postcapillary venules from adult rat 5 days after 910 rad gamma irradiation

Hale positive material is prominent at the base of the endothelial cells of the postcapillary venules.

Magnification x 600

D. Postcapillary venules from an adult rat after 4 days thoracic duct drainage.

The Hale positive material is prominent around the postcapillary venules.

Magnification x 600

All sections were stained with Hale stain
Small vessels were examined in the thymic cortex (Fig. 4.4A) and medulla (Fig. 4.4B), connective tissue, adipose tissue (Fig. 4.4C) and in skeletal and smooth muscle (Fig. 4.4D). Sometimes small amounts of acid mucosubstance could be identified on the surface of the endothelial cells of blood vessels in contact with the blood stream. Such material was never observed situated outside the vessel in a similar location to that noted around the postcapillary venules. The endothelial cells of these small vessels were frequently flattened with small compact nuclei. Some vessels within the thymus possessed larger endothelial cells with more prominent open nuclei, somewhat similar to the endothelial cells of the postcapillary venules. Although small amounts of acid mucosubstance could be identified within these endothelial cells none was present at the base of the cells as was observed around postcapillary venules.

4 ACID MUCOSUBSTANCE IN THE LYMPH NODE MEDULLA

The deep cervical and mesenteric lymph nodes from rats 1-15 weeks of age were examined. Hale positive material was found coating the meshwork formed by the reticuloendothelial cells of the lymph node medulla (Fig. 4.5A) and within the cytoplasm of the reticuloendothelial cells (Fig. 4.5B). This material could be removed completely from both sites by neuraminidase. Figure 4.5C is a high power view of this material coating the reticuloendothelial cells in the medulla of the mesenteric node, and figure 4.5D shows that the staining has been completely removed by neuraminidase treatment. A low power view of the cortex and medulla of a mesenteric node treated with heat-inactivated (Fig. 4.5E) or active (Fig. 4.5F) enzyme can be seen. The neuraminidase has removed the dark staining material from the medulla.
Acid mucosubstance associated with other small vessels

A. Thymic cortex of a 6 week old rat

The endothelial cells stain very faintly for Hale positive material. There is no concentration of material at the base of the endothelial cells as was seen with the postcapillary venules.

Magnification x 330

B. Thymic medulla of a 10 week old rat

The endothelial cells show a very small amount of Hale positive material associated with their cytoplasm. There is no material around the base of the endothelial cells.

Magnification x 530

C. Small venule in adipose tissue

There is a small amount of Hale positive material associated with the surface of the endothelial cells (arrow). The surface of the red cells within the vessels is faintly positive for Hale stain. Two mast cells within the tissue are strongly positive.

Magnification x 330

D. Diaphragm of a 12 week old rat

A small vessel (arrow) is situated among the muscle fibres. There is no Hale positive material around the endothelial cells. A small portion of a mast cell can be seen.

Magnification x 330

All sections were stained with Hale stain.
Acid mucosubstance in the lymph node medulla

A. The medulla of the deep cervical lymph node from an adult rat.
   Hale positive material can be seen forming a network lining the medullary sinuses.
   Magnification x 600

B. The medulla of the mesenteric node from an adult rat.
   Large amounts of Hale positive material are present within the reticuloendothelial cells.
   Magnification x 1320

C. The medulla of a mesenteric node after treatment with heat-inactivated neuraminidase.
   Hale positive material can be seen at the surface of the reticuloendothelial cells (arrow).
   Magnification x 1320

D. The medulla from the same node depicted in C after treatment with active neuraminidase.
   The Hale positive material has been completely removed.
   Magnification x 1320

E. A mesenteric lymph node treated with heat-inactivated neuraminidase.
   The medulla is situated between the two cortical areas (C). Dark staining Hale positive material is present within cells in the medulla.
   Magnification x 130

F. The same node depicted in E after treatment with active neuraminidase.
   The two cortical areas can be seen (C). The dark staining material has been removed from the medulla.
   Magnification x 130

All sections were stained with Hale stain
This investigation has shown the presence of sialic acid-containing mucosubstances associated with the high endothelial cells of the postcapillary venules of lymph nodes and Peyer's patches, and surrounding the reticuloendothelial cells of the medullary sinuses of lymph nodes. In contrast this material was not associated with blood vessels of a similar size in non-lymphoid tissues.

The reason for the decrease in intensity of staining after incubation with heat-inactivated enzyme is not known. Mowry's modification of Hale's stain is carried out at pH 1.05 and is said to be specific for the strongly acidic radicles namely sulphate, phosphate and carboxyl at this pH. The presence of the COOH group of sialic acid is inferred by the reduction in intensity of staining which occurs after neuraminidase treatment. Despite the non-specific loss of staining intensity, sialic acid can still be identified by comparing differences in staining after treatment with active and heat-inactivated enzyme. That some loss of staining occurs after incubation with heat-inactivated enzyme, stresses the necessity of including adequate controls in the histochemical identification of sialic acid.

The acid mucosubstance associated with the postcapillary venules was situated around the base of the high endothelial cells. Smith and Henon (1959) described a metachromatic staining reaction of the high endothelial cells of postcapillary venules with toluidine blue, which stains mast cells, cartilage and acid mucosubstances metachromatically. In their experiments, metachromatic staining occurred within the high endothelial cells themselves. Kruger (1968) mentioned that some high endothelial cells, and the basement membrane of postcapillary venules in mice stained with the Periodic acid Schiff reaction. No pictures were published of this material, and while it may be similar in nature, the acid mucosubstances described in the present experiments appear to be more substantial than would be expected if they were only situated in the basement membrane.
Electron microscopy of the endothelial cells of postcapillary venules shows an extensive golgi apparatus (Sugimura et al. 1964, Schoefl 1970), free ribosomes and abundant rough surfaced endoplasmic reticulum (Sugimura et al. 1964). The golgi is known to play an important role in the synthesis and excretion of the carbohydrate component of glycoproteins (Neutra and Leblond 1966). It would seem likely that the sialic acid-containing mucosubstance observed around the base of the high endothelial cells in these experiments has been produced by these cells. These are the only cells in the region with an extensive golgi, and the material itself is in close relation to the base of the cells. In certain areas where part of the postcapillary venule has a high, and another part a low endothelium, this mucosubstance is concentrated around the base of the high endothelial cells. Thoracic duct drainage and gamma irradiation, both procedures which deplete the traffic of lymphocytes recirculating through the postcapillary venules, did not decrease the amounts of Hale positive material observed. This does not support an alternative possibility that this material is transported to the base of the postcapillary venules by lymphocytes. It would also seem that the endothelial cells of the venules can accumulate such material in the absence of the stimulus of continuing lymphocyte migration, at least over the period of several days observed in the present experiments.

However, the occurrence of the material is related to lymphocyte migration. Acid mucosubstance was only found in association with the postcapillary venules. Other similarly sized vessels which do not support a large scale lymphocyte migration did not exhibit this material. It was noticed that some postcapillary venules situated at the periphery of Peyer's patches possessed a high endothelium on the side of the vessel in contact with the lymphoid tissue. In this case, the acid mucosubstance was concentrated on the high endothelial
side of the venule, which also appeared to have more lymphocytes migrating through it than the side with the low endothelium.

A possible function of the acid mucosubstance around the postcapillary venules is to prevent leakage of proteins and similar sized molecules from the bloodstream, while allowing lymphocytes to actively work their way through, by virtue of its gel like structure. Postcapillary venules are the site of migration of considerable numbers of lymphocytes from the bloodstream (Gowans and Knight 1964). It has been recently reported that the majority, if not all of these lymphocytes pass between the endothelial cells (Schoeffl 1971) contrary to the proposal of Marchesi and Gowans (1964), that the lymphocytes pass through the endothelial cells. Schoeffl (1970) has demonstrated the passage of the markers ferritin and carbon from the bloodstream, in association with lymphocytes passing between endothelial cells of the postcapillary venule. The basement membrane of the endothelial cells is very fragmented (Schoeffl 1971) probably due to the continuous passage of lymphocytes through it, and this would not itself appear to act as a barrier.

A second, but less likely possibility is that the lymphocytes pick up a glycoprotein coat as they migrate through this acid mucosubstance at the base of the postcapillary venules. There is much evidence that cells can passively acquire glycoproteins from their environment which then adhere to the cell surface as if they were part of it (Schlesinger 1970). It could be that the glycoprotein material that circulating lymphocytes have on their surface is picked up by passage through the postcapillary venules. Perhaps even the specificity or determinant for the cells to home to the postcapillary venule is given to the cells on migration through it, in the form of carbohydrate containing surface material. There are experimental findings against this idea. It will be shown that some lymphocytes after treatment with neuraminidase can
migrate into the thoracic duct lymph with their surface still modified (Chapter 5). If cells did obtain or renew their surface in passing through the postcapillary venules it would be expected that cells that appeared in the thoracic duct lymph would exhibit normal surface properties.

Sialyl-containing acid mucosubstances were identified coating the reticuloendothelial cells of the medullary sinuses of lymph nodes, and frequently appeared within the cells as well. The widespread intracellular and extracellular location of this material was observed only in the medulla, but not in the cortical regions of the lymph nodes. The great bulk of foreign material reaching the node is concentrated in the medulla. This has been well demonstrated by following the fate of radioactively labelled antigens (Ada et al. 1964, McDevitt et al. 1966). Surface glycoproteins of phagocytic cells have been implicated in the trapping and phagocytosis of foreign materials in in vitro studies of phagocytosis (Bona et al. 1968, Allen and Cook 1970). It is likely that the material demonstrated in the present experiments plays a similar role in vivo.
CHAPTER 5

THE MIGRATORY PROPERTIES OF THORACIC DUCT LYMPHOCYTES

It has been shown that treatment of thoracic duct lymphocytes with crude preparations of glycosidases or with the purified enzyme neuraminidase alters their migratory pattern on subsequent transplantation (Ginsbury and Ginsbury 1964, Woodcraft and Gasder 1969). This has led to the suggestion that sugars on the surface of the lymphocyte play an important role in determining the distribution of cells around the body. The aim of the present experiments was to examine the fate of cells transferred after neuraminidase treatment, with particular reference to any changes in their migratory properties.

RESULTS

1. LABELLING OF LYMPHOCYTES WITH \textsuperscript{51}Cr

Retention of label by lymphocytes in vitro

Neuraminidase treatment could alter the passage of certain cells through lymph nodes (Ginsbury et al., 1957) and consequently the cell membrane. If \textsuperscript{51}Cr labelling is to be used as a means of following the fate of neuraminidase treated lymphocytes, it is necessary to exclude the possibility that exposure of lymphocytes to neuraminidase could interfere with the retention of \textsuperscript{51}Cr by the cells. To test this point a single collection of \textsuperscript{51}Cr labelled thoracic duct lymphocytes was divided into two batches, one of which was treated with neuraminidase while the other was retained as a control. The release of \textsuperscript{51}Cr into the supernatant was measured at 90 minutes and at 4 hours. After 90 minutes incubation, 3.2\% of the radioactivities of control cells had been released into the supernatant compared with 1.2\% from the neuraminidase treated cells. By 4 hours, a further 3.7\% of the radioactivity of the control cells had been released, while 3.6\% had been released from neuraminidase treated cells. Thus, 93\% of the original label remained in association with both control and neuraminidase treated cells after 4 hours maintenance in vitro. \textsuperscript{51}Cr release, being
It has been shown that treatment of thoracic duct lymphocytes with crude preparations of glycosidases or with the purified enzyme neuraminidase alters their migratory pattern on subsequent transfusion (Gesner and Ginsburg 1964, Woodruff and Gesner 1969). This has led to the suggestion that sugars on the surface of the lymphocyte play an important role in determining the distribution of cells around the body. The aim of the present experiments was to examine the fate of cells transferred after neuraminidase treatment, with particular reference to any changes in their migratory properties.

**RESULTS**

1. **LABELLING OF LYMPHOCYTES WITH \(^{51}\)Cr**

   **Retention of label by lymphocytes in vitro**

   Neuraminidase treatment can alter the passage of certain ions (Glick and Glithens 1965), proteins (Glick et al. 1966) and an amino acid, alpha-aminoisobutyric acid, (Brown and Michael 1969) across the cell membrane. If \(^{51}\)Cr labelling is to be used as a means of following the fate of neuraminidase treated lymphocytes, it is necessary to exclude the possibility that exposure of lymphocytes to neuraminidase could interfere with the retention of \(^{51}\)Cr by the cells. To test this point a single collection of \(^{51}\)Cr labelled thoracic duct lymphocytes was divided into two batches, one of which was treated with neuraminidase while the other was retained as a control. The release of \(^{51}\)Cr into the supernatant was measured at 90 minutes and at 4 hours. After 90 minutes incubation, 3.3% of the radioactivity of control cells had been released into the supernatant compared with 3.2% from the neuraminidase treated cells. By 4 hours, a further 3.7% of the radioactivity of the control cells had been released, while 3.6% had been released from neuraminidase treated cells. That is 93% of the original label remained in association with both control and neuraminidase treated cells after 4 hours maintenance in vitro. \(^{51}\)Cr release, being
associated with death of the cells, is widely used as an index of cytotoxicity (Sanderson 1964, Wigzell 1965, Holm and Perlmann 1967).

It was concluded from this experiment that, over the period of observation, neuraminidase treatment of lymphocytes did not affect the retention of label by the cells. There was no evidence of any increase in cell death in the neuraminidase treated population of lymphocytes, as judged by an increased release of $^{51}$Cr as compared to the untreated lymphocytes.

**Retention of label by lymphocytes in vivo**

One criticism of experiments involving radioactive labelling techniques is the possibility that the label may be removed from the original cell inoculum and reincorporated in cells of the host. However, reutilization of $^{51}$Cr could not be demonstrated in an in vitro system using tissue culture (Holm and Perlmann 1967).

The following experiments were performed to determine whether $^{51}$Cr had been transferred from the cells originally labelled. They utilize the fact that labelled cells release their label into the supernatant when killed by complement-fixing cytotoxic isoantisera. $^{51}$Cr labelled thoracic duct lymphocytes were transfused into recipients with established thoracic duct fistulae. By examination of the labelled cells appearing in the thoracic duct lymph of the recipients, using allogeneic and semi-allogeneic transfer systems and appropriate isoantisera, it was possible to show that the label detected in cells draining from the thoracic duct was associated with the cell population originally labelled. In parallel with the $^{51}$Cr release studies, cytotoxicity testing by trypan blue uptake was performed as a check on the activity of the isoantisera.

Results in Table 5.1 show that 12-19 hours after injection of labelled cells, radioactivity is only released when the isoantisera was directed against transferred cells. Only 4% of the $^{51}$Cr was released by DA anti-Lewis serum when labelled DA cells were
### TABLE 5.1

**THE RELEASE OF $^{51}$Cr FROM LABELLED CELLS DRAINING IN THE THORACIC DUCT LYMPH 12-19 HOURS AFTER INJECTION**

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Serum</th>
<th>% Viability*</th>
<th>% $^{51}$Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{51}$Cr labelled DA cells in (DA x Lewis)F₁ recipient</td>
<td>DA anti Lewis</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Normal DA</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>$^{51}$Cr labelled (DA x Lewis)F₁ cells in DA recipient</td>
<td>DA anti Lewis</td>
<td>98</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Normal DA</td>
<td>99</td>
<td>6</td>
</tr>
</tbody>
</table>

* The viability of the entire cell population (unlabelled + labelled cells) obtained from the thoracic duct as determined by trypan blue uptake after treatment with serum.

150 - 200 x $10^6$ $^{51}$Cr labelled DA or (DA x Lewis)F₁ TDL were transferred intravenously to recipients with an established thoracic duct fistula. The recipient strain is indicated in the table. Thoracic duct lymphocytes draining from these recipients 12 - 19 hours after injection were incubated with serum as shown and $^{51}$Cr release was determined (methods). The release of $^{51}$Cr is expressed as a percentage of the radioactivity present in the cell population prior to treatment with serum.
draining from a (Lewis x DA)F₁ hybrid recipient. 56% of the label was released by DA anti-Lewis serum when labelled (Lewis x DA)F₁ cells were draining from a DA recipient.

A similar experiment was performed on labelled cells entering the thoracic duct between 57 and 67 hours after injection, to exclude the possibility that the label may be transferred to host lymphocytes with increasing time. Results in Table 5.2 show that all the radioactive label in the thoracic duct lymph was associated with the donor cells. These experiments show unequivocally that the cell associated label in the thoracic duct lymph remains on the donor cells that were originally labelled and that no label has been reutilized by host cells.

The migration index

As neuraminidase treatment of thoracic duct lymphocytes is known to markedly increase the migration of transfused lymphocytes to the liver and decrease migration to the spleen (Woodruff and Gesner 1969), this characteristic modification of cell behaviour was used as the basis for assessing persistence of enzyme mediated changes in these cells.

Comparison of the frequency of migration of the lymphocytes in a population to these 2 organs at 1 hour after transfer yielded a "migration index", namely:

\[
\frac{\% \text{ radioactivity localizing in liver @ 1 hour}}{\% \text{ radioactivity localizing in spleen @ 1 hour}}
\]

This index was useful in examining the migratory properties of very small numbers of cells, as, being a ratio of % distribution, it was independent of variation in the number of labelled cells injected intravenously. By employing this index it was feasible to determine the migratory properties of a few labelled cells mixed with a large excess of unlabelled cells, a situation which would arise when labelled transfused cells are being recovered in the thoracic duct lymph. That mixing an excess of unlabelled cells with a small number of labelled cells did not interfere with the migration index of the latter can be seen from Table 5.3.
# TABLE 5.2

**THE RELEASE OF $^{51}$Cr FROM LABELLED CELLS DRAINING IN THE THORACIC DUCT LYMPH 57-67 HOURS AFTER INJECTION**

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Serum</th>
<th>% Viability*</th>
<th>% $^{51}$Cr release</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{51}$Cr labelled DA cells in (DA x Lewis)F$_1$ recipient</td>
<td>Lewis anti-DA</td>
<td>62</td>
<td>63</td>
</tr>
<tr>
<td>As above</td>
<td>DA anti-Lewis</td>
<td>47</td>
<td>3</td>
</tr>
<tr>
<td>As above</td>
<td>Normal DA</td>
<td>99</td>
<td>3</td>
</tr>
</tbody>
</table>

* The viability of the entire cell population (labelled + unlabelled cells) obtained from the thoracic duct as determined by trypan blue uptake after treatment with serum.

150 x 10$^6$ $^{51}$Cr labelled DA TDL were transferred intravenously to (DA x Lewis)F$_1$ hybrid recipients with an established thoracic duct fistula. Thoracic duct lymphocytes draining from these recipients were incubated with serum as shown and $^{51}$Cr release was determined (methods). The release of $^{51}$Cr is expressed as a percentage of the radioactivity present in the cell population prior to treatment with serum.
TABLE 5.3

MIGRATION INDEX OF CONTROL OR NEURAMINIDASE TREATED CELLS WHEN MIXED WITH UNLABELLED CELLS

<table>
<thead>
<tr>
<th>Cell inoculum</th>
<th>Migration Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^6$ $^{51}$Cr Control</td>
<td>0.6 (0.4 - 0.8)*</td>
</tr>
<tr>
<td>$10^6$ $^{51}$Cr Neuraminidase</td>
<td>11 (9 - 12)*</td>
</tr>
<tr>
<td>$10^6$ $^{51}$Cr Control + 35 x $10^6$ Unlabelled</td>
<td>0.5</td>
</tr>
<tr>
<td>$10^6$ $^{51}$Cr Neuraminidase + 35 x $10^6$ Unlabelled</td>
<td>12</td>
</tr>
</tbody>
</table>

* 3 animals in each group (mean and range shown)

Thoracic duct lymphocytes were labelled with $^{51}$Cr and then treated with neuraminidase or left untreated as a control. $10^6$ lymphocytes were transferred to recipient rats intravenously. Other rats received similar inocula to which 35 x $10^6$ unlabelled normal thoracic duct lymphocytes had been added. The migration index of the labelled cells was determined in each case.
Inhibition of alterations in migratory pattern by exposure to excess sialic acid at the time of incubation with neuraminidase

It is important to exclude the effects of possible contaminant enzymes, if one is to attribute the changes in lymphocyte behaviour after incubation with neuraminidase to removal of surface sialic acid.

Cytotoxic and haemolytic phospholipases have been described as contaminants of a commercially prepared neuraminidase preparation derived from clostridium perfringens (Kraemer 1968). Although the neuraminidase preparation used in the present experiments was derived from vibrio cholera, and was stated to be free of contaminating proteases, aldolase or lecithinase C by the manufacturer, it is possible that the alterations observed in migratory patterns could reflect some alteration of the cell surface other than removal of sialic acid by neuraminidase. That this was not so was indicated by the efficiency with which sialic acid competitively inhibited the effects of neuraminidase treatment on lymphocyte migration (Table 5.4. and Fig. 5.1).

One unit of neuraminidase is defined as the amount of enzyme required to release 1 µg of N-acetylneuraminic acid from human α-acid glycoprotein in 15 minutes at 37°C (Mohr and Schramm 1960). Hence the 1 mg of sialic acid used in these experiments may be regarded as a forty fold excess.

2 DISTRIBUTION OF LYMPHOCYTES IN SYNGENEIC RECIPIENTS

Neuraminidase treatment of thoracic duct lymphocytes has been found by Woodruff and Gesner (1969) to alter the distribution of the lymphocytes in the tissues after transfusion. These authors reported that many enzyme treated cells became trapped in the liver initially, and that the selective accumulation of transfused cells in the lymph nodes and spleen that was observed with untreated lymphocytes was much less marked in the case of neuraminidase treated lymphocytes. The following experiments were undertaken to confirm these findings.
TABLE 5.4
INHIBITION OF THE EFFECT OF NEURAMINIDASE ON THE MIGRATION OF TDL BY EXCESS FREE SIALIC ACID

<table>
<thead>
<tr>
<th>Treatment of lymphocytes</th>
<th>% Localization at 1 Hour after Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>1 mg sialic acid</td>
<td>17</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>38</td>
</tr>
<tr>
<td>Neuraminidase + 0.5 mg sialic acid</td>
<td>31</td>
</tr>
<tr>
<td>Neuraminidase + 1 mg sialic acid</td>
<td>23</td>
</tr>
</tbody>
</table>

38-50 x 10^6 DA thoracic duct lymphocytes were labelled with ^{51}Cr then incubated with 25 units of neuraminidase alone, 1 mg of sialic acid alone or a mixture of 25 units of neuraminidase plus sialic acid. Incubations were performed for 15 minutes at 37°C. After incubation the lymphocytes were transferred intravenously to 15 week old syngeneic female recipients. The distribution of radioactivity in the organs of these recipients was determined one hour after cell transfer.
Thoracic duct lymphocytes were treated as indicated in table 5.4. The decrease in the migration index as excess free sialic acid (NANA) was added to the incubation medium is depicted. The migration index approaches that of cells incubated in the absence of neuraminidase.
$^{51}$Cr labelled thoracic duct lymphocytes were injected intravenously into syngeneic recipients. 2 animals were killed at 1, 8 and 24 hours after injection and the distribution of radioactivity in the various organs was determined. The results are shown in Table 5.5.

There is a marked difference in distribution between control and enzyme treated cells. A much higher percentage of enzyme treated cells migrate to the liver, and a large proportion of these cells persist there for at least 24 hours. Fewer enzyme treated cells localize in the lymph nodes and spleen, a difference from untreated lymphocytes which also remains apparent 24 hours after transfer. The radioactive content of the spleens of rats receiving untreated cells diminishes from 1 to 24 hours whilst that of the lymph nodes increases. In contrast, there is less increase in lymph node radioactivity 24 hours after transfusion of neuraminidase treated cells.

These results are basically similar to those of Woodruff and Gesner (1969), with the exception that they found the distribution of labelled, neuraminidase treated lymphocytes to be similar to that of untreated lymphocytes by 28 hours after injection.

3 RECIRCULATION OF LYMPHOCYTES IN THE THORACIC DUCT LYMPH

Neuraminidase treatment of thoracic duct lymphocytes has been reported to cause a decreased recovery and delayed peak appearance of labelled cells from the thoracic duct lymph after the transfer of either allogeneic or syngeneic lymphocytes to rats (Woodruff and Gesner 1969). The following experiments examined the appearance of untreated (control) and neuraminidase treated lymphocytes in the thoracic duct lymph after transfusion. Five separate experiments were performed and the results of 4 are presented in Fig. 5.2. Of the 4 experiments presented, recipients and donors were syngeneic in 3 cases and semiallogeneic in the remaining one. All 5 experiments gave similar results. Following the injection
### Table 5.5

The distribution of control and neuraminidase treated lymphocytes in syngeneic recipients

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time after injection</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 Hour*</td>
<td>8 Hours*</td>
<td>24 Hours*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>ND</td>
<td>C</td>
<td>ND</td>
<td>C</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph nodes*</td>
<td></td>
<td>1.6</td>
<td>2.4</td>
<td>0.6</td>
<td>0.7</td>
<td>6.6</td>
<td>5.6</td>
<td>0.7</td>
<td>0.7</td>
<td>9.6</td>
<td>10.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td>28</td>
<td>29</td>
<td>8</td>
<td>7</td>
<td>20</td>
<td>23</td>
<td>6</td>
<td>5</td>
<td>18</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>13</td>
<td>11</td>
<td>50</td>
<td>49</td>
<td>12</td>
<td>12</td>
<td>48</td>
<td>47</td>
<td>12</td>
<td>13</td>
<td>41</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Small Bowel</td>
<td></td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td>0.05</td>
<td>0.05</td>
<td>0.02</td>
<td>0.02</td>
<td>0.07</td>
<td>0.08</td>
<td>0.02</td>
<td>0.02</td>
<td>0.2</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Results are expressed as the percentage of the total radioactivity injected recovered per organ.

DA thoracic duct lymphocytes were labelled with $^{51}$Cr, then incubated with neuraminidase (ND) or left untreated as a control (C). 50 x $10^6$ lymphocytes were injected intravenously into 16 week old syngeneic female recipients. 2 recipients from each group were killed at the times shown after cell transfer, and the distribution of radioactivity was determined.
FIGURE 5.2
THE MIGRATION OF CONTROL AND NEURAMINIDASE TREATED LYMPHOCYTES INTO THE THORACIC DUCT

A. B. (Lewis x DA)F₁ lymphocytes to (Lewis x DA)F₁ hybrid recipients

C. DA lymphocytes to DA recipient

D. DA lymphocytes to (Lewis x DA)F₁ hybrid recipient

Lymphocytes were collected within the first 24 hours after thoracic duct cannulation, labelled with ⁵¹Cr and then either treated with neuraminidase or left untreated as a control.

120 to 200 x 10⁶ of these lymphocytes were transfused into recipients with established thoracic duct fistulae of 24 hours duration.

The thoracic duct lymph of these recipients was examined for the reappearance of labelled cells over 6 hour intervals for the first 36 hours after the transfusion of labelled cells, and over 8-12 hour intervals thereafter.

The appearance of radioactivity is expressed as that percentage of the total radioactivity administered which reappeared in the thoracic duct lymph per hour. This value is recorded for each successive collection of lymph.

The total lymphocyte output (unlabelled + labelled cells) from each recipient is recorded at the top of each figure.

Each curve represents the results obtained from one recipient rat.

The cell viability prior to injection as assessed by trypan blue uptake was greater than 95% in all cases.
The decreased lymphocytic response to the lymph node in untreated animals (A) as compared to the lymphocytes of lymphoma treated lymphocytes (B) is noted over the first 60 hours.

---

**CONTROL**

---

**NDASE**
of untreated lymphocytes, the recovery of labelled cells from the thoracic duct rose steadily to a peak at 15-20 hours in all recipients. In contrast, after injection of neuraminidase treated cells there was a delayed and lower peak recovery of labelled cells occurring 30-35 hours after transfusion. The retardation of the reappearance of neuraminidase treated cells in the thoracic duct was particularly marked in the first 10 hours after transfusion. In all experiments, the total of labelled plus unlabelled hourly cell output from the thoracic duct of the host animal was of similar magnitude, and showed a steady decline over the duration of the experiment, regardless of whether neuraminidase treated or control lymphocytes had been transferred.

The decreased migration of neuraminidase treated cells into the thoracic duct lymph may be related to the decreased migration of neuraminidase treated lymphocytes to the lymph nodes noted in section 2 above.

4 MIGRATORY PROPERTIES OF TRANSFUSED LYMPHOCYTES THAT HAVE REAPPEARED IN THE THORACIC DUCT AFTER TRANSFUSION

The aim of these experiments was to examine the migratory properties of neuraminidase treated cells that reappeared in the thoracic duct lymph in order to answer the questions:

(i) Do the lymphocytes that reappear in the thoracic duct after the transfusion of neuraminidase treated cells have the migratory properties of normal lymphocytes?

(ii) Is the increasing migration of neuraminidase treated lymphocytes into the thoracic duct lymph that is noted over the first 30 hours after transfusion associated with any change in the migratory properties of these cells?

It has already been established that transfusion of normal lymphocytes together with neuraminidase treated lymphocytes does not alter the migratory properties of the latter (Table 5.3). To answer the
questions posed above, labelled cells that had migrated into the thoracic duct lymph were reinjected intravenously into syngeneic recipients, and the migration index of these cells was determined (Fig. 5.3). The results were quite clear cut. In all 5 cases the migration index of normal labelled cells reappearing in the thoracic duct lymph was less than 0.5 at all times examined up to 60 hours after transfusion. In contrast, those neuraminidase treated cells that reappeared during the first 10 hours after transfusion had a migration index of 3.5 - 5.5 (Fig. 5.3). In every case, the migration index of the labelled cells which had been treated with neuraminidase prior to transfusion, and had reappeared in the thoracic duct, decreased over the next 10 hours, so that by 20 hours it was approaching that of the control cells. The reversion of the migration index to normal coincided with the increasing appearance of neuraminidase treated cells in the thoracic duct lymph described in the preceding section (Fig. 5.2). By 30 hours, the migration index of labelled lymphocytes recovered from recipients of the labelled control or neuraminidase treated cell populations was similar (Fig. 5.3). Detailed results from three experiments in which (Lewis x DA)F1 lymphocytes were transfused into syngeneic recipients are shown in Table 5.6.

A similar experiment was performed to examine the change in the migration index of enzyme treated cells which reappear in the thoracic duct lymph, with the exception that neuraminidase treated DA lymphocytes were transferred to a (Lewis x DA)F1 recipient. This permitted the use of isoantisera to show that the labelled cells emerging 24-30 hours after the injection of neuraminidase treated lymphocytes were from the cell population originally injected (Table 5.7). From 24-30 hours after transfusion, all the label was associated with the neuraminidase treated cells at a time when the migration index was 0.8.

Several other points arise from examination of Table 5.6. Alterations in the node seeking propensity of neuraminidase treated lymphocytes follow a similar
FIGURE 5.3

THE MIGRATION INDEX OF CONTROL AND NEURAMINIDASE TREATED LYMPHOCYTES WHICH HAVE MIGRATED INTO THE THORACIC DUCT

A. B. C. (Lewis x DA)F₁ lymphocytes to (Lewis x DA)F₁ hybrid recipients

D. E. DA Lymphocytes to DA recipients

Lymphocytes were collected within the first 24 hours after thoracic duct cannulation, labelled with ⁵¹Cr and then either treated with neuraminidase or left untreated as a control.

120 to 200 x 10⁶ of these lymphocytes were transfused into primary syngeneic recipients with established thoracic duct fistulae of 24 hours duration.

Labelled cells reappearing in the thoracic duct of the primary recipients over the times indicated (hours), were reinjected intravenously into secondary recipients. The secondary recipients were killed 1 hour later in order to determine the migration index of the labelled cells. The migration index of these cells is recorded for each successive collection of lymph.

Each curve represents the result obtained from one recipient rat.
TABLE 5.6
THE DISTRIBUTION OF LABELLED LYMPHOCYTES IN A SECONDARY HOST AFTER MIGRATION INTO THE THORACIC DUCT OF A PRIMARY HOST

<table>
<thead>
<tr>
<th>Time of collection of TDL after injection into primary host (Hours)</th>
<th>CONTROL CELLS</th>
<th></th>
<th></th>
<th>NEURAMINIDASE TREATED CELLS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Spleen</td>
<td>% Liver</td>
<td>% Lymph nodes</td>
<td>Migr. index</td>
<td>% Spleen</td>
<td>% Liver</td>
</tr>
<tr>
<td>0-6</td>
<td>53</td>
<td>7</td>
<td>6</td>
<td>0.1</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>5</td>
<td>14</td>
<td>0.2</td>
<td>12</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>6</td>
<td>6</td>
<td>0.1</td>
<td>8</td>
<td>37</td>
</tr>
<tr>
<td>6-12</td>
<td>40</td>
<td>6</td>
<td>14</td>
<td>0.2</td>
<td>12</td>
<td>44</td>
</tr>
<tr>
<td></td>
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<td>5</td>
<td>14</td>
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<td>7</td>
<td>6</td>
<td>0.2</td>
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<td>33</td>
</tr>
<tr>
<td>12-18</td>
<td>38</td>
<td>7</td>
<td>9</td>
<td>0.2</td>
<td>18</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>6</td>
<td>11</td>
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<td>25</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>7</td>
<td>6</td>
<td>0.2</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>18-24</td>
<td>36</td>
<td>7</td>
<td>10</td>
<td>0.2</td>
<td>36</td>
<td>20</td>
</tr>
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<td></td>
<td>36</td>
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<td>7</td>
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<td></td>
<td>50</td>
<td>7</td>
<td>12</td>
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</tr>
<tr>
<td>24-30</td>
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<td>15</td>
<td>0.3</td>
<td>31</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>9</td>
<td>9</td>
<td>0.2</td>
<td>27</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>8</td>
<td>6</td>
<td>0.2</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td>30-42</td>
<td>30</td>
<td>13</td>
<td>7</td>
<td>0.4</td>
<td>38</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>11</td>
<td>7</td>
<td>0.2</td>
<td>42</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45</td>
<td>10</td>
</tr>
</tbody>
</table>

* values expressed as the percentage of the total radioactivity injected recovered per organ.

Lymphocytes were collected within the first 24 hours after thoracic duct cannulation of (Lewis x DA)F₁ hybrid rats. The cells were labelled with ⁵¹Cr and then either treated with neuraminidase or left untreated as a control. 100-200 x 10⁶ of these lymphocytes were injected intravenously into primary syngeneic recipients with an established thoracic duct fistula of 24 hours duration.

Labelled cells reappearing in the thoracic duct of the primary recipients over the periods indicated (hours) were reinjected intravenously into secondary recipients. The secondary recipients were killed 1 hour later and the distribution of radioactivity was determined. The migration index is also expressed as for each individual cell transfer.
TABLE 5.7

THE RELEASE OF $^{51}$Cr FROM LABELLED NEURAMINIDASE TREATED CELLS DRAINING IN THE THORACIC DUCT LYMPH 24-30 HOURS AFTER INJECTION

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Serum</th>
<th>% Viability * after treatment</th>
<th>% $^{51}$Cr release</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{51}$Cr labelled neuraminidase treated DA cells in (Lewis x DA)F1 recipient</td>
<td>Le anti-DA</td>
<td>55</td>
<td>64</td>
</tr>
<tr>
<td>As above</td>
<td>DA anti-Le</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>As above</td>
<td>Normal DA</td>
<td>100</td>
<td>8</td>
</tr>
</tbody>
</table>

* The viability of the entire cell population (labelled + unlabelled cells) obtained from the thoracic duct, as determined by trypan blue uptake after treatment with serum.

$^{120} \times 10^6$ DA thoracic duct lymphocytes were labelled with $^{51}$Cr and then treated with neuraminidase. These lymphocytes were injected intravenously into a (Lewis x DA)F1 hybrid recipient with an established thoracic duct fistula.

Thoracic duct lymphocytes draining from this recipient from 24 to 30 hours after injection were incubated with serum and $^{51}$Cr release was determined (methods).

The release of $^{51}$Cr is expressed as a percentage of the radioactivity present in the cell population prior to treatment with serum.

The migration index of the labelled, neuraminidase treated, cell population collected at this time was 0.8.
course to those observed with the migration index. The node seeking propensity of the labelled cells emerging from the recipients of the enzyme treated lymphocytes in the first 10 hours is far less than that of control cells. However this increases steadily so that by 30 hours the node seeking abilities of the two populations are similar (Table 5.6). Thus over a period of 30 hours after transfusion, neuraminidase treated lymphocytes that appear in the thoracic duct lymph show an increasing ability to home to lymph nodes and spleen along with decreasing accumulation in the liver, so that by 30 hours the migratory properties of members of the control and neuraminidase treated cell populations reappearing in the thoracic duct lymph are identical.

The experiments described in this section show that neuraminidase treated lymphocytes have a lesser total migration, and delayed peak appearance, in the thoracic duct lymph of syngeneic recipients. Over a period of 24 hours the population of neuraminidase treated lymphocytes that appears in the thoracic duct lymph regains migratory properties similar to untreated cells.

5 MIGRATORY PROPERTIES OF LYMPHOCYTES LOCALIZING IN THE SPLEEN

The experiments described in section 4 were concerned with those neuraminidase treated cells which migrate into the thoracic duct lymph after transfusion. The following experiments were intended to detect any migratory changes in those neuraminidase treated thoracic duct lymphocytes which localize in the spleen after intravenous injection.

Labelled, neuraminidase treated or control thoracic duct lymphocytes were injected intravenously into syngeneic primary hosts. These primary hosts were killed at either 1 or 24 hours after injection and the distribution of the labelled cells in their liver, lymph nodes and spleen was determined. A cell suspension was prepared from the spleen of each primary host at either 1 or 24 hours after the injection of labelled cells,
and this suspension was injected intravenously into syngeneic secondary hosts. Figure 5.4 presents a schematic representation of the steps involved. The distribution of the labelled cells from the spleens of the primary hosts in the spleen, lymph nodes and liver of the secondary hosts was determined at 1, 12 and 24 hours. The results of these experiments are shown in figures 5.5 A, B and C. In each case, the first column gives the distribution of the labelled thoracic duct lymphocytes in the relevant organ of the primary host at 1 and 24 hours after transfusion. The second and third columns show the distribution of labelled cells from the spleens of the primary hosts after transfer to secondary hosts. Column 2 gives the distribution on transfer to secondary hosts, of cells which had localized in the spleens of the primary hosts one hour after transfusion. Column 3 gives similar data for cells localizing in the spleens of the primary hosts 24 hours after transfusion.

The migration of labelled cells to the lymph nodes (Column 1, Figure 5.5A) spleen, (Column 1, Figure 5.5B) and liver (Column 1, Figure 5.5C) of the primary hosts is similar to that observed previously (Table 5.5). The increasing accumulation of labelled lymphocytes in the lymph nodes of recipients of control cells between 1 and 24 hours can be seen in Figure 5.5A, Column 1. This contrasts with the slight increase in the number of neuraminidase treated cells localized in the lymph nodes over a similar period (Fig. 5.5A, Column 1). Localization of cells in the lymph nodes, spleen and liver of secondary hosts will be considered separately.

The behaviour of labelled cells localized in the spleen of the primary host at 1 hour after transfusion of lymphocytes, is shown in Figure 5.5A, Column 2. When these cells are transferred to secondary hosts, their distribution to the lymph nodes was similar to that seen in the primary hosts after the initial transfer of lymphocytes. This was so with the labelled cells obtained from the spleens of recipients of either control or neuraminidase treated cell populations. Thus, neuraminidase treated cells that have localized in the
FIGURE 5.4

The steps involved in determining the migratory pattern of lymphocytes localizing in the spleen:

1. Label TDL with $^{51}$Cr
2. Primary host kill after 1, 24 hours
3. Spleen
4. Secondary host kill after 1, 12, 24 hours
FIGURE 5.5
THE MIGRATION OF NORMAL AND NEURAMINIDASE TREATED LYMPHOCYTES TO THE LYMPH NODES, SPLEEN AND LIVER OF PRIMARY AND SECONDARY HOSTS

A. Migration to the lymph nodes
B. Migration to the spleen
C. Migration to the liver

(Lewis x DA)F₁ thoracic duct lymphocytes were collected within the first 24 hours after thoracic duct cannulation, labelled with ⁵¹Cr and then either treated with neuraminidase or left untreated as a control.

The following procedures were then carried out with both control and neuraminidase treated lymphocytes, using male or female (Lewis x DA)F₁ hybrid recipients of 12-20 weeks of age.

80 x 10⁶ labelled lymphocytes were injected intravenously into 6 primary hosts. 3 primary hosts were killed 1 hour later and the other 3 were killed 24 hours later. The distribution of the labelled cells in the lymph nodes, spleens and livers of these primary hosts was determined at these times (Column 1 A, B, C), and is plotted as a mean and range (vertical bar), where sufficient discrepancy in values was present.

Pooled cell suspensions were prepared from the 3 spleens removed from the primary hosts at 1 or 24 hours. Portions of this suspension were then injected into 3 secondary hosts which were killed at either 1, 12 or 24 hours after the cell transfer, and the distribution of radioactivity in the lymph nodes, spleens and livers of these secondary hosts was determined.

The distribution of those labelled cells situated in the spleens of the primary hosts at either 1 or 24 hours, in the relevant organ of the secondary hosts, is shown in column 2 (1 hour) and column 3 (24 hours).

All values are expressed as a percentage of the total radioactivity injected into each recipient prior to determination of the distribution of the radioactivity.
The lymph nodes of the spleen treated with 24 hours of RNAse digest were slower in control than the control enzyme. The lymph node of the spleen treated with 24 hours of the primary enzyme was slower than the control enzyme and in a manner similar to the primary enzyme. The lymph node of the primary enzyme was similar to the control enzyme after the primary enzyme. The lymph node of the primary enzyme was similar to the control enzyme after the primary enzyme.
spleen of the primary hosts 1 hour after transfusion accumulate in the lymph nodes of the secondary host much more slowly than control cells (Fig. 5.5A, Column 2).

The migratory pattern of neuraminidase treated cells that have localized in the spleen of the primary host 24 hours after injection is quite different from that of the original population of thoracic duct lymphocytes and also from that of those cells which localized in the spleen of the primary host at 1 hour. Neuraminidase treated cells resident in the spleen of primary hosts at 24 hours, now migrate to the lymph nodes more readily than the control cell population (Fig. 5.5A, Column 3) and in a manner similar to the original control cell population.

The migratory properties to the spleens of secondary hosts, on retransfer of thoracic duct lymphocytes localizing in the spleens of primary hosts (Fig. 5.5B) follow a similar pattern to that observed above for migration to lymph nodes. The neuraminidase treated cells that localize in the spleens of primary hosts at one hour after transfusion migrate on retransfer in a similar fashion to the original neuraminidase treated population (Fig. 5.5B, Column 2). However those neuraminidase treated lymphocytes resident in the spleens of primary hosts 24 hours after transfusion, migrate on retransfer in a manner similar to the original control cell population (Fig. 5.5B, Column 3).

The localization of control cells in the liver is of interest. From Figure 5.5C, Columns 1 and 2 it can be seen that approximately 15% of both the original control cell population and of those control cells which have entered the spleen of the primary host at 1 hour migrate to the liver after transfusion. This proportion remains constant over 24 hours. However, 30% of the control cell population present in the spleen of the primary host 24 hours after injection migrates to the liver of the secondary host, soon after injection, and this percentage also remains constant over the next 24 hours (Fig. 5.5C, Column 3). Neuraminidase treated
cells derived from the spleens of primary hosts at 1 hour after transfusion, still migrate more readily to the liver than an equivalent population of control cells (Fig. 5.5C, Column 2). The population of cells that was originally treated with neuraminidase obtained from the spleens of primary hosts at 24 hours, migrates less readily to the liver than an equivalent population of control cells (Fig. 5.5C, Column 3).

These experiments show that the migratory properties of neuraminidase treated thoracic duct lymphocytes localizing in the spleen change over a period of 24 hours, to become more similar to the properties of untreated thoracic duct lymphocytes which localize in the spleen. A similar finding was obtained when migratory properties of that population of neuraminidase treated thoracic duct lymphocytes which migrate into the thoracic duct were examined (Section 4).

6 THE EFFECT OF THE LABELLING PROCEDURE ON LYMPHOCYTE MIGRATION

From a comparison of Tables 5.5 and 5.6 it was apparent that labelled control lymphocytes reappearing in the thoracic duct lymph (Table 5.6), exhibited an increased node and spleen seeking tendency on retransfer, than did a normal population of thoracic duct lymphocytes transferred directly after labelling (Table 5.5). To clarify this point, the results from several experiments have been summarized in Table 5.8 which records the localization of labelled thoracic duct lymphocytes one hour after transfer. This table compares the migration of thoracic duct lymphocytes which have been transferred directly after labelling, with those that have recirculated once through an intermediate host prior to determination of the migratory properties. The lymphocytes which have recirculated through an intermediate host after labelling migrate more readily to the spleen and lymph nodes than those transferred directly after labelling (Table 5.8).

This difference could be explained by the existence of a subpopulation of lymphocytes within the recirculating...
TABLE 5.8
THE MIGRATION OF THORACIC DUCT LYMPHOCYTES
TRANSFERRED DIRECTLY AFTER LABELLING COMPARED WITH
THOSE THAT HAVE RECIRCULATED THROUGH A PRIMARY HOST

<table>
<thead>
<tr>
<th>Organ</th>
<th>% Radioactivity</th>
<th>TDL</th>
<th>Recirculating TDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>24 ± 1</td>
<td>43 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>1.2 ± 0.3</td>
<td>11 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(6)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>14 ± 1</td>
<td>6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(12)</td>
<td></td>
</tr>
</tbody>
</table>

$^{51}$Cr labelled thoracic duct lymphocytes were injected intravenously into syngeneic recipients which were killed 1 hour later and the distribution of radioactivity determined (TDL).

Labelled thoracic duct lymphocytes were also injected intravenously into primary syngeneic recipients with established thoracic duct fistulae. Labelled lymphocytes which reappeared in the thoracic duct of these primary recipients were reinjected intravenously into secondary recipients which were killed 1 hour later and the distribution of radioactivity determined (recirculating TDL).

Results are expressed as the percentage of total radioactivity injected per organ. In each case the mean and standard error is recorded. The number of observations is given in brackets.
pool with a greater node and spleen seeking propensity than the original population. Alternatively it may reflect a process of screening altered cells from the original population. The second explanation implies that most thoracic duct lymphocytes in optimal condition would migrate in a similar fashion to those labelled cells which have migrated into the thoracic duct lymph.

In order to distinguish between these two possibilities an experiment was performed in which thoracic duct lymphocytes were labelled with $^{51}$Cr and the migratory pattern of a sample determined, while the remainder were injected into a syngeneic recipient with an established thoracic duct fistula. The lymphocyte population draining from the thoracic duct of this recipient at various intervals after infusion, was divided into two portions. One of these portions was reinjected without further treatment while the other portion was subjected to all of the manipulations entailed in labelling with $^{51}$Cr (methods), with the substitution of radioactive sodium chromate by a solution of non-radioactive sodium chromate. The concentration of 'cold' sodium chromate used was identical with that used for the original labelling incubation with radioactive chromate. After the simulated labelling procedure, the second portion of cells was reinjected into syngeneic recipients. The distribution of the two groups of cells is shown in Table 5.9, and may be compared with the distribution of the original population (23% spleen, 11% liver and 2.3% nodes) determined without prior passage through an intermediate host.

It can be seen that repetition of the incubation procedure with 'cold' chromate reduced the lymph node seeking propensity and increased the percentage of migration to the liver towards that seen in the original population. However, the percentage of cells localized in the spleen remained much greater than that seen in the original population of cells.
TABLE 5.9
THE EFFECT OF A SIMULATED LABELLING PROCEDURE ON THE MIGRATORY PROPERTIES OF LYMPHOCYTES

<table>
<thead>
<tr>
<th>Time of collection after injection (Hours)</th>
<th>Reinject after incubation with 'cold' sodium chromate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%Spleen %Liver %Nodes</td>
</tr>
<tr>
<td>0 - 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46 6 13.1</td>
</tr>
<tr>
<td>10 - 22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>51 7 15.6</td>
</tr>
<tr>
<td>22 - 32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38 7 14.3</td>
</tr>
</tbody>
</table>

(DA x Lewis)F₁ thoracic duct lymphocytes were labelled with $^{51}$Cr (at a concentration of 0.36 µg/ml of sodium chromate) and injected intravenously into a syngeneic recipient with an established thoracic duct fistula of 24 hours duration.

Labelled cells reappearing in the thoracic duct of this recipient at varying periods after injection (hours), were divided into 2 portions.

One portion was reinjected immediately (reinject), the other was reinjected intravenously, after a simulated labelling procedure with 'cold' sodium chromate (at a concentration of 0.36 µg/ml), into syngeneic recipients.

These recipients were killed at 1 hour after the injection of cells and the distribution of radioactivity in their organs was determined. The distribution is expressed as a percentage of the radioactivity injected.
It was concluded that the labelling procedure itself, presumably by altering cells, could reduce the extent of the migration to the lymph nodes seen at 1 hour after cell transfer. However, there appeared to be a subpopulation of thoracic duct lymphocytes which migrated more readily to the spleen than did the original population. The demonstration that the lymph node seeking ability, but not the spleen seeking ability of the cells can be altered by the manipulations involved in labelling suggests that there may be different factors controlling cell migration into these 2 lymphoid tissues.

7 DISTRIBUTION OF LYMPHOCYTES IN LEThALLY IRRADIATED RECIPIENTS

Experiments to be reported in a subsequent chapter are concerned with an examination of the immune response to sheep erythrocytes by lethally irradiated recipients restored with untreated or neuraminidase treated thoracic duct lymphocytes.

The following experiment was performed in order to confirm that neuraminidase treatment of thoracic duct lymphocytes produced a similar alteration in the distribution of these cells, after transfer to lethally irradiated (910 rad) recipients, as was seen after transfer to normal recipients. Control or neuraminidase treated TDL were transferred to recipients which had been lethally irradiated either 6 or 24 hours previously. The distribution of these cells in the recipients is shown in Table 5.10.

The first point to be noted is that neuraminidase treatment of the lymphocytes prior to transfer has produced similar changes in the distribution as was found after transfer to normal unirradiated recipients. Thus many more neuraminidase treated than control cells are found in the liver after transfusion and less in the spleen and nodes.

The second point is there is an increased lymph node localization of both neuraminidase treated and control lymphocytes in the irradiated recipients (Table 5.10) as
TABLE 5.10
DISTRIBUTION OF LYMPHOCYTES IN SYNGENEIC ANIMALS
IRRADIATED PRIOR TO INJECTION

<table>
<thead>
<tr>
<th>Organ</th>
<th>910 RAD 24 Hours before lymphocytes</th>
<th>910 RAD 6 Hours before lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 Hours*</td>
<td>24 Hours*</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>ND</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>9.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>Liver</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Small Bowel</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Time after injection of lymphocytes at which tissues were examined.

DA thoracic duct lymphocytes were labelled with $^{51}$Cr, then incubated with neuraminidase (ND) or left untreated as a control (C).

50 x $10^6$ lymphocytes were injected intravenously into 15 week old syngeneic female recipients which had received 910 rad gamma irradiation either 24 or 6 hours previously. These animals were killed at 8 and 24 hours after the cell transfer and the distribution of radioactivity was determined.

Results are expressed as a percentage of the total radioactivity injected recovered per organ.
compared to the localization of equivalent cells in normal recipients (Table 5.5). There was a 60% increase in localization of control lymphocytes in the lymph nodes after 8 hours, when animals which had been irradiated 24 hours prior to the injection of cells were compared with unirradiated rats. However, there was no increase in node localization at this time if the recipient had been irradiated 6 hours previously. Regardless of whether the recipients had been irradiated 6 or 24 hours prior to transfusion with untreated lymphocytes, some 70% more of the injected cells were localized in the lymph nodes after 24 hours than was the case with unirradiated recipients.

Similarly, recipients of neuraminidase treated cells showed no increase in lymph node accumulation of label, as compared with unirradiated rats at 8 hours after injection, if the recipients were irradiated 6 hours previously. However, there was a 75% increase in lymph node localization at 8 hours if they were irradiated 24 hours previously. The most marked differences noted were in the localization of neuraminidase treated lymphocytes in the lymph nodes of irradiated recipients 24 hours after infusion of the lymphocytes. In this situation, there was an increase in node localization of the order of 200% as compared to unirradiated recipients of similar cells (compare Tables 5.5 and 5.10).

8 DISTRIBUTION OF PARENTAL CELLS IN F₁ HYBRID RECIPIENTS

Parental strain lymphocytes can produce a fatal graft-versus-host reaction after intravenous injection into F₁ hybrid recipients. It is intended to compare the ability of control and neuraminidase treated lymphocytes to initiate such a reaction in Chapter 9.

The experiments described below were performed to determine whether there were any differences in the localization of $^{51}$Cr labelled parental cells in F₁ hybrid recipients as compared to their localization previously described in syngeneic recipients. In addition, the experiments were intended to confirm that treatment of the parental cells with neuraminidase prior to transfer
would alter their distribution in an F₁ hybrid recipient. It has previously been shown that neuraminidase treated parental (DA) lymphocytes recirculate to a lesser degree than untreated parental (DA) lymphocytes in the thoracic duct of a (Lewis x DA)F₁ hybrid recipient (Fig. 5.2D).

Labelled, neuraminidase treated or untreated parental (DA) thoracic duct lymphocytes were injected into (DA x Lewis)F₁ hybrids, and the distribution of radioactivity was determined (Table 5.11). The distribution of normal DA cells in the F₁ hybrid recipients resembled that obtained previously in syngeneic recipients (Table 5.5). The distribution of the transfused parental lymphocytes in F₁ hybrid recipients was modified in the same way by neuraminidase treatment as was their distribution in syngeneic recipients (Table 5.11 and 5.5).

As no difference could be detected between distribution of lymphocytes in syngeneic and semi-allogeneic situations, the distribution of parental type lymphocytes, specifically sensitized against hybrid tissues, was examined in hybrid rats. If any differences in the distribution of parental cells in F₁ recipients were to be detected by ⁵¹Cr labelling these would be expected to be magnified when the population of parental cells were sensitized against the F₁ strain. Thoracic duct lymphocytes from DA rats, sensitized against (Lewis x DA)F₁ tissues, were labelled and injected into F₁ hybrid recipients. As a control, the sensitized DA cells were also injected into syngeneic recipients. The migration of these sensitized cells to various organs was determined (Table 5.12). The two patterns are almost identical. There is a slightly greater proportion of sensitized DA cells migrating to the lymph nodes in F₁ as compared to DA recipients particularly after 32 hours. However a much greater number of experiments would be required to determine whether this was significant. The conclusion to be drawn is that there are no marked differences between the migratory patterns of parental strain lymphocytes sensitized against F₁ tissues after transfer to either parental or F₁ hybrid recipients.
**TABLE 5.11**

THE DISTRIBUTION OF CONTROL AND NEURAMINIDASE TREATED PARENTAL LYMPHOCYTES IN F₁ HYBRID RECIPIENTS

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time after injection</th>
<th>8 hours*</th>
<th>24 hours*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>ND</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>5.2</td>
<td>0.7</td>
<td>11.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>21</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>Liver</td>
<td>12</td>
<td>47</td>
<td>12</td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Small bowel</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.06</td>
<td>0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.5</td>
<td>0.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Results are expressed as the percentage of total radioactivity injected recovered per organ.

DA thoracic duct lymphocytes were labelled with $^{51}$Cr then incubated with neuraminidase (ND) or left untreated as a control (C). 50 x $10^6$ lymphocytes were injected intravenously into 20 week old (Lewis x DA)$F₁$ female recipients. The recipients were killed at either 8 or 24 hours after cell transfer and the distribution of radioactivity was determined.
TABLE 5.12
DISTRIBUTION OF PARENTAL (DA) LYMPHOCYTES
SENSITIZED AGAINST HYBRID TISSUES, IN $F_1$
HYBRID RECIPIENTS

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Hour*</td>
</tr>
<tr>
<td></td>
<td>DA (LexDA) $F_1$</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>2.3</td>
</tr>
<tr>
<td>Spleen</td>
<td>34</td>
</tr>
<tr>
<td>Liver</td>
<td>14</td>
</tr>
<tr>
<td>Lung</td>
<td>6</td>
</tr>
<tr>
<td>Small bowel</td>
<td>3</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Results are expressed as the percentage of total radioactivity injected recovered per organ.

Two 8 week old male DA rats were immunized with $2 \times 10^8$ $F_1$ (Lewis x DA) spleen cells intravenously and $3 \times 10^8$ (Lewis x DA) $F_1$ spleen cells intraperitoneally. 2 weeks later their thoracic ducts were cannulated and at the time of cannulation they were reimmunized with $2.5 \times 10^8$ (Lewis x DA) $F_1$ thoracic duct lymphocytes injected locally into the mesentery and $50 \times 10^6$ (Lewis x DA) $F_1$ thoracic duct lymphocytes intravenously.

$25 \times 10^6$ sensitized thoracic duct lymphocytes draining in the 1st 24 hours after cannulation were labelled and injected intravenously to 9 week old DA or (Lewis x DA) $F_1$ recipients. These animals were killed at 1, 16 or 32 hours after cell transfer and the distribution of radioactivity was determined.
It has been noted above (Section 4) that labelled thoracic duct lymphocytes which had migrated into the thoracic duct of a primary recipient show a greater propensity than the original population to home back to the lymph nodes when transferred to a secondary recipient. If a significant number of this lymph node seeking population of parental cells were pre-empted to take part in graft-versus-host activity in the tissues of a primary F₁ hybrid host, their deficiency from the thoracic duct lymph may be shown up on retransfer to a secondary host. Accordingly labelled parental DA lymphocytes which had been sensitized against (Lewis x DA)F₁ tissues were transferred to a primary (DA x Lewis)F₁ hybrid recipient with an established thoracic duct fistula. Labelled parental cells reappearing in the thoracic duct lymph of the primary host were retransferred to secondary hosts and their distribution in the lymph nodes and spleens of the secondary hosts was determined. As a control sensitized parental lymphocytes were also passaged through a syngeneic DA recipient.

The results are shown in Table 5.13. It can be seen that after the passage of sensitized parental cells through an F₁ hybrid recipient, the node seeking ability of these cells on retransfer to either F₁ or parental secondary hosts was reduced by 20% as compared to sensitized parental cells passaged through a parental recipient, and transferred to F₁ or parental secondary hosts. This finding suggests that there may be a preferential removal of the lymph node seeking population in the primary F₁ host though it would require greater numbers to be fully substantiated.

DISCUSSION

There are 5 main points to be discussed from the experiments described in the present chapter.

(i) Incubation of thoracic duct lymphocytes with neuraminidase alters the migration of the cells after transfer to recipient rats.
(ii) Transfused, neuraminidase treated lymphocytes which have entered the recipient's thoracic duct lymph
TABLE 5.13
THE DISTRIBUTION OF SENSITIZED PARENTAL LYMPHOCYTES IN SECONDARY HOSTS
AFTER PASSAGE THROUGH A PRIMARY HOST

<table>
<thead>
<tr>
<th>Primary Host</th>
<th>DA</th>
<th>(DA × Le) F₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary Host</td>
<td>DA*</td>
<td>F₁*</td>
</tr>
<tr>
<td>Spleen</td>
<td>38</td>
<td>33</td>
</tr>
<tr>
<td>Nodes</td>
<td>10.1</td>
<td>11.5</td>
</tr>
</tbody>
</table>

* Results are expressed as the percentage of total radioactivity injected recovered per organ.

DA thoracic duct lymphocytes sensitized against (DA × Lewis)F₁ hybrid tissues (as described under Table 5.12) were labelled and injected into DA or (DA × Lewis)F₁ primary hosts with established thoracic duct fistulae.

Labelled lymphocytes appearing in the thoracic duct of these primary hosts from 8 to 17 hours after cell transfer were reinjected into secondary DA or (DA × Lewis)F₁ hosts aged 9 weeks. These animals were killed 1 hour after cell transfer and the distribution of radioactivity was determined.
in the first 18 hours after transfer, still demonstrate an altered migratory pattern on retransfer to a secondary host.

(iii) A recovery of normal migratory properties occurs by 24 hours after transfer in the population of transfused, neuraminidase treated lymphocytes which appear in the thoracic duct lymph and in those resident in the spleen of recipients.

(iv) The migration of labelled transfused lymphocytes to the lymph nodes of lethally irradiated recipients was greater than that seen in normal recipients.

(v) Although no marked differences could be demonstrated between the localization of sensitized parental strain cells in syngeneic or F1 recipients, retransfer of these cells after passage through an F1 intermediate host suggested that some of the lymph node seeking population had been specifically removed in the F1 intermediate host.

The distribution of normal and neuraminidase treated lymphocytes in syngeneic recipients

The effect of neuraminidase treatment on the distribution of thoracic duct lymphocytes is similar to that observed by Woodruff and Gesner (1969), with the most prominent features being a decreased migration to the spleen and lymph nodes and an increased accumulation in the liver. However, by 28 hours after cell transfer, Woodruff and Gesner reported that there was an approximately equal recovery of radioactive label from the lymph nodes of recipients of both control and neuraminidase treated lymphocytes. As there was also a decrease in the radioactivity recovered from the liver by this time, they suggested that neuraminidase treated cells had emigrated from the liver and recirculated to the nodes. In contrast to these findings of Woodruff and Gesner, the present experiments showed that the proportion of injected radioactivity recovered from the lymph nodes obtained from recipients of cells incubated with neuraminidase, 24 hours after cell transfer, was only one fifth of that recovered in recipients of untreated cells. There was also three to four times as
much radioactivity in the liver of the recipients of neuraminidase treated cells at this time. There was some increase in lymph node radioactivity over the period of 24 hours in the recipients of neuraminidase treated cells, but this increase was much less than that observed in recipients of untreated cells. It could not be inferred from the node and liver localization patterns observed over the first 24 hours after transfusion that the migratory properties of neuraminidase treated cells returned to normal.

The pattern of appearance of neuraminidase treated cells in the thoracic duct lymph was quite different from that of the control cells. The peak appearance of control cells at 15-20 hours after transfusion corresponded to the peak appearance of labelled lymphocytes described in previous reports (Gowans 1959, Gowans and Knight 1964, Goldschneider and McGregor 1968b, Woodruff and Gesner 1969). The consistent peak appearance of neuraminidase treated cells at 30-35 hours was similar to the peak of 40 hours noted by Woodruff and Gesner in an experiment where labelled syngeneic cells were transferred. However, the absolute recovery of label in the present experiments was different from that obtained by Woodruff and Gesner. The proportion of injected radioactivity recovered from the thoracic duct over a period of about 70 hours was of the order of 20-30%, in the experiments of Woodruff and Gesner. Recovery of neuraminidase treated cells, although less than that of control cells, was also in this range.

In contrast, in the present experiments the total recovery over 72 hours was about 7% of the radioactivity injected on control cells and 3-4% of that on neuraminidase treated cells. This diminished migration of cells into thoracic duct lymph parallels the reduced lymph node migration seen in the present experiments when compared with those of Woodruff and Gesner.

It was demonstrated that the procedures of incubating and washing the cells entailed in labelling with $^{51}$Cr could affect their migratory pattern to some extent,
(Section 6), and this could explain some of the differences between the present results and those of Woodruff and Gesner (1969). The use of radioactive $^{51}$Cr at a concentration of 50 µc/ml in these experiments was the same as that quoted by other workers using $^{51}$Cr labelled lymphocytes for circulatory (Bainbridge, Brent and Gowland 1966, Zatz and Lance 1970, Heslop and Hardy 1971) or cytotoxicity (Wigzell 1965, Boyle 1968) studies. The concentration of sodium chromate of 0.3-0.36 µg/ml used in these experiments was 1.5 to 2 times that used by Bunting et al. (1963) and Woodruff and Gesner (1969) for labelling rat lymphocytes. This was a concentration of sodium chromate 300 times lower than the minimal level found to produce increased mechanical fragility of erythrocytes (Nechles, Weinstein and Le Roy 1953). No evidence was obtained of any cytotoxic effect of $^{51}$Cr in vitro. The viabilities of both the control and neuraminidase treated cell suspensions used in the present experiments were always greater than 95% when assessed by trypan blue uptake several hours after labelling, and the rate of elution of label from the enzyme treated cell suspension of 2% per hour was less than that quoted by other workers (Sanderson 1964, Wigzell 1965) as being normal for a viable cell population.

The demonstration that the cell labelling procedure itself can alter lymphocyte migration has important implications in the interpretation of experiments in which $^{51}$Cr labelled lymphocytes are transferred from a primary to a secondary host, in order to demonstrate a subpopulation of lymphocytes. It is necessary to obtain an assessment of the degree of alteration in migration produced by labelling procedures. Without this it may be difficult to determine whether any differences noted on retransfer of cells to secondary recipients are due to a true subpopulation of cells, or whether these differences merely reflect a recovery of the cell population from the effects of the labelling procedure prior to the original transfer.
The true migratory properties of thoracic duct lymphocytes may only be revealed after a biological screening which eliminates any cells whose circulatory ability, if not viability, has been affected by cell collection and labelling procedures. In this context it is of interest that the splenic localization of approximately 50% of the injected cells at one hour after transfer to a secondary host is 2-3 times that reported by other workers after a single transfer using $^{51}$Cr labelling techniques, (Bainbridge et al. 1966, Woodruff and Gesner 1968, 1969, Zatz and Lance 1970). Despite the simulated labelling procedure, thoracic duct lymphocytes which had migrated through a primary recipient still migrated to the spleen twice as readily as the original population by one hour after transfusion. In contrast the node seeking ability of these lymphocytes was diminished by the simulated labelling procedure (Section 6). This is similar to the observation that treatment of thoracic duct lymphocytes by trypsin reduced the localization of these cells in the lymph nodes but not in the spleen after transfer to recipient rats (Woodruff and Gesner 1968). Both observations point to the possibility that factors which determine migration to the lymph nodes are different from those which determine migration to the spleen.

The migratory properties of neuraminidase treated lymphocytes which enter the thoracic duct in the first 18 hours after transfusion

Transfused, neuraminidase treated lymphocytes which have entered the recipients' thoracic duct lymph in the first 18 hours after transfer, still possess an altered migratory pattern on retransfer to a secondary host. This demonstrates two points. Firstly, it excludes an impairment of metabolic processes required for entry into the recirculating pool as a cause for the altered migratory behaviour of lymphocytes treated with neuraminidase. This has been proposed as a possible explanation by Woodruff and Gesner (1969).
Secondly, it shows that cell surface modification produced by incubation with neuraminidase does not necessarily prevent migration into the thoracic duct. It seems reasonable to assume that such neuraminidase treated lymphocytes as appear in the thoracic duct have entered from the bloodstream into the lymph by way of the postcapillary venules of the lymph nodes. This assumption is substantiated by the findings of Gesner, Woodruff and McCluskey (1969) who showed, by means of autoradiography, that neuraminidase treated lymphocytes entered the nodes after transfusion by the same anatomical route as untreated cells. In the present experiments neuraminidase treated cells which had migrated from the bloodstream into the thoracic duct could be shown to still possess the migratory properties of the original enzyme treated population. This finding casts doubt on the suggestion that interference with a recognition mechanism between the endothelial cells of the postcapillary venules and the enzyme treated lymphocytes is the cause of changes in cell migration after neuraminidase treatment (Woodruff and Gesner 1969).

It would seem more probable that the alteration in cell migration after incubation in neuraminidase is due to surface changes which cause the lymphocytes to be preferentially retained in the liver and hence unavailable to enter lymphoid tissue and recirculate via the thoracic duct.

The experiments on the retransfer of labelled transfused lymphocytes also demonstrate that the neuraminidase treated lymphocytes, which home into the thoracic duct lymph and spleen soon after transfusion, are not a special subpopulation with normal migratory characteristics, but have similar migratory properties themselves to the whole population. The localization of lymphocytes in the spleen and thoracic duct lymph soon after transfusion would act as a selective factor for any subpopulation with normal migratory characteristics. If such a subpopulation was present in the neuraminidase treated cells, this population should show normal migratory properties on transfer to
secondary hosts. However, the cells from the neuraminidase treated population that enter the spleen and thoracic duct soon after transfusion, migrate in a similar fashion to the original neuraminidase treated population on transfer to secondary hosts.

The recovery of normal migratory properties by the neuraminidase treated cell population

A recovery of normal migratory properties was shown to occur by 24 hours after transfer in the population of transfused, neuraminidase treated cells which appear in the thoracic duct lymph and in those resident in the spleen (Sections 4 and 5). The increasing appearance of neuraminidase treated lymphocytes in the thoracic duct lymph over the first 30 hours after transfusion was paralleled by a decrease in the migration index, and an increase in node seeking ability of these cells on transfer to a secondary recipient. This return of normal migratory properties excludes the possibility that the increasing migration of neuraminidase treated cells into the thoracic duct can be explained by the release of cells with their cell surface still altered, from a place where they were previously sequestered such as the liver.

The most likely explanation for the change in migratory properties of the neuraminidase treated cells after a period of residence within the host is the recovery of the surface sialyl groups previously removed by neuraminidase. That sialic acid release by neuraminidase can explain the migratory changes, is shown by the ability of excess free sialic acid during incubation to inhibit the effect of neuraminidase treatment on the migratory properties of the cells. There is no question of reutilization of label by the host cells as the radioactivity detected in the thoracic duct was shown to be associated with the population of cells that was originally treated with neuraminidase.

An alternative explanation may be proposed, that the alterations in the migratory ability of neuraminidase treated cells appearing in the thoracic duct lymph reflect
a biological screening phenomenon. The changing migratory ability of the cell population would be due to removal by the host of cells damaged by neuraminidase treatment, rather than to the regaining of normal surface properties. Thus soon after transfusion, the cell population available for migration into the thoracic duct lymph contains a mixture of cells with normal and abnormal migratory properties. As the cells with abnormal migratory properties are removed (e.g. by spleen and liver), with time only those cells with a normal migratory pattern are available to migrate into the thoracic duct lymph, and so the migratory pattern of cells appearing in thoracic duct lymph returns to normal.

Such a proposition assumes that the cells from the neuraminidase treated population, appearing in the thoracic duct lymph with a normal migration index 30 hours or so after transfusion, were present in the transfused population. If this were so, it would be expected that these cells would have migrated as untreated cells after injection, with a peak appearance at 15-20 hours in the thoracic duct. This would not be consistent with the observed peak appearance at about 35 hours.

Another point favouring recovery of surface properties rather than biological screening as an explanation, is the changes noted in the migration of cells localizing in the spleen. It appears from these experiments that the spleen can remove or accumulate damaged cells from the circulating population. Of labelled normal thoracic duct lymphocytes which localize in the spleens of primary hosts at 1 hour after transfusion, 15% migrate to the liver on retransfer to a secondary host. Labelled normal thoracic duct lymphocytes localized in the spleen 24 hours after transfusion, when transferred to secondary hosts, show 30% localization in the liver. A likely explanation is the accumulation of damaged cells in the spleen of the primary host over the period after transfusion. At one hour after transfusion, cells would be distributed around the body in a random manner, while by 24 hours
the spleen has had time to accumulate damaged cells incapable of recirculating and to lose those recirculating cells to the lymph nodes. On retransfusion many of these damaged cells would localize in the liver. It is known that cells purposely damaged or killed *in vitro* localize predominantly in the liver after transfer (Woodruff and Gesner 1969, Heslop and Hardy 1971). If the changes in migratory properties of the neuraminidase treated cell population are due to removal of damaged cells it would also be expected that the spleen would screen out these damaged cells from the circulation. Thus, on retransfer of the neuraminidase treated cell population localized in the spleen at 24 hours after transfusion, many of these damaged cells should localize in the liver. However, the neuraminidase treated cell population in the spleen at 24 hours migrates *less* readily to the liver, and *more* readily to the lymph nodes and spleen than the control cell population present in the spleen at that time. These observations do not support the explanation that changes observed in the migratory properties of neuraminidase treated cells with time can be accounted for by the screening of damaged cells.

The time taken for recovery of normal surface properties by the neuraminidase treated cells is about 24 hours. The migratory properties of labelled, transfused, neuraminidase treated cells reappearing in the thoracic duct lymph of primary recipients from 18-24 hours were approaching those of the control cell population (Table 5.6). This period of recovery could also explain the observation that the labelled neuraminidase treated cell population resident in the spleens of primary recipients 24 hours after transfer, shows a *greater* homing tendency to the spleen and lymph nodes than the untreated labelled cell population resident in the spleen at 24 hours (Fig. 5.5). The migration of this neuraminidase treated cell population after transfer to a secondary host is very similar to that of untreated labelled cells resident in the spleen of the primary host one hour after transfer (Fig. 5.5). The neuraminidase treated lymphocytes present
in the spleen at 24 hours have recently recovered their normal migratory properties. Thus they are able to migrate on retransfer as the control cells which had localized in the spleen of a primary host at 1 hour after transfer were able to do. By 24 hours some of the control cell lymph node seeking population has had time to leave the spleen and enter the recirculating lymphocyte pool, and hence at this time the population of neuraminidase treated cells in the spleen will show a greater tendency to home to the lymph nodes than do the control cells, after transfer to secondary recipients.

The migration of lymphocytes in lethally irradiated recipients

The increased migration of control and neuraminidase treated lymphocytes to the lymph nodes of lethally irradiated recipients was of interest. In contrast, Lance and Taub (1969) did not observe any appreciable increase in the localization of $^{51}$Cr labelled lymph node cells in recipient mice irradiated with 900 rad, 48 hours prior to infusion of the cells. The most likely explanation for the increase noted in the present experiments is that reduction of the recirculating lymphocyte population has reduced competition for migration across the postcapillary venules. There is a rapid and severe depletion of recirculating lymphocytes in rats receiving 300 rad total body irradiation (Everett et al. 1964). A decrease in the number of cells in the thoracic duct lymph was reported by 4 hours after irradiation while by 24 hours the cell output approaches its minimum value of roughly 15% of the preirradiation level. Similarly, the cellular content of the cervical node had reached a minimum by 30 hours after 300 rad irradiation (Benninghoff et al. 1969). It is certain that the 910 rad given in the present experiments would have drastically depleted the recirculating lymphocyte pool by 24 hours after irradiation. The migration of lymphocytes from the blood stream into the lymph nodes via the postcapillary venules is probably a competitive process. Due to the depletion of the recirculating
lymphocyte population by 90% or more after irradiation (Everett, Caffrey and Rieke 1964), there will be much less competition from host lymphocytes for migration across the postcapillary venules by the transfused lymphocytes, and therefore a greater node localization of injected cells will be observed.

The present results imply that the process of migration of lymphocytes into the lymph nodes via the postcapillary venules is unaffected by irradiation. This point has been confirmed by Everett, Caffrey and Rieke (1964) who showed that labelled small lymphocytes can migrate into the thoracic duct of irradiated recipient rats. In addition Hall and Morris (1964) demonstrated that 2000 rad local irradiation to the popliteal node in sheep caused a temporary fall in lymphocyte output from that node, but this cell output returned to normal levels by 20 hours after irradiation.

The distribution of parental cells in F₁ hybrid recipients

The present experiments demonstrated that incubation of parental lymphocytes with neuraminidase produced changes in the distribution of these cells after transfer to F₁ hybrid recipients, similar to those observed after transfer to syngeneic recipients. However, it was not possible to detect any marked differences in the distribution of normal parental cells in F₁ hybrid recipients or syngeneic recipients even if the parental cells had been sensitized against the F₁ hybrid recipient prior to transfer.

It was noted that after the passage of sensitized parental cells through an F₁ recipient, the node seeking ability of these cells on retransfer to either F₁ or parental hosts was reduced by 20% as compared to parental cells passaged through a parental recipient. This finding could suggest a preferential removal of the lymph node seeking population in the primary F₁ host as compared to the primary DA host. It would require greater numbers of experiments to be fully substantiated. In a more extensive series of experiments Ford (1971) arrived at
a similar conclusion. He was able to demonstrate differences in the fate of parental and F₁ lymphocytes after transfer to an F₁ recipient, using a double labelling technique. Populations of parental and F₁ lymphocytes were labelled, one with ³H uridine, the other with ¹⁴C, and a mixture of the two was injected into F₁ recipients. There was a constant deficit of parental cells in the recipients' thoracic duct and a surplus in the spleen as compared to the F₁ cells. The figures were compatible with a sequestration of 14% of the parental population in the spleen of the F₁ recipient, which is comparable to the figure obtained in the present experiments.

The experiments described in this chapter have shown that treatment of thoracic duct lymphocytes with neuraminidase can alter their migratory behaviour after transfer to syngeneic, semi-allogeneic or lethally irradiated recipients. It was considered that the decreased migration of enzyme treated lymphocytes to the lymph nodes and into the thoracic duct lymph was due to the fact that the enzyme treated lymphocytes had been sequestered in the liver, rather than that removal of cell surface sialic acid had interfered with a specific recognition process occurring at the endothelial surface of the postcapillary venules. The migratory properties of labelled enzyme treated lymphocytes obtained from the spleen or thoracic duct were demonstrated to return to normal over the first 24 hours after transfusion.
CHAPTER 6
THE MIGRATORY PROPERTIES OF THYMOCYTES

It has been shown previously (Chapter 3) that thymus cell populations differed from thoracic duct lymphocytes in that only half as much kiwi was on a cell for cell basic could be released by treatment with neuraminidase. Migration studies have shown that labelled rat thymus cells migrated in a different fashion to thoracic duct lymphocytes after intravenous transfer (Goldmannsifer and McGregor 1968 and L. Barney and Casper 1970). This difference in migratory properties of thoracic duct lymphocytes and thymocytes may be related to surface differences between the two types of lymphocyte.

Whilst the thymus cell population as a whole possesses different migratory and circulatory characteristics from thoracic duct lymphocytes, experiments in mice have indicated once more there exists a subpopulation of thymus cells and ready to redistribute (Lancé and Lancé 1970, Lancé, Cooper and Boyce 1971, Half 1972).

The following experiments were performed to confirm the results of other workers on the distribution of labelled thymus cells and to provide a comparison with the results of the previous chapter on the migration of thoracic duct lymphocytes. In addition some properties of the subpopulation of thymus cells which migrated into the thoracic duct were examined.

RESULTS
The distribution of normal thymocytes in syngeneic recipients

The distribution of labelled thymus cells after intravenous injection into syngeneic recipients is shown in Table 41. Thymus cells were retained in the lungs to a much greater extent than thoracic duct lymphocytes at 1 hour after injection. However, this retention was transient. At all times examined, the migration of thymus cells to the spleen and lymph nodes was less, and to the liver more, than that of thoracic duct
THE MIGRATORY PROPERTIES OF THYMOCYTES

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Whilst the thymus cell population as a whole possesses different migratory and circulatory characteristics from the thoracic duct lymphocytes, experiments in mice have indicated that there exists a subpopulation of thymus cells which are mature and ready to recirculate (Lance and Taub 1969, Zatz and Lance 1970, Lance, Cooper and Boyse 1971, Raff 1971).

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### TABLE 6.1
THE DISTRIBUTION OF THYMUS CELLS IN SYNGENEIC RECIPIENTS

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Hour</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>0.14</td>
</tr>
<tr>
<td>Spleen</td>
<td>5</td>
</tr>
<tr>
<td>Liver</td>
<td>22</td>
</tr>
<tr>
<td>Lung</td>
<td>22</td>
</tr>
<tr>
<td>Small Bowel</td>
<td>1.1</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.02</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Results are expressed as the percentage of total radioactivity injected recovered per organ.

Thymus cells obtained from 8 week old (Lewis x DA)F1 female hybrids were labelled with $^{51}$Cr. 40 - 50 x $10^6$ thymocytes were injected intravenously into 8 week old syngeneic female recipients. Recipients were killed at 1, 8 or 24 hours after cell transfer and the distribution of radioactivity was determined. The results from 2 separate experiments are presented. Viability of the cells prior to injection as assessed by trypan blue uptake was greater than 95% in both experiments.
lymphocytes. The migratory pattern of the normal thymus cells was similar to that seen after treatment of thoracic duct lymphocytes with neuraminidase.

The migration of thymocytes into the thoracic duct

The decreased migration of injected thymus cells to the lymph nodes suggested that their appearance in the thoracic duct would be much less than that of injected thoracic duct lymphocytes. This was confirmed by three experiments in which labelled thymus cells were transfused to syngeneic recipients with established thoracic duct fistulae. The appearance of these labelled cells in the thoracic duct is shown in figure 6.1. The total recovery of labelled thymus cells from the thoracic duct was only one tenth of the recovery after transfusion of labelled thoracic duct cells (Chapter 5). There was rise to a peak recovery at about 26 hours which occurred at a later time than the peak recovery of normal thoracic duct lymphocytes.

Retransfer of labelled thymus cells

The labelled thymus cells that migrated into the thoracic duct lymph were reinjected into secondary recipients and their distribution in these recipients at 1 hour after injection was determined. Table 6.2 shows that those thymus cells that can migrate into the thoracic duct of the primary recipients possessed vastly different migratory properties from the original population, notably there was a ten fold increase in propensity to home to the spleen and a forty to fifty fold increase in lymph node seeking ability, as compared with the distribution in a primary host (Table 6.1). In fact thymocytes which have entered the thoracic duct behave in a similar fashion on retransfer to labelled normal thoracic duct cells which have been passaged through a primary recipient (Chapter 5).
FIGURE 6.1
THE MIGRATION OF THYMUS CELLS
INTO THE THORACIC DUCT

A. B. C. (Lewis x DA)F₁ thymocytes to (Lewis x DA)F₁ hybrid recipients

Thymus cells obtained from 8 week old (Lewis x DA)F₁ hybrid rats were labelled with ⁵¹Cr. 300-500 x 10⁶ of these lymphocytes were injected intravenously into syngeneic recipients with an established thoracic duct fistula of 24 hours duration.

The thoracic duct lymph of these recipients was examined for the appearance of labelled cells over 8 hour intervals for the first 32 hours after the transfusion of labelled cells, and over 12-14 hour intervals thereafter.

The appearance of radioactivity is expressed as the percentage of the total radioactivity administered which appeared in the thoracic duct lymph per hour. This value is recorded for each successive collection of lymph.

The total lymphocyte output (labelled + unlabelled cells) from each recipient is recorded at the top of each figure.

Each curve represents the results obtained from one recipient rat.

The cell viability prior to injection as assessed by trypan blue uptake was greater than 95% in all three experiments.
TABLE 6.2
THE DISTRIBUTION OF LABELLED THYMOCYTES
IN A SECONDARY HOST AFTER MIGRATION INTO THE
THORACIC DUCT OF A PRIMARY HOST

<table>
<thead>
<tr>
<th>Time of collection after injection into primary host (Hours)</th>
<th>Distribution in secondary host*</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Spleen</td>
<td>% liver</td>
</tr>
<tr>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>6 - 14</td>
<td>44</td>
</tr>
<tr>
<td>14 - 22</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>53</td>
</tr>
<tr>
<td>22 - 30</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>30</td>
</tr>
</tbody>
</table>

* Results expressed as the percentage of total radioactivity injected recovered per organ.

Thymus cells obtained from 8 week old (Lewis x DA)F₁ hybrid rats were labelled with $^{51}$Cr. 300-500 x 10⁶ of these lymphocytes were injected intravenously into primary syngeneic recipients with an established thoracic duct fistula of 24 hours duration.

Labelled thymocytes collected from the thoracic duct of the primary recipients over the periods indicated (hours), were reinjected intravenously into secondary recipients. The secondary recipients were killed 1 hour later and the distribution of radioactivity was determined.
DISCUSSION

These experiments have confirmed the observation that rat thymocytes localize in the lungs and liver more readily than thoracic duct lymphocytes at early intervals after injection (Goldschneider and McGregor 1968b), Berney and Gesner 1970). The migration of normal thymus cells after transfer to a primary host was reminiscent of that seen after transfer of neuraminidase treated thoracic duct lymphocytes, with a high proportion of cells going to the liver, and a much smaller number to the spleen and nodes as compared to normal thoracic duct lymphocytes. The peak appearance of thymocytes in the thoracic duct lymph at 26 hours was also later than the peak appearance of normal thoracic duct lymphocytes. Thus two different types of lymphocyte, with less surface sialic acid than normal thoracic duct lymphocytes, exhibit similar migratory tendencies. Although the correlation is indirect, it may be that differing amounts of sialic acid on the cell surface of thymocytes and normal thoracic duct lymphocytes are responsible for the different migratory properties of the two populations.

The total recovery of the injected thymus cells was 1/10th of that obtained after the injection of thoracic duct cells. If thoracic duct lymphocytes are considered to be predominantly a recirculating population it appears that 10% of the population of thymus cells also have similar characteristics, on the basis of their recovery from the thoracic duct in the present experiments. This is the same as the figure calculated for the percentage of thymus cells with the migratory properties of peripheral lymphocytes, obtained in rats using tritiated uridine as a cell label (Goldschneider and McGregor 1968b), and mice using $^{51}$Cr (Lance and Taub 1969) or tritiated adenosine (Parrott and de Sousa 1969). The difference between these experiments and the current ones was that, apart from the experiments of Goldschneider and McGregor (1968b), the conclusions were drawn from the proportion of thymus cells localizing in lymph nodes.
after transfer, while the current experiments have directly examined migration into the thoracic duct.

The migratory properties of thymocytes appearing in the thoracic duct were quite different from those of the original population. This strongly implies the existence of a subpopulation in the thymus with the homing characteristics of thoracic duct lymphocytes. There is other evidence for the existence of a subpopulation of thymocytes with different migratory and metabolic properties. Mouse thymus cells localizing in lymph nodes show migratory behaviour on retransfer similar to thoracic duct lymphocytes (Lance and Taub 1969, Zatz and Lance 1970). The experiments of Goldschneider and McGregor (1968b) demonstrated that thymocytes localizing in lymphoid tissues were a special population which utilized tritiated uridine in a similar manner to thoracic duct lymphocytes, but different from that of the remainder of the thymus cell population.

The differences noted in the present experiments, between the migratory properties of those labelled thymus cells entering the thoracic duct lymph and the original population, are unlikely to be due solely to the screening phenomenon after labelling which has been demonstrated to occur in the case of thoracic duct lymphocytes (Chapter 5, section 6). In the case of thoracic duct lymphocytes, the increase in node and spleen seeking ability after passage through a primary recipient was 5 fold and 2 fold respectively. However, the increase in node seeking ability of the thymus cell population entering the thoracic duct was 40-50 fold and of the spleen seeking ability was 8-10 fold, as compared to the original population. This is too great a difference to be accounted for by the effects of the labelling procedure alone. A 10 fold increase in lymph node seeking ability, after transfer to secondary recipients, was described in those $^{51}$Cr labelled thymus cells which localized in the lymph nodes of primary recipient mice (Lance and Taub 1969, Zatz and Lance 1970). However
these workers did not indicate the degree to which their cell labelling procedure could affect migration.

The acquisition of the migratory properties of recirculating lymphocytes by thymus cells may be related to changes in surface structure. A decrease in susceptibility to the cytotoxic effect of anti-\(\theta\) (Raff 1971) or anti-TL (Lance, Cooper and Boyse 1971) antisera has been noted in those mouse thymocytes which localize in the peripheral lymphoid tissues after \(^{51}\)Cr labelling and intravenous injection. In view of the known increased susceptibility of thymus or lymph node cells to the cytotoxic action of anti-\(\theta\) antiserum, after treatment of the cells with neuraminidase (Schlesinger and Amos 1971), it is interesting to speculate whether the decreased cytotoxic effect of anti-\(\theta\) antiserum on thymocytes localizing in lymphoid tissue is due to an increase in the surface sialic acid of these cells.
CHAPTER 7

THE MAINTENANCE OF NEURAMINIDASE TREATED LYMPHOCYTES ON RAT EMBRYO MONOLAYERS

It has been shown that lymphoid cells derived from the lymph nodes or thoracic duct lymph of rats can be maintained in vitro on rat embryo fibroblast monolayers (Ginsburg and Sachs 1965). The behavior of the cultured lymphocytes varies with the nature of the monolayer donors, syngeneic or allogeneic. Cultures on a syngeneic monolayer is characterized by a relatively better survival of small lymphocytes, and by the appearance of varying numbers of large lymphoid cells which differentiate to histiocytes ormacrophage-like lymphocytes. In contrast, when lymphocytes and monolayer donors are both from a randomly bred colony, or when lymphocytes are from rats of certain inbred strains, and monolayers from randomly bred rats, lymphoid cell aggregation occurs at an early stage followed by the transformation of lymphocytes to blast cells. A type of reaction of this nature in explants of blastocysts, accompanied by the production of large numbers of blast cells. This type of reaction occurs with syngeneic and with certain allogeneic combinations, and has been designated the 'graft reaction culture' (Ginsburg 1970). It has been suggested that these features of blast cell transformation and lysis of the monolayer are an in vitro model of the graft-vs-host reaction (Ginsburg and Sachs 1965).

Such a tissue culture system would lend itself to the examination of several facets of the behavior of neuraminidase treated lymphocytes. In particular, the capacity of thoracic duct lymphocytes to transform into blast cells after treatment with neuraminidase could be observed. Additionally, as the survival of lymphocytes on these monolayers is much superior to that during maintenance in nutrient media alone, it could be a suitable means of maintaining neuraminidase-treated lymphocytes for a period in vitro, to determine whether their migratory properties could return to normal.
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Such a tissue culture system would lend itself to the examination of several facets of the behaviour of neuraminidase treated lymphocytes. In particular, the capacity of thoracic duct lymphocytes to transform into blast cells after treatment with neuraminidase could be observed. Additionally, as the survival of lymphocytes on these monolayers is much superior to that during maintenance in nutrient media alone, it could be a suitable means of maintaining neuraminidase treated lymphocytes for a period in vitro, to determine whether their migratory properties could return to normal.
These observations would be of interest in light of the findings in Chapter 5 which indicated that a return of normal migratory properties to the neuraminidase treated lymphocytes had occurred in vivo.

**RESULTS**

1 **PRELIMINARY EXPERIMENTS TO ESTABLISH OPTIMAL MONOLAYERS**

Preliminary experiments were conducted to find out the optimum number of embryonic cells and the time needed to establish a suitable monolayer. Cell numbers of $10^5$, $10^6$, $10^7$ embryonic cells were plated in 4 ml of medium in Falcon plastic bottles. It was found that $10^6$ cells formed a suitable monolayer after about 7 days culture. Satisfactory monolayers could also be formed by plating $10^7$ cells, re-trypsinizing these cells after 3 or 4 days and replating out $5-10 \times 10^5$ cells to each culture. Such monolayers would satisfactorily support $50-60 \times 10^6$ thoracic duct lymphocytes, and this was the standard number added to the monolayers in the following experiments.

In order to establish the viability and functional capabilities of thoracic duct cells after maintenance on the monolayer, (Lewis x DA)$F_1$ thoracic duct lymphocytes were maintained on syngeneic monolayers for 72 hours. After this period, the viability as determined by trypan blue uptake was 81%. 100 x $10^6$ viable cells were injected intravenously together with sheep erythrocytes into a (Lewis x DA)$F_1$ hybrid recipient which had been given 730 rad irradiation 24 hours previously. Serum haemolysin titrations were performed daily. The peak serum haemolysin titre of 1024 was the same as the mean of 5 similarly treated animals each of which had received an equal number of freshly collected, unincubated thoracic duct lymphocytes.

2 **BEHAVIOUR OF (LEWIS X DA)$F_1$ THORACIC DUCT LYMPHOCYTES ON OUTBRED MONOLAYERS**

50 x $10^6$ thoracic duct lymphocytes from (Lewis x DA)$F_1$ hybrid rats, either untreated or previously incubated with neuraminidase, were plated onto outbred rat embryo monolayers. The cells were observed daily for 14 days and, at intervals, smears of a small sample of the floating
cell population were made using a cytocentrifuge. There was no difference in the behaviour of neuraminidase treated and control lymphocytes. The sequence of aggregation, and large cell formation was similar to that described by Ginsburg and Lagunoff (1968). Thus, lymphoid aggregates were clearly visible by 48 hours after plating, and large cells became prominent by 6 days. At this stage, after removal of the free floating lymphocytes from the monolayers, aggregates of lymphoid cells remained attached to the fibroblasts. The aggregates started to decrease in size about 12 days after plating and disappeared by 16 days. 20 days after the plating of thoracic duct lymphocytes the monolayers were still intact.

Smears of cells from the cultures were made on 1, 3, 6, 8, and 14 days after plating (Fig. 7.1). Examination of these confirmed the observations made on living cells with phase contrast, namely that very few large blast type cells were present in the first 3 days after plating (Fig. 7.1A and B) but that such cells were starting to appear by 6 days (Fig. 7.1D and C) and were plentiful at 14 days (Fig. 7.1E and F). Figure 7.2 shows the percentage of blast cells in the cell smears obtained from the cultures. Treatment with neuraminidase has not inhibited the ability of thoracic duct cells to transform into such cells. It was also concluded that the alterations produced in the cell surface by neuraminidase did not affect the ability of the lymphocytes to aggregate in clumps, and did not appear to alter their survival on the monolayers.

3 BEHAVIOUR OF (LEWIS X DA)F₁ THORACIC DUCT LYMPHOCYTES ON SYNGENEIC MONOLAYERS

Neuraminidase treated or untreated control thoracic duct lymphocytes from (Lewis x DA)F₁ hybrid rats, did not form aggregates when plated on syngeneic monolayers. After removal of free floating lymphocytes by gentle agitation followed by aspiration of the medium, very few lymphocytes remained attached to the monolayers. This contrasts with the behaviour observed when the
FIGURE 7.1
The morphology of control and neuraminidase treated (Lewis x DA)F₁ thoracic duct lymphocytes after maintenance on outbred rat embryo monolayers.

A. Control lymphocytes at 24 hours
The cell population consists predominantly of small lymphocytes with occasional granulocytes or medium lymphocytes. Some erythrocytes can also be seen.

B. Neuraminidase treated lymphocytes at 24 hours
The cell population is similar to that seen in A.

C. Control lymphocytes at 8 days
Some large blast cells are present (arrows).

D. Neuraminidase treated lymphocytes at 8 days
Some large blast cells are present (arrows).

E. Control lymphocytes at 14 days
There is a greater proportion of blast cells present.

F. Neuraminidase treated lymphocytes at 14 days
There is a greater proportion of blast cells present.

McNeal's Tetrachrome Magnification x 330
FIGURE 7.2

THE PERCENTAGE OF BLAST CELLS IN THE CELL POPULATION
OBTAINED FROM CULTURES OF THORACIC DUCT LYMPHOCYTES
ON RAT EMBRYO MONOLAYERS

50-60 x 10^6 untreated control or neuraminidase
-treated (Lewis x DA) F1 thoracic duct lymphocytes
were plated on outbred rat embryo monolayers. Smears
were made from the free floating cell population of
the same culture, at the times shown after plating,
in each case. The percentage of blast cells in the
smears was counted.
cells were plated on outbred rat embryo monolayers described above, where the formation of cell aggregates which adhered to the monolayer was a prominent feature.

4 MIGRATION OF LYMPHOCYTES AFTER MAINTENANCE ON MONOLAYERS

The aim of the present experiments was to see if the migratory pattern of neuraminidase treated cells would revert towards normal after a period of maintenance on the monolayer. A positive result would suggest that regeneration of normal surface properties had occurred.

The lymphocytes were labelled with $^{51}$Cr before or after plating on the monolayer, and their distribution in syngeneic recipients was determined at various intervals after plating. All the following experiments were performed utilizing (Lewis x DA)$F_1$ thoracic duct lymphocytes and (Lewis x DA)$F_1$ hybrid rat embryo monolayers. After removal from the monolayer, the lymphocytes were injected into syngeneic (Lewis x DA)$F_1$ hybrid male or female recipients aged from 7 to 10 weeks.

Change in Migration with time on the monolayer

(Lewis x DA)$F_1$ thoracic duct lymphocytes were labelled with $^{51}$Cr, treated with neuraminidase or left as control, and $50 \times 10^6$ cells plated onto $F_1$ monolayers. Labelled cell samples were withdrawn from the monolayers at the times shown in Table 7.1, injected into syngeneic $F_1$ recipients, and their distribution determined. The distribution may be compared with that of the same cell population which was transferred immediately after labelling without maintenance on the monolayer (Table 7.1).
TABLE 7.1

DISTRIBUTION OF THORACIC DUCT CELLS
IN SYNGENEIC RECIPIENTS AFTER MAINTENANCE
ON RAT EMBRYO MONOLAYERS. THE CELLS
WERE LABELLED WITH $^{51}$Cr PRIOR TO
PLATING ON THE MONOLAYERS

<table>
<thead>
<tr>
<th>Hours on monolayer</th>
<th>CONTROL*</th>
<th>NEURAMINIDASE*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>Not maintained on monolayer</td>
<td>18</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>18</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>24</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>48</td>
<td>29</td>
<td>12</td>
</tr>
</tbody>
</table>

* % distribution of label at 1 hour after transfer to syngeneic recipients

The distribution of neuraminidase treated lymphocytes remained different from that of the control cells regardless of the period of incubation on a monolayer, with greater migration to the liver and less to the spleen and lymph nodes. The proportion of both control and enzyme treated lymphocytes localizing in the lymph nodes was greater between 6 and 24 hours than in the original samples.

Retreatment of lymphocytes with neuraminidase after 24 hours on monolayer

If an alteration in migratory pattern could be shown by retreating cells with neuraminidase after a period on the monolayer, this would be suggestive of some resynthesis of surface sialic acid.

In order to confirm that retreatment of lymphocytes per se with neuraminidase did not further alter migration, a freshly collected sample of thoracic duct lymphocytes was treated with neuraminidase and divided into two parts, one of which was reincubated with neuraminidase.
immediately. The distribution of these cells, after one or two incubations with neuraminidase, in syngeneic recipients was then determined. Retreatment immediately with enzyme did not appreciably alter the migration of the cells (Table 7.2).

**TABLE 7.2**

**DISTRIBUTION OF THORACIC DUCT CELLS**
**AFTER 1 OR 2 TREATMENTS WITH NEURAMINIDASE**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Neuraminidase* Treatment x 1</th>
<th>Neuraminidase* Treatment x 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>5.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Liver</td>
<td>36</td>
<td>37</td>
</tr>
</tbody>
</table>

* % distribution of label at 1 hour after transfer to syngeneic recipients.

The following experiments were performed to determine whether retreatment of lymphocytes with neuraminidase after incubation on the monolayers could again alter their localization after transfer to syngeneic recipients. Thoracic duct cells were labelled, treated with neuraminidase or left untreated as a control, and plated on monolayers. Cells from both the control and neuraminidase treated groups were removed at 24 hours, half the cells from each group were then treated with neuraminidase, the other half was left untreated. The cells were then injected intravenously into syngeneic recipients and their distribution to the liver, spleen and lymph nodes was determined (Table 7.3).
TABLE 7.3
INCUBATION OF LYMPHOCYTES WITH NEURAMINIDASE
AFTER 24 HOURS ON MONOLAYER. THE CELLS
WERE LABELLED WITH $^{51}$Cr PRIOR TO
PLATING ON MONOLAYER

<table>
<thead>
<tr>
<th>Treatment Prior to Plating on Monolayer</th>
<th>Control*</th>
<th>Neuraminidase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment After 24 Hours on Monolayer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>23</td>
<td>43</td>
</tr>
<tr>
<td>Spleen</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Nodes</td>
<td>3.1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* % distribution of label at 1 hour after transfer to syngeneic recipients

As noted in a previous experiment (Table 7.1) the migratory capacity of the neuraminidase treated population did not return to that of a similarly treated control cell population. In both cases, treatment with neuraminidase after 24 hours on the monolayer, altered the migratory pattern of the cells, so that after treatment the two populations distributed in a similar manner.

Labelling of cells after period on monolayers

Experiments similar to those described above, were performed, with the difference that the lymphocytes were labelled with $^{51}$Chromium after maintenance on the monolayers. Control or neuraminidase treated thoracic duct lymphocytes were plated onto monolayers and removed 24 or 72 hours later, labelled with $^{51}$Cr and half of the control and neuraminidase treated cell populations were then treated with neuraminidase. The viability of the labelled cells was determined using trypan blue. Table 7.4 shows the migration of these cells after injection into syngeneic recipients.
TABLE 7.4

INCUBATION OF LYMPHOCYTES WITH NEURAMINIDASE
AFTER 24 HOURS ON MONOLAYER. THE CELLS WERE
LABELLED WITH $^{51}$Cr AFTER REMOVAL FROM MONOLAYER

<table>
<thead>
<tr>
<th>Treatment prior to plating on monolayer</th>
<th>Control*</th>
<th>Neuraminidase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment after 24 hours on monolayer</td>
<td>Control</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td></td>
<td>Neuraminidase</td>
<td>Control</td>
</tr>
<tr>
<td>Liver</td>
<td>12</td>
<td>45</td>
</tr>
<tr>
<td>Spleen</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>Nodes</td>
<td>5.6</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* % distribution of label at 1 hour after transfer to syngeneic recipients.

Viability as assessed by trypan blue uptake after removal from the monolayer was 90% for the control lymphocytes and 91% for the neuraminidase treated lymphocytes.

After 24 hours on the monolayer the cell population which was originally treated with neuraminidase still migrates in a different fashion to the control population. However, treatment of both these populations with neuraminidase after removal from the monolayer altered their migratory pattern, resulting in a greater migration to the liver and less to the nodes and spleen than the equivalent untreated population (Table 7.4).

As the migratory capacity of neuraminidase treated lymphocytes had not completely returned to that of the control population by 24 hours, incubations on the monolayer were extended to 72 hours. The in vitro regeneration of normal cell surface properties by other types of neuraminidase treated lymphoid cells was shown to take at least 4 days (Pardoe et al. 1970, Grothaus et al. 1971). Lymphocytes were maintained for 72 hours on monolayers and then labelled with $^{51}$Cr and injected into syngeneic recipients to determine their migratory properties. Table 7.5 shows the results of a representative experiment of this type. Differences in migration between...
TABLE 7.5
DISTRIBUTION OF LYMPHOCYTES AFTER 72 HOURS ON THE MONOLAYER.
THE CELLS WERE LABELLED WITH $^{51}$Cr AND TREATED AS INDICATED,
AFTER REMOVAL FROM MONOLAYER

<table>
<thead>
<tr>
<th>Treatment prior to plating on monolayer</th>
<th>Organ</th>
<th>Treatment after removal from monolayer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Spleen</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Nodes</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>10</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>Spleen</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Nodes</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>10</td>
</tr>
</tbody>
</table>

*% distribution of label at 1 hour after transfer to syngeneic recipients. Viability as assessed by trypan blue uptake after removal from the monolayer was 79% for control lymphocytes and 83% for neuraminidase treated lymphocytes.
the original neuraminidase treated and control cell populations remained after 72 hours incubation on monolayers. Treatment with neuraminidase can alter the distribution of both populations and excess sialic acid can partially prevent this.

Four separate experiments of the type shown in Table 7.5 were performed and the migration indices of lymphocytes removed from monolayers were calculated before and after treatment with neuraminidase. Table 7.6 shows the mean and standard error of the migration index. The migration indices of the control, 2.3, and neuraminidase treated populations, 5.6, were still different after 72 hours on the monolayer, with fewer of the control population settling in the liver and more in the spleen. An excess of free sialic acid in the incubation medium can largely prevent the alteration in the migration index produced by neuraminidase treatment of the cells in both cases (Table 7.6).

**DISCUSSION**

The experiments described above have examined the ability of neuraminidase treated thoracic duct lymphocytes to transform into blast cells when cultured on allogeneic rat embryo monolayers, and the changes in their migratory properties when cultured on syngeneic monolayers. After maintenance on the monolayers for 72 hours, the lymphocytes retained their normal functional capacity as judged by their ability to restore the immune response to an irradiated rat.

(Lewis x DA)\(F_1\) thoracic duct lymphocytes maintained on an outbred embryo monolayer behaved in a manner similar to that described by Ginsburg and Lagunoff (1968). These workers cultured lymphocytes from outbred Sprague-Dawley or inbred Lewis strain rats on outbred Sprague-Dawley embryo monolayers. The identical behaviour of the neuraminidase treated and control lymphocytes in the present experiments indicated that the removal of cell surface sialic acid had not affected the ability of the lymphocytes to form lymphoid aggregates and transform
TABLE 7.6

THE MIGRATION INDEX OF LYMPHOCYTES AFTER 72 HOURS ON MONOLAYER, THE CELLS WERE LABELLED WITH $^{51}$Cr AFTER REMOVAL FROM MONOLAYER

<table>
<thead>
<tr>
<th>Treatment prior to plating on monolayer</th>
<th>Control</th>
<th>Neuraminidase</th>
<th>Neuraminidase + 2 mg. sialic acid</th>
<th>Neuraminidase + 2 mg. sialic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment after removal from monolayer</td>
<td>Control</td>
<td>Neuraminidase</td>
<td>Control</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>Migration* Index</td>
<td>2.3 $\pm$ .4 (4)</td>
<td>11.4 $\pm$ 1.3 (4)</td>
<td>4.0 $\pm$ 1.2 (3)</td>
<td>5.6 $\pm$ .8 (4)</td>
</tr>
<tr>
<td></td>
<td>11.5 $\pm$ 1.8 (4)</td>
<td>6.6 $\pm$ .6 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Migration index was determined 1 hour after injection of lymphocytes into syngeneic recipients and is expressed as mean $\pm$ standard error. The number of experiments is shown in brackets.
into blast cells. Some process of "recognition" occurs in aggregate formation as this is not noticed when cells are plated onto syngeneic monolayers. However, it was not possible to determine from the present experiments whether the cell surface coat had been reacquired prior to the participation of the lymphocytes in these activities.

A comparison of the migratory abilities of neuraminidase treated and control lymphocytes after maintenance on syngeneic monolayers demonstrated two main points:

(i) The neuraminidase treated thoracic duct lymphocytes migrated in a different fashion to a similarly maintained control cell population at all times examined.

(ii) The migratory pattern of the cells which were originally treated with neuraminidase could be altered by retreatment with the enzyme after 24 or 72 hours maintenance on the monolayer.

Removal of sialic acid from the cell surface is the most likely explanation of the alteration in migration seen after treatment of cells removed from monolayers with neuraminidase. The nature of the change in migration is similar to that seen when freshly collected thoracic duct lymphocytes are treated with neuraminidase, with increased migration to the liver and decreased migration to the spleen and lymph nodes. This change could be prevented by the presence of excess free sialic acid at the time of enzyme treatment. Reincubation of lymphocytes with neuraminidase immediately after the initial treatment did not further alter the migration to the liver or spleen.

Could the changes observed be accounted for by elimination of a subpopulation due to death and disintegration, from the neuraminidase treated cells, leaving cells with more normal migratory properties? A mechanism such as this could explain the observation that the node seeking ability of both normal and
neuraminidase treated lymphocytes increased 3-4 fold after 6 hours maintenance on the monolayers (Table 7.1). Some damaged cells may release label, thus increasing the percentage of label retained by the viable cells. An alternative explanation is that the process of labelling and washing the cells, temporarily affects the ability of the cells to migrate to the nodes at 1 hour after transfer, as was demonstrated in Chapter 5. Maintenance in favourable conditions allows reversal of this process, and thus after 6 hours on the monolayer a greater percentage of cells will migrate to the nodes soon after transfer. This is a much more likely explanation of the increase in node seeking ability of lymphocytes seen after short term maintenance on the monolayers. To increase the node seeking proportion of the transferred inoculum by a factor of 3-4 fold, by means of loss of label from cell populations localizing elsewhere, would require release of about 70% of the label. Experiments in Chapter 5 demonstrated that only 7% of label was released after 4 hours maintenance in vitro.

The crucial point in favour of some reacquisition of surface sialic acid is that retreatment of cells with neuraminidase can alter the migration index so that the migration indices of the original control and neuraminidase treated populations are similar (Table 7.6). This indicates that the migration of some cells in the originally neuraminidase treated cell population has become susceptible to further treatment by neuraminidase, presumably by removal of cell surface sialic acid.

The evidence that some regeneration of normal surface properties took place after maintenance for 24 hours in vitro is in accord with the findings of Chapter 5, which indicated that neuraminidase treated lymphocytes regained normal migratory properties by 24 hours in vivo. However, even after 72 hours in vitro, the migratory properties of control and neuraminidase treated cell populations were different. This could be because conditions for regeneration are more favourable in vivo than in vitro. More likely is the possibility that only
a proportion of the cell population regains normal surface properties. Such a population would be selected out in vivo for the lymphoid pools examined, that is the spleen and thoracic duct lymph. In vitro, any proportion of the cell population not regaining a normal surface will still be present when subject to test at 24 or 72 hours, whereas in vivo such a population may be selectively removed by other organs such as the liver.
CHAPTER 8

THE IMMUNE RESPONSE TO SHEEP ERYTHROCYTES
OF IRRADIATED RATS RESTORED WITH NORMAL OR NEURAMINIDASE TREATED LYMPHOCYTES

Irradiated rats injected with thoracic duct lymphocytes have been used to follow the course of an immune response to sheep erythrocytes (SABC). The thoracic duct lymphocytes transferred are the precursors of the antibody-forming cells derived in response to antigenic stimulation (McGregor et al., 1961). Two parameters of the response can be readily quantitated, the appearance of plaque-forming cells (PFC) in the spleen and changes in the serum haemolysin. The experiments to be described utilize such a system to compare the immune responses of recipients of normal or neuraminidase treated thoracic duct lymphocytes.

There are several factors which can influence the rate of appearance of PFC in the spleen, the variation in the induction period of individual cells after exposure to antigen (Storal et al., 1965). In view of the alterations produced in the migratory properties of lymphocytes after treatment with neuraminidase, it is possible that such treatment prior to transfer would alter the recruitment of lymphocytes from the recirculating pool during an immune response. Apart from influencing the involvement of lymphocytes in an immune response by this means, neuraminidase treatment might also affect the ability of lymphocytes to be stimulated by antigen. Such differences may be reflected in the appearance of splenic PFC in rats injected with normal or compared with neuraminidase treated lymphocytes. Another possibility to be considered is that the splenic PFC response and serum haemolysin may not run parallel if antibody formation occurs in other organs, as may be the case were the localization of injected lymphocytes is altered by treatment with neuraminidase.
THE IMMUNE RESPONSE TO SHEEP ERYTHROCYTES OF IRRADIATED RATS RESTORED WITH NORMAL OR NEURAMINIDASE TREATED LYMPHOCYTES

Irradiated rats injected with thoracic duct lymphocytes have been used to follow the course of an immune response to sheep erythrocytes (SRBC). The thoracic duct lymphocytes transferred are the precursors of the antibody-forming cells derived in response to antigenic stimulation (McGregor et al., 1967). Two parameters of the response can be readily quantitated, the appearance of plaque-forming cells (PFC) in the spleen and changes in the serum haemolysin. The experiments to be described utilize such a system to compare the immune responses of recipients of normal or neuraminidase treated thoracic duct lymphocytes.

There are several factors which can influence the rate of appearance of plaque-forming cells in the spleen, namely cell division (Sterzl and Silverstein, 1967), recruitment of lymphocytes from the recirculating lymphocyte pool (Ford and Gowans, 1967, Ford, 1968) and variation in the induction period of individual cells after exposure to antigen (Sterzl et al., 1965). In view of the alterations produced in the migratory properties of lymphocytes after treatment with neuraminidase, it is possible that such treatment prior to transfer would alter the recruitment of lymphocytes from the recirculating pool during an immune response. Apart from influencing the involvement of lymphocytes in an immune response by this means, neuraminidase treatment might also affect the ability of lymphocytes to be stimulated by antigen. Such differences may be reflected in the appearance of splenic PFC in rats injected with normal as compared with neuraminidase treated lymphocytes. Another possibility to be considered is that the splenic PFC response and serum haemolysin may not run parallel if antibody formation occurs in other organs, as may be the case when the localization of injected lymphocytes is altered by treatment with neuraminidase.
Experiments on the migratory properties of neuraminidase treated lymphocytes indicated that cell surface changes can be reversed after a period in vivo (Chapter 5). If removal of the cell surface sialic acid compromises the ability of infused lymphocytes to react to sheep red blood cells in the irradiated host, then a period of residence within the host prior to antigenic stimulation may enable the changes to be reversed.

The experiments described below were carried out to examine the effect of removal of surface sialic acid on the ability of lymphocytes to undertake an immune response to sheep red blood cells after transfer to an irradiated host. To examine possible changes in the response of neuraminidase treated cells after a period of residence in vivo, the times of both lymphocyte injection and antigenic challenge were varied in relation to that of the irradiation.

RESULTS

1 COLONIZATION OF THE IRRADIATED HOST'S SPLEEN BY INJECTED LYMPHOCYTES

Experiments on the distribution of thoracic duct lymphocytes in lethally irradiated recipients (Chapter 5), showed that treatment with neuraminidase reduced by 3/4 the number of lymphocytes present in the host's spleen 8 hours after transfer. By 24 hours after transfer, the number of transferred cells in the spleens of recipients of treated lymphocytes were still less than, although closer to, the number of untreated lymphocytes located there. The ability of neuraminidase treated cells to colonize the host spleen is obviously important in the subsequent assessment of the immune response in the spleen. The ability of neuraminidase treated and control lymphocytes to colonize the spleen of irradiated hosts was compared by determining the numbers of nucleated cells in the spleens of rats 6 or 7 days after receipt of 910 rad irradiation and the injection of $10^8$ thoracic duct lymphocytes plus $10^8$ sheep erythrocytes (SRBC). In each case, neuraminidase treated and control lymphocytes were from a single collection of lymph. The number of nucleated cells in
the spleen 6 or 7 days after irradiation would be expected to reflect the capacity of the transferred lymphocytes to migrate to the spleen and colonize it by dividing. The number of splenic cells may be compared with that obtained from 11 irradiated rats which were given SRBC alone 6 hours after irradiation but no lymphocytes (Table 8.1).

### TABLE 8.1

THE COLONIZATION OF IRRADIATED SPLEENS 
BY CONTROL OR NEURAMINIDASE TREATED LYMPHOCYTES

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Splenic cell content x 10^3</th>
<th>6 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRBC + control TDL</td>
<td></td>
<td>156 ± 14 (22)</td>
<td>160 ± 16 (18)</td>
</tr>
<tr>
<td>SRBC + neuraminidase treated TDL</td>
<td></td>
<td>145 ± 14 (22)</td>
<td>140 ± 12 (18)</td>
</tr>
<tr>
<td>SRBC alone</td>
<td></td>
<td>59 ± 8 (11)</td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as mean ± S.E. The number of observations in each group is recorded in brackets.

DA rats were injected intravenously with 10^8 neuraminidase treated or control syngeneic thoracic duct lymphocytes and sheep erythrocytes within 24 hours after 910 rad irradiation. The numbers of nucleated cells present in the spleens of the irradiated recipients were determined 6 or 7 days later.

It can be seen that neuraminidase treated and control lymphocytes have a similar capacity to colonize the spleen of the irradiated host. These experiments do not exclude the unlikely possibility that the splenic cell content has been restored by some indirect effect of the transferred cells rather than by direct colonization.
When normal thoracic duct lymphocytes are administered to irradiated rats, and sheep red blood cells are injected after the lymphocytes, the peak plaque-forming cell response in the spleen occurs 6 days after the administration of antigen (Ellis et al., 1967). This peak of splenic plaque-forming cell activity 6 days after antigen is a constant finding when irradiated rats are restored with thoracic duct lymphocytes, and occurs 1-2 days later than the peak splenic plaque-forming cell response in normal unirradiated animals injected with sheep erythrocytes alone (Ellis et al., 1967).

It was found that $10^8$ control or neuraminidase treated lymphocytes were equivalent in ability to restore a plaque-forming cell response to irradiated recipients, when the lymphocytes were given 6 hours after irradiation and the sheep red blood cells were given 24 hours after irradiation (Table 8.2a). The time course of the response in the recipients of the neuraminidase treated and control lymphocytes was similar with a clear peak occurring on the sixth day after administration of antigen.

If removal of cell surface sialic acid compromises the ability of infused lymphocytes to react to SRBC in the irradiated host, either by alteration of the ability of individual cells to respond to antigenic stimulus or by altered migration of the population, a period of residence in the host may enable the changes to be reversed. If antigen was given at the same time as neuraminidase treated cells, instead of 18 hours later as in Section 2, a difference in the peak splenic PFC response of irradiated recipients of control or neuraminidase treated lymphocytes may become evident.

To test this, neuraminidase treated lymphocytes and SRBC were injected together into irradiated recipients 6 hours after irradiation. There was no significant
TABLE 8.2
PLAQUE-FORMING CELL RESPONSE AT 6 DAYS IN IRRADIATED RATS
INJECTED WITH NORMAL OR NEURAMINIDASE TREATED THORACIC
DUCT LYMPHOCYTES AND SHEEP ERYTHROCYTES

<table>
<thead>
<tr>
<th>KEY</th>
<th>Lymphocytes</th>
<th>Antigen Time of injection (hours)</th>
<th>Antigen Time of injection (hours)</th>
<th>Treatment of lymphocytes</th>
<th>No. of Expts.</th>
<th>Splenic + nucleated cells x 10⁶</th>
<th>PFC/10⁶ splenic cells</th>
<th>Total splenic + PFC x 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Control</td>
<td>6</td>
<td>24</td>
<td>Control</td>
<td>5</td>
<td>66 ± 6</td>
<td>1654±871</td>
<td>91±37</td>
</tr>
<tr>
<td></td>
<td>Ndase</td>
<td>6</td>
<td>24</td>
<td>Ndase</td>
<td>7</td>
<td>94 ± 5</td>
<td>1221±364</td>
<td>119±38</td>
</tr>
<tr>
<td>b</td>
<td>Ndase</td>
<td>6</td>
<td>6</td>
<td>Ndase</td>
<td>4</td>
<td>78 ± 7</td>
<td>1599±554</td>
<td>131±47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>24</td>
<td>Control</td>
<td>5</td>
<td>99 ± 7</td>
<td>1435±243</td>
<td>141±25*</td>
</tr>
<tr>
<td>c</td>
<td>Control</td>
<td>24</td>
<td>24</td>
<td>Ndase</td>
<td>11</td>
<td>81 ± 4</td>
<td>469±104</td>
<td>40±10*</td>
</tr>
<tr>
<td></td>
<td>Ndase</td>
<td>24</td>
<td>24</td>
<td>Ndase</td>
<td>11</td>
<td>81 ± 4</td>
<td>469±104</td>
<td>40±10*</td>
</tr>
<tr>
<td>d</td>
<td>Control</td>
<td>24</td>
<td>48</td>
<td>Control</td>
<td>8</td>
<td>220 ± 18</td>
<td>1570±436</td>
<td>389±126</td>
</tr>
<tr>
<td></td>
<td>Ndase</td>
<td>24</td>
<td>48</td>
<td>Ndase</td>
<td>9</td>
<td>177 ± 14</td>
<td>1609±381</td>
<td>290±73</td>
</tr>
<tr>
<td>e</td>
<td>Control</td>
<td>24</td>
<td>6</td>
<td>Control</td>
<td>11</td>
<td>151 ± 7</td>
<td>1298±314</td>
<td>189±43*</td>
</tr>
<tr>
<td></td>
<td>Ndase</td>
<td>24</td>
<td>6</td>
<td>Ndase</td>
<td>10</td>
<td>123 ± 9</td>
<td>279±81</td>
<td>33±9*</td>
</tr>
<tr>
<td>f</td>
<td>Control</td>
<td>48</td>
<td>6</td>
<td>Control</td>
<td>4</td>
<td>166 ± 15</td>
<td>592±167</td>
<td>94±25</td>
</tr>
<tr>
<td></td>
<td>Ndase</td>
<td>48</td>
<td>6</td>
<td>Ndase</td>
<td>3</td>
<td>152 ± 6</td>
<td>191±17</td>
<td>29±3</td>
</tr>
</tbody>
</table>

+ Results expressed as mean ± standard error.
* Difference between these results is significant at the 1% level.

DA strain rats were injected intravenously with 10⁸ untreated (control) or neuraminidase treated (Ndase) syngeneic thoracic duct lymphocytes, at varying times (hours) after 910 rad gamma irradiation. 10⁸ sheep erythrocytes were injected at the times shown. Splenic plaque-forming cells (PFC) were determined 6 days after the administration of lymphocytes or erythrocytes (whichever were injected last).
difference between the response of recipients of simultaneously injected neuraminidase treated lymphocytes and antigen (Table 8.2b) and that of recipients challenged with antigen 18 hours after injection of neuraminidase treated lymphocytes (Table 8.2a).

In another experimental variation, lymphocytes and antigen were injected together 24 hours after irradiation. The splenic plaque-forming cell response in recipients of neuraminidase treated lymphocytes was significantly less 6 days later than that in recipients of control lymphocytes (Table 8.2c). Further experiments were performed in order to determine whether the difference in response at six days between rats receiving neuraminidase treated and control lymphocytes, represented a lower peak response with similar kinetics, or a delayed response. In the latter case, rats receiving neuraminidase treated cells would be expected to attain a peak response later than rats receiving control cells. The results are shown in figure 8.1A. The response of recipients of neuraminidase treated cells is delayed, as evidenced by the lesser numbers of splenic plaque-forming cells on the fifth and sixth days. From the 7th day onwards the responses are identical. Recipients of neuraminidase treated cells did not show a clear peak in the PFC response as was seen in recipients of untreated lymphocytes.

After intravenous injection of sheep erythrocytes, the antibody response in normal animals occurs mainly in the spleen (Rowley 1950) and the serum haemolysin determination reflects antibody-forming activity in the spleen. Neuraminidase treated lymphocytes migrate in lesser numbers than control lymphocytes to the spleen, but in larger numbers to the liver. It is possible that the decreased response noted in the spleens of recipients of neuraminidase treated cells may be compensated for by an increased response elsewhere. In order to compare the total response of recipients of control and neuraminidase treated lymphocytes, serum haemolysins were measured after administration of
FIGURE 8.1

IMMUNE RESPONSE OF RATS INJECTED WITH LYMPHOCYTES AND ANTIGEN 24 HOURS AFTER IRRADIATION

A. Splenic Plaque-Forming Cells.

$10^8$ control or neuraminidase treated DA thoracic duct lymphocytes plus sheep erythrocytes were administered intravenously to syngeneic recipients 24 hours after 910 rad irradiation. Splenic PFC were determined at the times shown. Results are expressed as mean ± standard error (4 - 11 rats at each point).

B. Serum Haemolysin.

$10^8$ control or neuraminidase treated (DA x Lewis)F₁ thoracic duct lymphocytes plus sheep erythrocytes were administered intravenously to syngeneic recipients 24 hours after 730 rad irradiation. Serum haemolysins were determined at the times shown. Results are expressed as mean ± standard error (5 rats in each group).
The response of untreated lymphocytes is a reflection of the response of the whole animal in vivo (Chapter 9). The results show that removal of small acid from lymphocytes prior to injecting them has suppressed the subsequent response of the irradiated host if lymphocytes and antigen were simultaneously administered 24 hours after irradiation. This diminished response is probably related to changes in lymphocyte behavior: either an altered migration or an inability to be stimulated by antigen. A portion of residence within the host may enable recovery of neuraminidase-treated lymphocytes so that they can give a response equivalent to that of control lymphocytes having participated in the specific PFC response (Table 9.3 and cases 8-10).
lymphocytes and antigen together, 24 hours after irradiation (Fig. 8.1B). The haemolysin response is delayed in recipients of neuraminidase treated cells, but by the eighth day the response resembles that of recipients of untreated lymphocytes. The response as measured by serum haemolysin parallels that of the splenic plaque-forming cells. It appears that the early decrease in splenic plaque-forming cell response in recipients of neuraminidase treated lymphocytes is a reflection of the response in the whole animal.

4 IMMUNE RESPONSE OF IRRADIATED RATS INJECTED WITH LYMPHOCYTES 24 HOURS AND ANTIGEN 48 HOURS AFTER IRRADIATION

The change in migratory properties of neuraminidase treated lymphocytes is reversed by a period of residence in vivo (Chapter 5). The above experiments show that removal of sialic acid from lymphocytes prior to injecting them has compromised the subsequent response of the irradiated host if lymphocytes and antigen were simultaneously administered 24 hours after irradiation. This diminished response is probably related to changes in lymphocyte behaviour, either an altered migration or an inability to be stimulated by antigen. A period of residence within the host may enable recovery of neuraminidase treated lymphocytes so that they can give a response equivalent to that of control lymphocytes. To test this proposition, lymphocytes were given 24 hours after irradiation and sheep red cells 48 hours after irradiation.

The splenic PFC response (Table 8.2d) and serum haemolysin response (Fig. 8.2) are not significantly different for rats receiving control and neuraminidase treated cells. The higher total splenic PFC response, noted for both neuraminidase treated and control cells, is probably a reflection of the increased cellularity of the host spleens. For an undetermined reason the animals used in the experiment had spleens which were much more cellular than usually seen after irradiation.
10⁸ control or neuraminidase treated (DA x Lewis)F₁ thoracic duct lymphocytes were administered intravenously to syngeneic recipients 24 hours after 730 rad irradiation. Sheep erythrocytes were given 48 hours after irradiation. Serum haemolysins were determined at the times shown. Results are expressed as mean ± standard error (4 rats in each group).
and the injection of $10^8$ thoracic duct lymphocytes. The number of PFC/10^6 spleen cells is of the same order as for the other experiments. The serum haemolysin response (Fig. 8.2) is the same at all times for neuraminidase treated and control cells. In both cases serum antibody is detectable at 4 days when TDL have been allowed a period of residence within the host prior to the administration of antigen. When TDL are given together with antigen, serum haemolysin does not appear until 5 days (Fig. 8.1B). The changes responsible for the differences in response between neuraminidase treated and control lymphocytes noted in Section 3 have been reversed by a period of 24 hours residence in the host.

5 IMMUNE RESPONSE OF IRRADIATED RATS INJECTED WITH ANTIGEN 6 HOURS AND LYMPHOCYTES 24 OR 48 HOURS AFTER IRRADIATION

As the changes produced by neuraminidase treatment of lymphocytes appear reversible, the question arises as to the cause of the lesser peak plaque-forming cell response observed in recipients of neuraminidase treated lymphocytes when lymphocytes and antigen have been given together 24 hours after irradiation. One factor may be inability of host macrophages to process and retain antigen normally as a result of irradiation. Handling of antigen by macrophages has been shown to be affected by irradiation (Gallily and Feldman 1967, Mitchison 1969) and there is a latent period, whereby antigens presented to phagocytes soon after irradiation are more effective immunogens than those presented at later times (Mitchison 1969, Talmage et al. 1970).

Neuraminidase treated cells require a longer time than normal cells to migrate into the spleen in large numbers. Antigen presented to host macrophages 24 hours after their irradiation may not be processed or retained in immunogenic form as efficiently as antigen given 6 hours after irradiation. If this is so, the stimulatory ability of the macrophages may be diminished by the time neuraminidase treated lymphocytes migrate into the spleen when lymphocytes and antigen are administered together.
24 hours after irradiation (Table 8.2c). Normal lymphocytes, as they migrate into the spleen earlier than neuraminidase treated lymphocytes, may be more efficiently stimulated, and hence give the differences in PFC response noted.

In the following experiments antigen was administered 6 hours after irradiation, followed 24 hours after irradiation by thoracic duct lymphocytes.

The reasons for this timing were:

(i) to eliminate any deficiency of antigen handling by macrophages present at later times after irradiation.

(ii) To determine the period for which antigen can be retained in an immunogenic form by the host.

When lymphocytes were pretreated with neuraminidase the splenic PFC at six days was depressed (Table 8.2e). The kinetics of the full response is shown in Figure 8.3A. There is a 24 hour delay in the induction of the splenic PFC response which reaches a peak similar to that of rats receiving the control cells. By the eighth day the splenic PFC response of recipients of neuraminidase treated cells is higher than that of rats receiving control cells. In each case the response differs from the response when antigen is given with or after cells. Thus, in recipients of both control and enzyme treated lymphocytes there is no clear peak in the PFC response on the sixth day but a more diffuse peak. The serum haemolysin response when SRBC are given at 6 hours and TDL at 24 hours after irradiation is seen in Figure 8.3B. There is some decrease in titres on the fifth, sixth and seventh days with neuraminidase treated cells, but from the eighth day onwards titres are identical.

In another experiment antigen was given 6 hours and lymphocytes 48 hours after irradiation. The results in Table 8.2f show that even if antigen is given 42 hours prior to cells, lymphocytes can still be stimulated to give a sizeable response. Again the number of splenic PFC on the sixth day is decreased in those animals receiving neuraminidase treated lymphocytes.
FIGURE 8.3

IMMUNE RESPONSE OF RATS INJECTED WITH ANTIGEN
6 HOURS AND LYMPHOCYTES 24 HOURS
AFTER IRRADIATION

A. Splenic Plaque-Forming Cells

Sheep erythrocytes were administered intravenously to DA strain rats 6 hours after 910 rad irradiation. $10^8$ syngeneic control or neuraminidase treated thoracic duct lymphocytes were given intravenously 24 hours after irradiation. Splenic PFC were determined at the times shown. Results are expressed as mean ± standard error (4 - 11 rats at each point).

B. Serum Haemolysin

Sheep erythrocytes were administered intravenously to (Lewis x DA)$_F_1$ hybrid rats 6 hours after 730 rad irradiation. $10^8$ syngeneic control or neuraminidase treated thoracic duct lymphocytes were given intravenously 24 hours after irradiation. Serum haemolysins were determined at the times shown. Results are expressed as mean ± standard error (8 rats each group).
6 THE INHIBITION OF THE EFFECT OF NEURAMINIDASE ON THE REDUCTION OF THE PFC RESPONSE BY AN EXCESS OF FREE SIALIC ACID IN THE INCUBATION MEDIUM

The capacity of an excess of sialic acid to inhibit the reduction in PFC ability of lymphocytes was tested. The situation chosen was that in which neuraminidase treatment of lymphocytes produced the most marked depression of PFC potential, namely when SRBC were administered 6 hours and lymphocytes 24 hours after irradiation.

Collections of TDL were divided into 3 groups which were incubated in a volume of 1 cc of phosphate buffered saline with either 50 units of neuraminidase, 50 units of neuraminidase plus 2 mg of sialic acid or 2 mg of sialic acid alone. After washing, the cells were transferred to irradiated recipients which had been injected with SRBC 6 hours after irradiation. The splenic PFC response was assayed 6 days later. The results are shown in Table 8.3.

<table>
<thead>
<tr>
<th>Treatment of lymphocytes</th>
<th>Number of recipients</th>
<th>Total splenic PFC x 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialic Acid + Neuraminidase</td>
<td>6</td>
<td>138 ± 23</td>
</tr>
<tr>
<td>Sialic Acid</td>
<td>6</td>
<td>155 ± 50</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>6</td>
<td>41 ± 11*</td>
</tr>
</tbody>
</table>

* This difference is significant at the 1% level

Thoracic duct lymphocytes were incubated in a volume of 1 cc with 50 units of neuraminidase and/or 2 mg of sialic acid for 30 minutes. After incubation 10^8 lymphocytes were injected intravenously into syngeneic recipients which had received 910 rad irradiation 24 hours previously and sheep erythrocytes 18 hours previously. Splenic plaque-forming cells were determined 6 days after the injection of lymphocytes, and are expressed as mean ± standard error.
The decrease in splenic PFC at six days in recipients of lymphocytes incubated with neuraminidase is inhibited if there is excess free sialic acid present. This strongly supports the idea that the experimental changes noted are due to removal of cell surface sialic acid rather than to some other effect of incubation with the enzyme.

7 THE ABILITY OF NEURAMINIDASE TREATED LYMPHOCYTES TO ABROGATE TOLERANCE TO SHEEP ERYTHROCYTES

The transfer of allogeneic TDL together with SRBC to recipients tolerant of SRBC can abrogate the tolerance of the recipient, as indicated by the appearance of haemolysin forming cells which are predominantly of host origin (McCullagh 1970b). When parental cells are transferred to a tolerant $F_1$ recipient a recognition of "foreignness" between the donor cells and those of the host is a prerequisite for abrogation. It is necessary that the SRBC be present close to the time that allogeneic cells are transferred for an optimal abrogation response to occur. If administration of antigen is delayed by more than one hour after allogeneic cells the response obtained is greatly reduced (McCullagh 1972). It appears that the necessary interaction between host and donor cells of different genetic strain occurs soon after administration of the cells. Such a system provides a useful means of determining whether neuraminidase treated parental lymphocytes can take part in this recognition soon after injection. At this time it is known that their migratory characteristics, and probably cell surface have not reverted to normal. This information will also be of relevance to experiments to be reported in the following chapter on the ability of neuraminidase treated lymphocytes to undertake graft-versus-host reactions.

It was found that $2.5 \times 10^7$ normal parental thoracic duct lymphocytes were sufficient to abrogate the SRBC tolerance of $F_1$ hybrid recipients, though the response became smaller as less than $3 \times 10^8$ lymphocytes were administered (McCullagh 1972). As the frequency
of migration of neuraminidase treated cells to the spleen at early intervals after injection is only $1/3$ that of control cells, doses of up to $6 \times 10^8$ parental cells were given in the present experiments, to ensure that there would be a reasonable number of parental cells present in the spleen at the time when interaction between host and donor cells was necessary for abrogation.

Normal or neuraminidase treated DA thoracic duct lymphocytes were transferred together with $10^8$ SRBC, to (DA x Lewis)\textsuperscript{F}1 hybrid recipients tolerant of sheep erythrocytes. Their spleens were removed and assayed for PFC 92 hours after injection, a time at which it was found that a maximal response of the previously tolerant host occurred as judged by the numbers of splenic PFC. The ability of neuraminidase treated lymphocytes to abrogate tolerance was equivalent to that of normal lymphocytes (Table 8.4).

**TABLE 8.4**

<table>
<thead>
<tr>
<th>Treatment of lymphocytes</th>
<th>Number of lymphocytes ($\times 10^8$)</th>
<th>Total splenic PFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.0</td>
<td>638,000</td>
</tr>
<tr>
<td>Control</td>
<td>3.0</td>
<td>886,000</td>
</tr>
<tr>
<td>Control</td>
<td>1.5</td>
<td>62,000</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>6.0</td>
<td>1,835,000</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>6.0</td>
<td>483,000</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>2.2</td>
<td>763,000</td>
</tr>
</tbody>
</table>

Each (DA x Lewis)\textsuperscript{F}, hybrid rat, tolerant of sheep erythrocytes, received \textsuperscript{1}either control or neuraminidase treated DA strain thoracic duct lymphocytes, together with $10^8$ sheep erythrocytes, by the intravenous route. Their spleens were examined for plaque-forming cells 92 hours later.
DISCUSSION

The results presented above show that, in general, treatment of thoracic duct lymphocytes with neuraminidase does not compromise their ability to differentiate for antibody formation in an irradiated recipient, though the immune response mounted in the recipient may be modified, depending on the relative times of lymphocyte and antigen administration after irradiation.

Thoracic duct lymphocytes injected into irradiated recipients are the precursors of the antibody-forming cells derived in response to antigenic stimulation (McGregor et al. 1967).

Neuraminidase treated lymphocytes are as capable of restoring the splenic PFC response as untreated lymphocytes in the following situations:

(i) when the lymphocytes are given together with antigen 6 hours after irradiation.

(ii) when the lymphocytes are given 6 hours after and antigen 24 hours after irradiation.

(iii) when the lymphocytes are given 24 hours after irradiation and antigen a further 24 hours later.

The peak splenic PFC response is diminished when neuraminidase treated lymphocytes and antigen are given together 24 hours after irradiation, and a delayed but equivalent response is obtained when antigen is administered 6 hours and neuraminidase treated lymphocytes 24 hours after irradiation.

The following discussion is divided into two parts. The differences in the kinetics of the response when the times of administration of normal lymphocytes and antigen are varied are discussed first. Then factors contributing to the dissimilarities between the responses produced by normal and neuraminidase treated lymphocytes will be considered.
The immune response in recipients of normal lymphocytes

The peak haemolysin and peak plaque-forming cell responses of irradiated rats receiving normal thoracic duct lymphocytes occur one to two days later than those of normal animals. In the present experiments serum haemolysin was not detected until 5 days after simultaneous administration of normal lymphocytes and SRBC. However haemolysin could be detected after 4 days if the cells had undergone a period of 24 hours residence in the host prior to administration of antigen. This suggests that migration of normal cells to a site in the spleen appropriate for stimulation and differentiation is a limiting factor in the time of initiation of the response. After a 24 hour period of residence within the irradiated host, cells are available for induction into antibody formation as soon as antigen has been processed, and thus serum antibody can be detected earlier. The time taken by immunocompetent cells to localize in appropriate sites within the host animal would also explain the delayed time course of the immune response in irradiated animals restored with an injection of lymphocytes as compared with the response of normal unirradiated animals.

The different kinetics of the splenic plaque-forming cell response when irradiated recipients are given thoracic duct lymphocytes 18 hours after antigen as compared to administration of antigen with or after the lymphocytes may be due to decrease in efficiency of the recruitment process. When antigen is administered prior to the lymphocytes, there is a diffuse peak plaque-forming cell response occurring on day 6 and day 7 in contrast to the clear cut peak when antigen is given with the lymphocytes. When antigen is given together with or after thoracic duct lymphocytes efficient recruitment of antigen-reactive cells would result in the synchronous induction of cells into the immune response. Hence a clear cut peak of plaque-forming cells would be noted 6 days later. In contrast when antigen is given 18 hours prior to cells, efficiency of recruitment is
waning by the time lymphocytes are transferred (Ford 1968b), and the circulating lymphocytes are exposed to the inductive influence of antigen in a more random manner, thus resulting in a broad peak response.

Dissimilarities in the responses of animals restored with normal or neuraminidase treated lymphocytes

The differences in splenic PFC response observed between rats receiving control and neuraminidase treated lymphocytes are probably due to a combination of two factors;

(i) as a consequence of the alteration in migration of neuraminidase treated lymphocytes, fewer lymphocytes are available for recruitment into the immune response at times soon after cell transfer.

(ii) a decrease in the stimulatory ability of the antigen processing mechanism of the host with time after irradiation, such that by the time neuraminidase treated lymphocytes are available, less efficient stimulation results.

Macrophages are necessary for the induction of an immune response to sheep red blood cells (Mosier 1967, Roseman 1969, Pierce and Benacerraf 1969, McCullagh 1970c). The mechanism by which the macrophages function in the response is unresolved. It has been suggested that they have a role in processing of antigen and the transfer of information to immunocompetent lymphoid cells (Cohn 1968, Fishman 1969). However, experiments by Palmer (1970), suggest that the function of the macrophage is to break down the sheep red cells to a size suitable for the stimulation of lymphocytes.

There is evidence that antigen processing by macrophages is susceptible to irradiation (Galli1y and Feldmann 1967), and there are suggestions of a latent period for this effect. Mitchison (1969) studied the ability of protein antigens bound to mouse peritoneal exudate cells to elicit an immune response after transfer to normal mice, and the effect of irradiation of the peritoneal cell donor on this process. 600 rad
irradiation of the peritoneal cell donors 2 days prior to the exposure of these cells to antigen, reduced the serum antibody response of the recipient mice by approximately 90%. There was a progressive diminution in the antibody response, as the interval between irradiation of peritoneal cells and their exposure to antigen was increased from 0 to 2 days, indicating that the effects of irradiation on the antigen handling mechanism of the peritoneal cells took time to become established. A similar observation has been recorded by Donaldson et al. (1956) who found that the intracellular digestion of phagocytosed chicken erythrocytes by mouse peritoneal cells was not suppressed until 2 days after 350 rad irradiation. Talmage et al. (1970) have shown a delayed effect of irradiation on the adherent cell, presumably a macrophage, necessary for an in vitro response to sheep red blood cells. The ability of these cells to support a plaque-forming cell response to sheep red blood cells decreased markedly when they were utilized 24 hours after 500 rad irradiation as compared with utilization at 2 hours. Thus it appears that irradiation has a delayed effect on macrophages impairing their ability to process and/or retain antigenic material in an immunogenic form.

This effect of irradiation on the host macrophages could partially explain differences in the response noted between recipients of control and neuraminidase treated lymphocytes. There are less neuraminidase treated than control lymphocytes in the spleens of irradiated recipients at 8 and 24 hours after the administration of thoracic duct lymphocytes (Chapter 5). Normal migratory properties return to the cells after about 24 hours. The magnitude of the splenic PFC response when antigen was administered at 6 hours and lymphocytes at 48 hours after irradiation, indicated that the antigen injected at this time could be retained in an immunogenic form for at least 42 hours. However, if lymphocytes are given together with antigen 24 hours after irradiation, delayed effects of irradiation on
the host are operative by the time migration of neuraminidase treated lymphocytes through the spleen has recovered, and a smaller peak response is observed. Different migratory patterns of neuraminidase treated cells are another factor which could contribute to the observed response. In the early stages of the immune response, plaque-forming cells increase rapidly in number in the spleen (exponential phase), and this increase is correlated with the rise in serum antibody (Urso and Mackinodan 1963). The rapid doubling time, of less than 6 hours, of antibody-forming cells in the spleen during the exponential phase (Ellis et al. 1967), is a result of both cell division of antibody-forming cells (Sterzl and Silverstein 1967), and recruitment of lymphocytes by the spleen from the recirculating pool (Ford and Gowans 1967, Ford 1968b). The major period of lymphocyte recruitment probably occurs within 24 hours of the injection of sheep erythrocytes (Ford 1968b). Neuraminidase treatment of lymphocytes, by altering the migratory pattern, can affect both the number of lymphocytes in the spleen available for stimulation, and the number that can be recruited from the recirculating lymphocyte pool.

There was a lower peak PFC response observed when enzyme treated lymphocytes were administered simultaneously with antigen 24 hours after irradiation as compared with that obtained with normal lymphocytes. However the later phases of the response are equivalent. This can be explained by a combination of the delayed effects of irradiation on antigen processing (discussed above) together with unavailability of the neuraminidase treated lymphocytes for recruitment from the recirculating pool. Recruitment of lymphocytes may be important in the early rapidly rising phase of the immune response (Ford 1968b), but then the most important factor becomes division of antibody-forming cells. Thus, alteration of the early recruitment and induction may not necessarily affect the later magnitude of the response. This response is similar for both control and neuraminidase treated cells.
administered with antigen 24 hours after irradiation as judged by haemolysin formation and splenic plaque-forming cells in the later phases of the response.

The lag in the induction of the response noted when antigen is given prior to neuraminidase treated cells could also be due to an alteration in migratory pattern. In this case antigen is given 6 hours after irradiation, before the irradiation has reduced the efficiency of antigen processing, and 18 hours later when lymphocytes are given, antigen is ready for stimulation of the cells. Thus lymphocytes will be stimulated as they migrate through the spleen, and the delayed response with neuraminidase treated cells is due to the known decrease in splenic migration and recirculation early after transfusion. The return of normal migratory properties by 24 hours gives rise to an equivalent but delayed response.

Another possible explanation for the observed differences in behaviour of neuraminidase treated cells is that these cells are unable to respond to antigenic stimulation when their surface has been altered, and that the time lag noted is directly due to the time taken to regain a normal surface. There is no clear evidence on the ability of neuraminidase treated cells to respond to antigenic stimulus. Results obtained from the abrogation of tolerance by neuraminidase treated lymphocytes show that these cells can take part in the 'recognition' reaction that is a necessary prerequisite for abrogation, within an hour after transfusion. However, lymphocytes whose ability to make antibody has been lost by incubation with mitomycin-C can participate in the allogeneic interactions necessary to abrogate tolerance to the same extent as normal lymphocytes (McCullagh, 1972).

These results suggest that neuraminidase treated cells can take place in immunological reactions whilst their cell surface is in an altered state, but do not directly answer the questions:
(i) Can neuraminidase treated lymphocytes be stimulated by antigen soon after treatment?

(ii) If so are they able to respond by differentiation into antibody-forming cells?

If this is in fact so, the equivalent response of neuraminidase treated and control cells when given with antigen 6 hours after irradiation would suggest that recovery of ability to be stimulated and respond must occur within several hours. This does not accord with the period of 20 hours or so necessary for the return of normal migratory properties which has been previously demonstrated (Chapter 5).

These experiments provide an in vivo corroboration of the results of Ford and Gowans (1967) obtained using the isolated, perfused rat spleen preparation, that the migration of lymphocytes through the spleen is an important factor in the immune response of that organ to sheep erythrocytes. The 24 hour delay in the induction of the immune response, that was noted when enzyme treated lymphocytes were administered after antigen correlates nicely with the time taken for the transfused enzyme treated lymphocytes to recover their normal migratory properties (Chapter 5). Transfusion into an irradiated recipient followed by stimulation with antigen is a commonly used model to assess the immunological capacity of experimentally modified lymphocytes. The experiments described in this chapter indicate that factors other than changes in the immunological capacity of individual lymphocytes must be considered, when evaluating the significance of the results obtained using such a model.
THE CAPACITY OF THORACIC DUCT LYMPHOCYTES INCUBATED WITH NEURAMINIDASE TO INITIATE AND SUSTAIN GVH REACTIONS

The following experiments examine the effect of removal of the cell surface sialic acid by treatment with neuraminidase, or the ability of parental type thoracic duct lymphocytes to initiate graft-versus-host (GVH) reactions in F1 hybrid recipients. The GVH reaction was studied after two different routes of lymphocyte administration:

(i) The systemic reaction produced by the intravenous injection of cells with their subsequent dissemination throughout the tissues of the host animal.
(ii) The local reaction, produced when cells are injected locally into tissue.

RESULTS

CHAPTER 9

THE CAPACITY OF THORACIC DUCT LYMPHOCYTES INCUBATED WITH NEURAMINIDASE TO INITIATE AND SUSTAIN GVH REACTIONS

As the severity of the GVH syndrome decreases because of the number of parental cells in the inoculum and the initial weight of the host animal (Ratan 1966), a group of different sized rats were examined. The results are shown in Figure 9.1. Of 5 rats ranging from 30-40 grams in initial weight, all 5 were dead by the 16th day after receipt of 10^9 parental thoracic duct cells that had been incubated without enzyme (Fig. 9.1A). In contrast, only 1 of the 7 that received neuraminidase treated cells was alive by the 20th day (Fig. 9.1B). A marked difference can be seen in the degree of wasting shown by the two groups (Fig. 9.1B). A similar reduction in the severity of the graft-versus-host reaction is apparent.
The following experiments examined the effect of removal of the cell surface sialic acid by treatment with neuraminidase, on the ability of parental type thoracic duct lymphocytes to initiate graft-versus-host (GvH) reactions in F₁ hybrid recipients. The GvH reaction was studied after two different routes of lymphocyte administration;

(i) The systemic reaction produced by the intravenous injection of cells with their subsequent dissemination throughout the tissues of the host animal.
(ii) The local reaction, produced when cells are injected locally into tissues.

RESULTS

1 THE ACUTE, SYSTEMIC GRAFT-VERSUS-HOST REACTION

The severity of runting

The course of a graft-versus-host reaction in the F₁ hybrid recipients is reflected by changes in body weight (Simonsen 1962). 10⁸ control or neuraminidase treated DA strain thoracic duct lymphocytes were injected intravenously into each of a group of (Lewis x DA)F₁ recipients, and the subsequent course of the GvH syndrome was followed by daily weighing.

As the severity of the GvH syndrome depends both on the number of parental cells in the inoculum and the initial weight of the host animal (Gowans 1962), 2 groups of different sized rats were examined. The results are shown in figure 9.1. Of 5 rats ranging from 57-80 grams in initial weight, all 5 were dead by the 16th day after receipt of 10⁸ parental thoracic duct cells that had been incubated without enzyme (Fig. 9.1A). In contrast only 1 of the 7 that received neuraminidase treated cells had died by the 20th day (Fig. 9.1A). A marked difference can be seen in the degree of wasting shown by the two groups (Fig. 9.1A). A similar reduction in the severity of the graft-versus-host reaction is apparent
FIGURE 9.1

WEIGHT CHANGES IN F₁ HYBRID RATS INJECTED WITH NORMAL OR NEURAMINIDASE TREATED PARENTAL LYMPHOCYTES

$10^8$ control or neuraminidase treated DA thoracic duct lymphocytes were injected intravenously into (Lewis x DA)$F₁$ recipients. The body weight changes after injection were followed on alternate days and are expressed as a percentage of the body weight at the time of injection (mean ± standard error).

A. RECIPIENTS OF 57-80 GRAMS WEIGHT

There were 5 recipients (4 male, 1 female) of control lymphocytes and 7 recipients (6 male, 1 female) of neuraminidase treated lymphocytes. The mean weight of recipients of control lymphocytes was 75 grams and of recipients of neuraminidase treated lymphocytes was 71 grams.

B. RECIPIENTS OF 89-112 GRAMS WEIGHT

There were 8 recipients in each group (7 female, 1 male). The mean weight of recipients of control lymphocytes was 103 grams and of recipients of neuraminidase treated lymphocytes was 97 grams.
in larger rats, of which the initial weight ranged from 89-112 grams, after injection of neuraminidase treated lymphocytes (Fig. 9.1B). The rats depicted in figure 9.2 are littermates, 15 days after the injection of \(10^8\) neuraminidase treated (top), or untreated (bottom) DA strain lymphocytes. Initially each animal weighed approximately 100 grams. The animal which had received untreated lymphocytes shows obvious signs of GvH disease, namely dermatitis of the ears and paws, ruffling of the fur and a hunched posture. The recipient of \(10^8\) neuraminidase treated lymphocytes appears normal.

However, differences between the two groups of animals were noticeable prior to the onset of wasting. The first abnormality noticed in rats which had received normal cells was a marked erythema of the ears and paws, which developed on the 5th or 6th day after lymphocyte injection. This manifestation of the GvH syndrome was reduced or entirely absent in recipients of enzyme treated cells. Thus it was quite clear that incubation of parental strain thoracic duct lymphocytes with neuraminidase altered the course of the acute GvH syndrome, when these lymphocytes were administered by the intravenous route to \(F_1\) hybrids. The subsequent fate of recipients of neuraminidase treated lymphocytes is discussed further in section 2.

**Histological changes in the spleen in the early stages of runting**

Histological changes are detectable in the spleens of \(F_1\) hybrid rats in receipt of parental strain lymphocytes as early as 12-24 hours after the injection of thoracic duct lymphocytes (Gowans 1962). The most typical histological feature is the appearance of pyronophilic cells in the white pulp of the spleen around the central arteriole. These cells become increasingly prominent during the following four days with the result that the white pulp around the central arteriole assumes a lacy appearance when examined at low power, instead of the relatively homogeneous appearance, normally associated with large numbers of small lymphocytes. Radioisotopic
FIGURE 9.2
Comparison of two F₁ hybrid littermates which received control or neuraminidase treated, parental thoracic duct lymphocytes.

The animals weighed about 100 grams at the time of injection of $10^8$ control (bottom) or neuraminidase treated (top) DA lymphocytes. The pictures were taken 15 days later. Features of GvH disease are present in the animal which had received control cells (bottom) but are absent in the animal injected with neuraminidase treated cells (top).
labelling studies have shown that these pyroninophilic cells are derived from the injected thoracic duct lymphocytes (Gowans 1962). In order to compare the changes in the spleen produced by normal and neuraminidase treated cells, 4 week old (Lewis x DA)F₁ rats were injected intravenously with \(10^8\) enzyme treated or control untreated lymphocytes, their spleens being removed and examined histologically at either 16 or 36 hours after injection of the cells. The results are shown in figure 9.3.

Figure 9.3A shows the white pulp of the spleen from a normal rat, while figures 9.3B and 9.3C compare the white pulp 16 hours after the injection of untreated (Fig. 9.3B) or neuraminidase treated (Fig. 9.3C) lymphocytes. In the spleens of rats receiving either untreated or neuraminidase treated thoracic duct lymphocytes, the presence of blast cells in the white pulp produces a lacy appearance of the white pulp surrounding the central arteriole. The neuraminidase treated lymphocytes that reach the spleen are capable of undergoing transformation to large pyroninophilic cells as early as 16 hours after injection. There is no obvious difference in the early histological changes in the spleen following the injection of untreated or neuraminidase treated thoracic duct lymphocytes.

2 THE CHRONIC, SYSTEMIC GRAFT-VERSUS-HOST REACTION

Animals which received \(10^8\) neuraminidase treated DA strain parental lymphocytes were observed over a period of months for delayed effects attributable to the injected lymphocytes. The appearance of delayed effects might suggest that the decrease in severity of the acute GvH reaction represented a different anatomical pattern of attack by parental lymphocytes, rather than an impairment of the immunological reactivity required to initiate a GvH reaction. If this were so, a chronic GvH syndrome might be produced in rats injected with neuraminidase treated lymphocytes.
FIGURE 9.3

The appearance of the white pulp in the spleens of F₁ hybrid rats 16 hours after the intravenous injection of control or neuraminidase treated parental lymphocytes.

A. Control uninjected rat

The white pulp surrounding the central arteriole (arrow) has a relatively homogeneous appearance.

B. Rat which had received control lymphocytes

The white pulp surrounding the central arteriole (arrow) has a "lacy" appearance due to the presence of large blast cells.

C. Rat which had received neuraminidase treated lymphocytes.

The white pulp surrounding the central arteriole (arrow) has a lacy appearance similar to that noted in the recipient of control lymphocytes.

Haematoxylin and Eosin

Magnification x 380
Oliner et al. (1961) have described recovery from acute GvH disease occurring occasionally in mice, with relapse occurring many months later. Streilein (1971) reported a chronic GvH syndrome characterized by lymphoid tissue atrophy in F₁ hybrid hamsters that survived acute GvH reactions. A chronic GvH syndrome occurring in F₁ hybrid mice, injected with divided doses of parental strain spleen cells from 6 weeks of age, has been described (Armstrong et al. 1967, Armstrong et al. 1970). Many animals survived up to 24 months of age and exhibited a relatively normal weight gain. Histological features included an early enlargement of lymphoid follicles in the spleen and lymph nodes, followed by a marked plasmacytosis at 3 to 6 months. After 6 months a significant proportion of the animals developed malignant lymphomas.

Apart from those mentioned above, few observations on chronic GvH syndromes have been recorded owing to the difficulty inherent in its production, that most experimental animals die in the acute stage of the disease.

To assess GvH activity, weight changes, peripheral blood counts, quantitation of the recirculating lymphocyte pool and histological changes were recorded over a period of months, in (Lewis x DA)F₁ hybrid recipients of 10⁸ neuraminidase treated DA strain, parental thoracic duct lymphocytes.

**Weight changes**

Figure 9.4 shows the fate of 7 female rats, ranging in initial weight from 70-104 grams at the time of injection of neuraminidase treated parental cells. The weight gain of these animals may be compared with the mean weight gain of 6 normal rats. Over the 5 month period of observation 2 animals died after a period of weight loss with clinical features suggestive of GvH disease. The surviving animals showed variable degrees of dermatitis and alopecia, and were prone to respiratory infections. Wheezy breathing and chronic cough were
7 female $F_1$ (Lewis x DA) hybrid rats were injected at the age of 4-5 weeks with $10^8$ neuraminidase treated DA thoracic duct lymphocytes.

The weight gain of individual rats which had received neuraminidase treated cells.

The mean weight gain of 6 normal $F_1$ hybrid female rats.
common features. The death of 2 animals and the signs exhibited by the others, suggested that the neuraminidase treated lymphocytes were producing chronic disease in the recipients.

After 6 to 8 months, many animals with chronic GvH syndrome had lost fur, particularly in a rim surrounding the eyes, around the mouth, and to a variable extent over the dorsum and abdomen. The tail also lost its normal scaly skin and became white and smooth. Figure 9.5B demonstrates an extreme example of these changes in a rat 15 months after the induction of chronic GvH disease and should be compared with a normal animal of similar age (Fig. 9.5A).

**Haematologic changes**

Various cellular changes have been described in the blood of animals with GvH disease. These occurred mainly in the acute phase of the disease. Billingham et al. (1962) described a mild anaemia with progressive leukocytosis, predominantly due to an increase of granulocytes, in the blood of F₁ rats inoculated at birth with parental strain lymphocytes. These changes were noted from the 12th to the 18th day, following injection of cells, by which time most recipients had died. Anderson and Nowell (1966) described a rapidly progressive thrombocytopenia and agranulocytosis associated with bone marrow hypoplasia following the injection of parental strain lymphocytes into the thyroid gland of F₁ hybrids. This syndrome occurred 21-28 days after the injection of cells, and terminated in death. Oliner et al. (1961) described lymphopenia in adult F₁ hybrid mice, inoculated with parental spleen cells. The lymphopenia varied according to the degree of weight loss. If weight was regained the lymphopenia disappeared. Streilein (1971) noted a progressive decrease of the blood lymphocyte count with time in F₁ hamsters that survived acute GvH disease.

To determine normal values in rats of similar age to those with chronic GvH disease, the red cell count,
A comparison between a normal (Lewis x DA)F<sub>1</sub> hybrid rat and one suffering from chronic GvH disease at the age of 16 months.

A. Normal rat aged 16 months.

B. Rat with chronic GvH disease for 15 months. Loss of fur from the back and snout can be seen. The small portion of the tail visible is white and smooth due to loss of the normal scaly skin.
white cell count and haemoglobin were determined on four 8 week old (Lewis x DA)F₁ and four 16 week old (Lewis x DA)F₁ female rats. The results were pooled and the mean and range of the observations are expressed as a horizontal line and a bar on the left hand side of Figure 9.6A.

Weekly peripheral blood examinations were performed on 4 F₁ hybrid animals injected with either control or enzyme treated thoracic duct lymphocytes at 4-5 weeks of age. Examinations were made from 7-12 weeks and at 20 weeks after injection of lymphocytes.

The results (mean and range) are shown in figure 9.6A. Whereas the haemoglobin and red cell counts tend to lie within the normal range throughout, there is a consistent depression of the leukocyte count. The maximum leukocyte count of any experimental animal was never as high as the lowest leukocyte count of the normal animals. The degree of suppression of circulating white cells is similar in both groups of animals, though there is some selective influence in the case of animals receiving untreated lymphocytes as only those surviving the acute runting syndrome could be examined.

Differential counts were performed on blood smears, the white blood cells being divided into two groups, granulocytes and 'mononuclear cells'. Figure 9.6B shows the % of the blood leukocyte count attributable to granulocytes. Although the variation is greater, the mean percentage of granulocytes in the peripheral blood of animals receiving either neuraminidase treated or control cells, is similar to those of the normal animals. Thus the decreased leukocyte count noted above is due to a depression of both granulocytic and mononuclear elements of the blood.

Despite the lack of early runting, the injection of neuraminidase treated lymphocytes evokes a depression of leukocytes in the peripheral blood similar to that produced by untreated cells. That the abnormalities are confined predominantly to the leukocytic elements of the blood, with a relative sparing of the erythroid
FIGURE 9.6
EXAMINATION OF THE PERIPHERAL BLOOD OF RATS WITH CHRONIC GVH SYNDROME

A. Haemoglobin, red cell count and white cell count.

The values for normal (Lewis x DA)F<sub>1</sub> female rats are shown as a mean (horizontal line) and range (vertical bar on left hand side of the graph).

The values from (Lewis x DA)F<sub>1</sub> female rats injected with 10<sup>8</sup> neuraminidase treated or control DA thoracic duct lymphocytes, at intervals from 7 to 20 weeks after injection are shown as a mean and range (vertical bar).

<table>
<thead>
<tr>
<th>Haemoglobin (Hb)</th>
<th>gms/mm&lt;sup&gt;3&lt;/sup&gt; x 10&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red cell count (RBC)</td>
<td>million/mm&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>White cell count (WBC)</td>
<td>thousand/mm&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

B. The percentage of the peripheral white cell count attributable to granulocytes.

The results are expressed as a mean and range (vertical bar).
A

WEEKS AFTER INJECTION

Hb

RBC

WCC

B

WEEKS AFTER INJECTION

% POLYMORPHS

NORMAL

CONTROL TDL

NDASE TDL
elements, suggests that a preferential attack of the parental cells on the white cells or their stem cell precursors has occurred in the $F_1$ host.

**Histological changes**

The pathological changes of lymphoid tissue in GvH disease have been summarized by Simonsen (1962), and are characterized by the disappearance of the normal lymphocytes and their concomitant replacement by the proliferation of other cell types. The new cells are predominantly pyroninophilic. Splenomegaly occurs at an early stage.

Tissues of (Lewis x DA)$F_1$ hybrids, which had been injected with $10^8$ neuraminidase treated parental lymphocytes at the age of 4-5 weeks were examined histologically at 1, 2, 3, 5, 6, 10 and 15 months after cell injection. The predominant findings were variable degrees of enlargement of the nodes and disorganization of the normal structure of lymph nodes and spleen. Atrophy and cellular depletion of the thymus, and depletion of lymphocytes within the lymph nodes and spleen were also prominent in all of the rats examined. The period in months referred to in the following description relates to the time after parental cell injection.

**Thymus**

The changes produced in the thymus in the chronic GvH syndrome are illustrated in figure 9.7. Two months or more after induction of GvH disease, the thymus showed marked cellular depletion, with virtually no small lymphocytes remaining. Scattered plasma cells could be identified among the reticular elements of the thymus. All the examples depicted in figure 9.7 were taken from animals that were killed, to exclude the complication of changes in the thymus produced by a terminal illness, which may occur in animals that die.
FIGURE 9.7

The thymic changes in the chronic GvH syndrome

A. Normal thymus from a 10 week old rat.
   Prominent cortical areas can be seen packed with small lymphocytes.

B. Normal thymus from a 20 week old rat
   The cortical areas are still prominent although not as prominent as those seen in the thymus of a 10 week old rat.

C. Thymus from a rat with chronic GvH syndrome of 6 weeks duration.
   Note the thinning and cellular depletion of the cortex as compared to A which depicts the thymus from a normal rat of the same age.

D. Thymus from a rat with chronic GvH syndrome of 7 weeks duration.
   The thymus shows 'cortico-medullary inversion' due to the marked loss of small lymphocytes from the cortical areas.

E. Thymus from a rat with chronic GvH syndrome of 11 weeks duration.
   The thymus shows an extensive depletion of small lymphocytes. There is disorganization of the normal lobular structure.

F. Thymus from a rat with chronic GvH syndrome of 6 months duration.
   Atrophy and fibrosis are present. The picture depicts about one quarter of the whole thymus.

Haematoxylin and Eosin  
Magnification x 85
**Lymph nodes and Peyer's patches**

One of the most striking features noted was the marked depletion of small lymphocytes in the cortical and paracortical areas of the lymph nodes. There were very few germinal centres present (Fig. 9.8B, C and D). Changes occurring in the deep cervical lymph node have been selected for illustration (Fig. 9.8), but similar changes were observed in the superficial cervical, inguinal and mesenteric nodes. After 15 months the nodes were atrophic with a fibrotic cortical area (Fig. 9.9A). The subcapsular space was obliterated and large cystic spaces within the nodes were frequent (Fig. 9.8D).

Similar depletion of lymphoid cells and absence of germinal centres was noted in the Peyer's patches.

**Spleen**

Spleens were enlarged in the first 2 months after parental cell transfer. Varying degrees of disorganization of normal follicular architecture were seen. At later times enlargement was less marked or absent. Large numbers of small cells with dark nuclei were frequently present in the red pulp (Fig. 9.9B). This appearance is consistent with that observed in GvH disease by other workers (Miller and Howard 1964, Armstrong et al. 1967) and was attributed to extramedullary haemopoiesis. At 15 months the spleen showed almost complete disorganization of normal structure and fibrotic bands were prominent (Fig. 9.9C). Decreased cellularity, particularly of the small lymphocytic elements surrounding the central arteriole, was consistently observed.

**Liver**

In some cases the liver showed mononuclear cell infiltration in the portal areas (Fig. 9.9D). Such an infiltration has been described as a feature of GvH reactions in adult mice (Gorer and Boyse 1959). The infiltrations reported in the mice were more extensive than those currently observed in rats.
FIGURE 9.8

The morphology of the deep cervical lymph node in the chronic GvH syndrome

A. A normal node from a 10 week old rat.
   Note the prominent germinal centres in the cortex surrounded by closely packed small lymphocytes and the prominent paracortical area.

B. A node from a rat with chronic GvH syndrome of 7 weeks duration.
   Only a small rim of cortex (arrow) can be seen. The rest of the node consists entirely of medulla. Note the absence of both the paracortical area and the germinal centres.

C. A node from a rat with chronic GvH syndrome of 6 months duration.
   The changes are similar to those in B. There is a complete absence of germinal centres and the paracortical area. The arrow identifies the cortico-medullary junction.

D. A node from a rat with chronic GvH syndrome of 15 months duration.
   The normal structure is completely disorganized with no recognizable cortex or medulla. Large cystic structures filled with blood can be seen.

Haematoxylin and eosin       Magnification x 35
The histological features of the chronic GvH syndrome in the lymph node, spleen and liver.

A. Cortical area of an inguinal lymph node from a rat with chronic GvH syndrome of 15 months duration.

The extensive fibrosis can be seen as pale areas between the dark nuclei.
Masson's trichrome Magnification x 350

B. The spleen from a rat with chronic GvH syndrome of 11 weeks duration.

The white pulp (arrow) is depleted of small lymphocytes. Extra-medullary haemopoiesis can be seen as groups of small dark nuclei interspersed among large pale cells in the red pulp.
Haematoxylin and eosin Magnification x 130

C. The spleen from a rat with chronic GvH syndrome of 15 months duration.

A central arteriole (arrow) is situated in the centre of the picture. There is a marked depletion of lymphocytes in the red and white pulp which become difficult to distinguish. Bands of fibrosis are scattered throughout the spleen.
Haematoxylin and eosin Magnification x 130

D. The liver from a rat with chronic GvH syndrome of 11 weeks duration.

The portal area shows infiltration by mononuclear cells.
Haematoxylin and eosin Magnification x 350
Recirculating lymphocyte population

The consistent leukocyte depletion in the peripheral blood together with the lymphocytic depletion of the tissues, suggested that the recirculating lymphocyte population of animals with chronic GvH syndrome was reduced.

This was confirmed by cannulating the thoracic duct in 5 animals which had been injected with $10^8$ neuraminidase treated parental cells at 4-5 weeks of age. The cannulations were performed 4-10 weeks after injection of parental cells. The cell output from the thoracic ducts of these animals is shown in figure 9.10A. For comparison the mean cell output of 3 normal $F_1$ hybrid animals of similar age is recorded.

The initial output of cells from animals with chronic GvH syndrome is approximately one half of that of the normal animals. After a period of rapid decrease in cell output during the first day or so, the cell output of the control animals more nearly approaches that of the animals with chronic GvH disease. It has been shown that the size of the lymphocyte pool that recirculates is similar to that number of cells which can be collected from the thoracic duct during the period of rapid fall in output (Gowans and Knight 1964). This pool is depleted to a greater extent in the chronic GvH syndrome, than that pool from which the continual low level output is derived after the recirculating cells have been withdrawn. There was nothing unusual about the morphology of the lymphocyte population obtained from the thoracic duct of animals with chronic graft-versus-host disease. The great majority of the cells appeared to be typical small lymphocytes (Fig. 9.10B).

In summary it appears that neuraminidase treatment of parental lymphocytes markedly reduces the severity of the acute runting when these lymphocytes are transferred to $F_1$ hybrid recipients. However these animals develop a chronic GvH syndrome which is
FIGURE 9.10

THE OUTPUT AND MORPHOLOGY OF CELLS OBTAINED FROM THE THORACIC DUCT OF RATS WITH CHRONIC GVH SYNDROME

A. THE HOURLY LYMPHOCYTE OUTPUT FROM THE THORACIC DUCT

- Mean lymphocyte output of 3 normal F₁ (Lewis x DA) male rats at the age of 12 weeks.
- Lymphocyte output of 5 individual F₁ (Lewis x DA) male rats injected with 10⁸ neuraminidase treated parental (DA) lymphocytes 4-10 weeks previously.

B. THE CELL POPULATION FROM THE THORACIC DUCT OF A RAT WITH CHRONIC GVH SYNDROME OF 11 WEEKS DURATION

The great majority of nucleated cells have the appearance of typical small lymphocytes.

McNeal's tetrachrome  
Magnification x 530
characterized by a decrease in size of the recirculating lymphocyte pool with lymphoid cell depletion of the spleen, lymph nodes and thymus.

3 THE LOCAL GRAFT-VERSUS-HOST REACTION

The decrease in the ability of neuraminidase treated parental lymphocytes to produce acute GvH disease may be due to inability of these cells to recognize or respond to 'foreigness' in the hybrid recipient. Alternatively, the less severe runting may reflect a modification in lymphocyte distribution within the host animal, without any loss of immunological capacity on the part of the parental cells. Local reactions, where the cells are injected into a given site and the response is followed, provide a way of distinguishing between these two possibilities.

Local graft-versus-host reactions have been described in the rat in the skin (Ford 1967), and under the kidney capsule (Elkins 1964), but are difficult to quantitate in these situations. The normal lymphocyte transfer reaction or GvH reaction in the skin of the rat is greatly reduced in intensity as compared to that seen in the skin of guinea pigs or sheep. Histological changes are minimal or absent and hence it is not a suitable location in which to visualize the response and fate of injected cells.

Local GvH reaction in the retroperitoneal adipose tissue

An assay was developed which depended on the increase in weight of a lymph node draining the site of injection of parental lymphocytes. A volume of 0.1 ml of a lymphocyte suspension was injected into the retroperitoneal fat overlying the psoas muscle 0.8 cm caudal to where the iliolumbar artery enters the adipose tissue. When $10^7$ parental cells were injected into the adipose tissue of F₃ hybrid recipients a tiny node or nodes, the lateral iliac, which drain this area, underwent considerable enlargement and a macroscopically visible red lesion was produced in the adipose tissue 7 days later (Fig. 9.11A).
FIGURE 9.11
The local GvH reaction in the retroperitoneal adipose tissue.

A. A macroscopic view of the inflammatory lesion (L) and the enlarged lateral iliac nodes (N). The reaction was photographed 7 days after the injection of $10^7$ parental (DA) thoracic duct lymphocytes into the adipose tissue of a (Lewis x DA)$_1$ hybrid recipient. This lesion is situated in the left iliac fossa.

B. A comparison of a lateral iliac node draining a local GvH reaction produced by $10^7$ parental lymphocytes (top) with an unstimulated node from the opposite side of the same animal (bottom). The nodes were taken 7 days after the injection of lymphocytes.

Haematoxylin and eosin Magnification x 25
Figure 9.11B compares a node draining a local GvH reaction with an unstimulated node from the opposite side of the same animal.

In order to quantitate this response the weight of the node and largest diameter of the lesion at the injection site were recorded. As an indication of normal variation to be expected in the lateral iliac node weight, normal nodes from 21 hybrid rats had a mean weight of $1.6 \pm 0.1$ mg (Mean ± standard error).

To provide a control for the injection of parental lymphocytes, $10^8$ (Lewis x DA)F$_1$ thoracic duct lymphocytes were injected into the retroperitoneal fat of (Lewis x DA)F$_1$ hybrid recipients. Seven days after injection there were no visible lesions in any of the experiments. The node weight was $1.6 \pm 0.1$ mg (mean and standard error of 5 experiments), that is, the same as in untreated rats. This indicated that the injection procedure itself did not affect the size of the node.

Activity of thoracic duct lymphocytes

A dose response curve was constructed, the node weight being measured after a range of doses of injected Lewis thoracic duct cells, increasing from $10^5$ to $10^7$ lymphocytes. The results are given in figure 9.12 as the mean and standard error of the node weight for each group of 8 or 9 lesions. The increase in node weight is proportional to the dose of cells injected, $10^7$ Lewis thoracic duct lymphocytes producing a twenty fold increase in node weight.

A dose of $10^7$ cells invariably produced a visible inflammatory response at the site of injection when examined 7 days later. $10^6$ cells produced a visible, but smaller lesion in 5 out of 10 experiments, whilst $10^5$ cells produced a macroscopic lesion in only 1 out of 10 cases.

Activity of spleen and thymus cells

The GvH activity of $10^7$ Lewis spleen or thymus cells was compared with that of $10^7$ thoracic duct cells.
The enlargement produced by Lewis strain thoracic duct lymphocytes (TDL), spleen or thymus cells 7 days after injection into F₁ (Lewis x DA) hybrid rats. For comparison the mean weight of a normal unstimulated node is included.

Node weights are plotted on a log₁₀ scale and expressed as mean ± standard error.

Nodes draining 8 or 9 lesions from recipients of thoracic duct lymphocytes and 4 lesions from recipients of spleen or thymus cells are included in each point.
As judged by this assay spleen cells were of similar potency to thoracic duct cells, whilst thymus cells were only 1/10th as effective (Fig. 9.12). Similar conclusions on the relative GvH activity of different lymphoid cell populations have been reached by Billingham et al. (1962 and Elkins (1964).

Activity of neuraminidase treated thoracic duct lymphocytes

The local assay allows two parameters of the GvH activity of lymphocytes to be observed, namely their ability to excite a local response in the tissue and their ability to cause enlargement in a draining node. The adipose tissue is a particularly suitable site to observe the local response microscopically because of its own simplicity of structure. To examine the ability of neuraminidase treated cells to initiate a GvH reaction, $10^7$ untreated or neuraminidase treated, Lewis or DA, thoracic duct lymphocytes were injected into the retroperitoneal fat of $F_1$ hybrid recipients, and the size of the lesion produced and the weight of the draining lateral iliac node were measured 7 days later. The results are shown in table 9.1. Utilizing cells from either Lewis or DA strain donors, enzyme treated and control lymphocytes produced an equivalent local response as measured by the node weight and size of the lesion. Histologically the lesions produced were similar. Figure 9.13 compares the site of injection of either $10^7$ neuraminidase treated or control, parental thoracic duct lymphocytes with that of $10^8 F_1$ hybrid thoracic duct lymphocytes at 7 days.

Numerous pyroninophilic blast cells could be seen among the mononuclear cell infiltrate in the adipose tissue when either normal or neuraminidase treated parental cells were injected (Fig. 9.13D).

The foot pad assay

A GvH assay based on a similar principle of increase in node weight has been described in rats by Ford, Burr and Simonsen (1970). This assay depends on the enlargement
TABLE 9.1
A COMPARISON OF THE LOCAL GVH ACTIVITY OF 10^7 CONTROL OR NEURAMINIDASE TREATED THORACIC DUCT LYMPHOCYTES

<table>
<thead>
<tr>
<th>Lymphocytes injected</th>
<th>Node weight(^+) (mg)</th>
<th>Lesion size(^+) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Lewis</td>
<td>21.1 ± 4.6(^*)</td>
<td>7.4 ± 0.5 (8)</td>
</tr>
<tr>
<td>Neuraminidase treated Lewis</td>
<td>31.4 ± 5.0(^*)</td>
<td>7.7 ± 0.2 (8)</td>
</tr>
<tr>
<td>Control DA</td>
<td>26.9 ± 4.4(^o)</td>
<td>9.7 ± 0.3 (14)</td>
</tr>
<tr>
<td>Neuraminidase treated DA</td>
<td>17.5 ± 3.0(^o)</td>
<td>9.8 ± 0.4 (14)</td>
</tr>
</tbody>
</table>

\(^+\) Values are expressed as mean ± standard error. The number of observations in each group is recorded in brackets.

\(^o\) Difference between these results is not significant at the 5% level.
The local GvH lesion produced by control or neuraminidase treated thoracic duct lymphocytes in the retroperitoneal adipose tissue of a (Lewis x DA)\textsubscript{F\textsubscript{1}} hybrid rat.

The peritoneal surface of the tissue is at the top of A, B and C.

A. The site of injection of $10^8$ syngeneic F\textsubscript{1} lymphocytes at 7 days.

There is no inflammatory reaction. Some small lymphocytes can still be identified among the fat cells (arrow).

Haematoxylin and eosin  
Magnification x 35

B. The site of injection of $10^7$ control parental lymphocytes at 7 days.

The section shows intense cellular infiltration among the fat cells and many dilated blood vessels.

Haematoxylin and eosin  
Magnification x 35

C. The site of injection of $10^7$ neuraminidase treated parental lymphocytes at 7 days.

The lesion has the same appearance as that produced by control lymphocytes.

Haematoxylin and eosin  
Magnification x 35

D. Cellular detail of the lesion produced by neuraminidase treated parental cells at 7 days.

Numerous pyroninophilic 'blast' cells are present among the fat cells.

Methyl green and pyronin  
Magnification x 600
of the popliteal node following injection of lymphocytes into the hind foot of rats. This assay was used to compare the GvH activity of neuraminidase treated and control thoracic duct lymphocytes. DA thoracic duct lymphocytes, collected on the 2nd day after cannulation, and either treated with neuraminidase or left untreated as a control, were injected subcutaneously with a tuberculin syringe into the foot pads of 7 week old (Lewis x DA)F₁ hybrid recipients (methods). There were 5 animals in each group. A parallel line assay was performed, utilizing the calculated regression lines to obtain the potency ratio of control to neuraminidase treated cells (Fig. 9.14). The potency ratio of control to neuraminidase treated cells was 1:1.1. This was not a significant difference. Thus the results of both the retroperitoneal fat and foot pad assays indicate that the local GvH activity of an injected population of lymphocytes is unimpaired by neuraminidase treatment.

4 THE FATE OF NEURAMINIDASE TREATED PARENTAL CELLS AFTER TRANSFER TO F₁ HYBRIDS

Simonsen (1962) has summarised 3 possible alternatives as to the fate of the parental cells in GvH reactions.

(i) The grafted cells maintain indefinitely their reactivity against the host.

(ii) The grafted cells commit "allergic suicide" in the first host.

(iii) The cells lose reactivity to the host but retain immunological competence in respect other strains.

Animals suffering from chronic GvH syndrome produced by neuraminidase treated parental cells were examined to determine whether reactive parental cells could be identified. Three test systems were used, skin grafting, transfer of thoracic duct lymphocytes to sublethally irradiated F₁ hybrids, and the local foot pad assay.

Skin grafting

Six (Lewis x DA)F₁ hybrid males which had received 10⁸ neuraminidase treated DA thoracic duct lymphocytes at 4-5 weeks of age were grafted 6-9 weeks
FIGURE 9.14

A COMPARISON OF THE GVH ACTIVITY OF CONTROL AND NEURAMINIDASE TREATED THORACIC DUCT LYMPHOCYTES USING THE FOOT PAD ASSAY

The popliteal node enlargement, 7 days after injection of doses of 0.33, 1 and 3 million control or neuraminidase treated parental (DA) thoracic duct lymphocytes into the hind foot pads of F1 (Lewis x DA) rats is recorded.

Values are expressed as the mean ± s. error of the log10 of the node weight in mgm (5 in each group).

For comparison the weight of the popliteal node 7 days after the injection of 27 x 10^6 syngeneic F1 (Lewis x DA) thoracic duct lymphocytes is recorded in the bottom left hand corner of the graph.
later with full thickness Lewis and DA skin grafts simultaneously. All of the DA grafts healed in well and by 2-3 weeks after grafting were growing fur. The Lewis grafts healed in satisfactorily, but after 2-3 weeks, 3 of the 6 grafts felt firmer than the DA grafts and showed some localized ulceration and scab formation. However, by 3 weeks fur growth was present on these Lewis grafts. The other 3 Lewis grafts healed well and grew fur satisfactorily (Fig. 9.15A). All the grafts were followed for at least 4 months. The 3 Lewis grafts that had showed some early, localized ulceration remained intact and grew fur for this duration of time. The fur growth of these grafts was not as luxuriant as the DA control grafts, and the grafts themselves underwent some degree of contraction. Figure 9.15B and C compares DA (B) and Lewis (C) grafts that have been in place for 4 months on an animal with chronic GvH disease. There are fewer appendages such as hair follicles and sweat glands in the dermis of the Lewis graft. The epithelium of the Lewis graft is slightly thinner than that of the DA graft.

The different courses of Lewis and DA grafts on some of the F\textsubscript{1} hybrids may reflect underlying parental cell activity. However there was no evidence of an acute rejection process, as all 6 Lewis grafts were showing fur growth 4 months after grafting.

**Runting of sublethally irradiated hybrid rats**

If there were sufficient parental lymphocytes present in the thoracic duct lymph of F\textsubscript{1} hybrid hosts suffering from chronic GvH syndrome, transfer of these cells to a sublethally irradiated F\textsubscript{1} hybrid host may be one way to demonstrate them. Sublethal irradiation has been shown to greatly increase the susceptibility of F\textsubscript{1} hybrid rats to GvH disease produced by parental cells (McGregor 1968), and is therefore a useful system to detect relatively small numbers of parental cells.

In preliminary experiments it was found that 5 million DA thoracic duct lymphocytes caused runting...
FIGURE 9.15

DA and Lewis skin grafts on (Lewis x DA)F₁ hybrid rats with chronic GvH syndrome produced by 10⁸ DA strain neuraminidase treated lymphocytes.

A. DA (top) and Lewis (bottom) skin grafts at 7 weeks after grafting. The F₁ hybrid rat received the parental cells at 4 weeks of age and was grafted 8 weeks later.

Both grafts are healthy.

B. DA skin graft after 4 months in situ on a rat with chronic GvH disease.

The skin appears normal.

Haematoxylin and eosin Magnification x 35

C. Lewis skin graft from the same rat as the DA graft depicted in B, after 4 months in situ.

Note that the hair follicles and sweat glands are reduced in number as compared to figure B. The epithelium also appears to be thinner than that of the DA graft.

Haematoxylin and eosin Magnification x 35
invariably, and death frequently after intravenous transfer to (Lewis x DA)\(_1\) hybrid rats weighing 60-90 gm. The hybrid rats received 455 rad sublethal irradiation prior to cell transfer. Intravenous doses of 10 and 20 million DA cells caused more severe runting.

Varying numbers of thoracic duct lymphocytes (depicted on the graph) from (Lewis x DA)\(_1\) hybrids, 4-10 weeks after the transfer of neuraminidase treated parental DA cells, were injected intravenously into sublethally irradiated recipients. Figure 9.16 shows the weight changes of 6 hybrids after transfer of thoracic duct lymphocytes from 5 different animals with chronic GvH syndrome. 4 animals showed no evidence of any GvH disease, whilst 2 animals showed an arrest in weight gain between days 16-24, a time when runting becomes evident in more severe cases of GvH disease. Both of these animals exhibited erythema of the paws at this time. They subsequently gained weight satisfactorily and showed no further signs of GvH disease.

From these experiments it would appear that, if parental lymphocytes with activity against the \(F_1\) recipients had been present in the thoracic duct lymph, they would contribute less than 5% of the total lymphocyte pool. This estimate is made from the numbers of lymphocytes transferred from animals suffering from chronic GvH syndrome, (from 50-100 \(x\) \(10^6\)), and the fact that 5 \(x\) \(10^6\) DA lymphocytes are known to invariably cause runting after inoculation into sublethally irradiated (Lewis x DA)\(_1\) hybrids weighing 60-90 grams.

**Foot pad assay**

The foot pad assay for GvH activity (Ford et al. 1970) was used in an attempt to detect cells of parental type in the thoracic duct lymph of \(F_1\) hybrids manifesting the chronic GvH syndrome. Such an assay can detect 0.3 \(x\) \(10^6\) DA parental cells when injected into a (Lewis x DA)\(_1\) host (Fig. 9.14).
FIGURE 9.16

WEIGHT CHANGES OF (LEWIS x DA)F₁ HYBRID RATS AFTER TRANSFER OF THORACIC DUCT LYMPHOCYTES FROM (LEWIS x DA)F₁ RATS WITH CHRONIC GVH DISEASE

6 Sublethally irradiated (455 rad) (Lewis x DA)F₁ hybrid rats received an intravenous injection of 45 - 78 x 10⁶ thoracic duct lymphocytes (recorded on graph), from 5 different (Lewis x DA)F₁ hybrids with chronic GvH syndrome. The chronic GvH syndrome had been produced by injection of 10⁸ neuraminidase treated DA thoracic duct lymphocytes 6 - 10 weeks prior to thoracic duct cannulation.

The weights of the recipients of TDL from animals with chronic GvH disease were recorded at 4 day intervals after injection. No animals died from GvH disease.
Thoracic duct lymphocytes, draining from (Lewis x DA)$F_1$ hybrids with the chronic GvH syndrome, were injected into foot pads of 6-8 week old (Lewis x DA)$F_1$ hybrid recipients. As a control, equal numbers of normal (Lewis x DA)$F_1$ thoracic duct lymphocytes were injected into the opposite foot pad of each animal. The weights of the popliteal nodes on the two sides were compared 7 days later. If it is assumed that the parental cells present have the same level of GvH activity as normal DA thoracic duct lymphocytes, an estimate can be made of the percentage of parental cells present by reference to the dose response curve (Fig. 9.14). Even if the DA cells were sensitized against Lewis strain tissues, the error introduced would be small as the factor of immunization for strong AgB combinations in less than 2 (Ford and Simonsen 1971).

In every case tested, there was a significant difference in popliteal node size between the 2 sides (Table 9.2). The nodes challenged with cells from $F_1$ animals undergoing a chronic GvH reaction were always larger than those challenged with normal $F_1$ cells. The $F_1$ hybrid animal cannulated 6 months after parental cell transfer had been bearing DA and Lewis skin grafts for 4 months at the time of cannulation. Fur growth on the Lewis graft was not as profuse as that on the DA graft.

Reactive cells, presumably of parental origin, were demonstrated in the thoracic duct lymph of $F_1$ hybrids, in all of the series of rats examined from 1 to 9 months after injection of neuraminidase treated parental cells. These cells comprised from 2 to 5% of the lymphocyte population obtained from the thoracic duct.

Activity against a third party strain

The experiments described above imply that there are DA lymphocytes with activity against Lewis tissues circulating in $F_1$ hybrids with chronic GvH disease. It is possible that DA cells reactive against (Lewis x DA)$F_1$
TABLE 9.2
THE GvH ACTIVITY OF THORACIC DUCT LYMPHOCYTES FROM (LEWIS X DA)F₁ HYBRID RATS WITH CHRONIC GvH DISEASE

<table>
<thead>
<tr>
<th>Time after parental cell transfer (months)</th>
<th>Number of lymphocytes injected into each foot pad (x 10⁶)</th>
<th>Popliteal node weight* (mg)</th>
<th>Probability</th>
<th>% of parental lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Chronic GvH</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>7.1±1.2</td>
<td>20.3±2.4</td>
<td>.001</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>7.2±0.7</td>
<td>20.3±0.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>1.5</td>
<td>12.9</td>
<td>6.4±0.1</td>
<td>12.8±0.7</td>
<td>.002</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>4.7±0.5</td>
<td>14.9±0.3</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>6</td>
<td>9.2</td>
<td>4.6±0.4</td>
<td>10.5±1.3</td>
<td>.01</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>5.6±0.6</td>
<td>22.2±1.9</td>
<td>.0001</td>
</tr>
</tbody>
</table>

* mean ± standard error (4 or 5 in each group)

![This percentage of parental lymphocytes in the thoracic duct lymph was calculated by reference to the dose response curve for DA cells injected into the foot pads of (Lewis x DA)F₁ recipients (Figure 9.14).](image)

Male (Lewis x DA)F₁ rats were injected intravenously with neuraminidase treated DA strain thoracic duct lymphocytes at 4-5 weeks of age. At varying times after this (1st column of table), thoracic duct lymphocytes were obtained from these F₁ animals and injected into the footpads of (Lewis x DA)F₁ recipients. As a control an equal number of (Lewis x DA)F₁ thoracic duct lymphocytes from a normal rat were injected into the opposite foot pad. The popliteal nodes were removed and weighed 7 days later.
tissues may be selectively removed from the recirculating pool in the (Lewis x DA)F₁ hosts. If this were so, then a greater degree of parental cell reactivity may be shown by lymphocytes from (Lewis x DA)F₁ hybrids with chronic GvH syndrome against F₁ hybrids of DA and a third party strain. F₁ hybrids of the DA and Hooded (H) strains were used to test the activity of thoracic duct cells from (Lewis x DA)F₁ hybrids, in which a chronic GvH syndrome had been produced by the injection of $10^8$ neuraminidase treated DA cells.

Two test systems were tried, the foot pad assay and the runting of sublethally irradiated hybrids.

It was found that the foot pad assay was unsatisfactory in attempts to demonstrate parental cell activity using (DA x H)F₁ hybrid recipients. Reaction between (Lewis x DA)F₁ cells and the (DA x H)F₁ host masked any reactivity between parental DA cells and the (DA x H)F₁ host. Accordingly, (DA x H)F₁ hybrids were immunized with two intraperitoneal injections of 6-10 x $10^7$ Lewis spleen cells, in an attempt to eliminate reactivity of the (Lewis x DA)F₁ cells against the (DA x H)F₁ host. The first injection was given at the age of 3 weeks and the second 6 days later. These animals are subsequently referred to as 'immune' (DA x H)F₁ hybrids.

The popliteal node weight of 'immune' (DA x H)F₁ recipients produced by injection into the foot pad of 33 million (Lewis x DA)F₁ thoracic duct lymphocytes from normal donors was 6.8 ± 0.5 (mean ± s. error). This may be compared with a node weight of 26.7 ± 5.6 produced by the injection of the same number of lymphocytes into normal non-immune (DA x H)F₁ recipients.

However it was not possible to detect any significant difference in the node enlargement produced by 13 million normal or 'chronic GvH' thoracic duct lymphocytes from (Lewis x DA)F₁ hybrids when transferred to 'immune' (DA x H)F₁ recipients (table 9.3). In both cases the node enlargement produced was greater than that produced by one million parental (DA) cells (table 9.3).
TABLE 9.3
RESULTS OF THE FOOT PAD ASSAY
USING 'IMMUNE' (DA x H)F₁ HYBRID RECIPIENTS

<table>
<thead>
<tr>
<th>Lymphocyte donor</th>
<th>Number of Lymphocytes transferred (x 10^6)</th>
<th>Popliteal node weight* (mgm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (Lewis x DA)F₁</td>
<td>13</td>
<td>10.2 ± 1.8</td>
</tr>
<tr>
<td>Chronic GvH (Lewis x DA)F₁</td>
<td>13</td>
<td>7.8 ± 1.2</td>
</tr>
<tr>
<td>Normal DA</td>
<td>1</td>
<td>4.2 ± 0.5</td>
</tr>
</tbody>
</table>

* mean ± standard error (5 or 6 in each group)

Consequently it is possible that well over 10% of parental type cells could remain undetected among the lymphocytes from animals with chronic GvH syndrome using this form of assay.

Further experiments were performed using sublethally irradiated (DA x H)F₁ hybrids. Doses of 43 to 78 x 10^6 cells from animals with chronic GvH syndrome caused severe runting and early death in sublethally irradiated (DA x H)F₁ recipients (Fig. 9.17A). Both effects were largely prevented by preimmunization of the (DA x H)F₁ hybrid recipients against Lewis tissues (Fig. 9.17B) suggesting that the runting syndrome was due to the (Lewis x DA)F₁ lymphocytes in the inocula. It was found that 10-15 x 10^6 parental DA lymphocytes were necessary to produce signs of GvH disease in sublethally irradiated 'immune' (DA x H)F₁ hybrid recipients (table 9.4).
FIGURE 9.17
WEIGHT CHANGES OF \((DA \times H)_{F_1}\) HYBRID RATS AFTER TRANSFER OF THORACIC DUCT LYMPHOCYTES FROM \((LEWIS \times DA)_{F_1}\) RATS WITH CHRONIC GVH DISEASE

A. **Non-Immune**

Sublethally irradiated (455 rad), \((DA \times H)_{F_1}\) hybrid rats received intravenous doses of \(43-78 \times 10^6\) thoracic duct lymphocytes from 2 different \((LEWIS \times DA)_{F_1}\) rats with chronic GVH syndrome.

The \((LEWIS \times DA)_{F_1}\) hybrid rats had been injected with \(10^8\) neuraminidase treated DA thoracic duct lymphocytes 6 and 7 weeks prior to thoracic duct cannulation.

The weights of the \((DA \times H)_{F_1}\) hybrid rats were recorded at 4 day intervals after the transfer of lymphocytes. All 5 animals had died by day 20 with features of GVH disease. The cell dose received by each animal is recorded on the weight curve of that animal.

B. **Immune**

\((DA \times H)_{F_1}\) rats were immunized with Lewis spleen cells. The sublethally irradiated (455 rad) \((DA \times H)_{F_1}\) hybrid rats received intravenous doses of \(45-78 \times 10^6\) thoracic duct lymphocytes from 5 different \((LEWIS \times DA)_{F_1}\) rats with chronic GVH syndrome. The \((LEWIS \times DA)_{F_1}\) hybrid rats had been injected with \(10^8\) neuraminidase treated DA thoracic duct lymphocytes from 5-10 weeks prior to thoracic duct cannulation.

The weights of the immune \((DA \times H)_{F_1}\) rats were recorded at 4 day intervals after the transfer of lymphocytes. Two animals died from GVH disease (broken lines).
TABLE 9.4

THE GvH ACTIVITY OF NORMAL PARENTAL (DA) THORACIC DUCT LYMPHOCYTES AFTER TRANSFER TO 'IMMUNE' (DA x H)F₁ HYBRID RATS

<table>
<thead>
<tr>
<th>No. lymphocytes x 10⁶</th>
<th>Recipient*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. inoculated</td>
<td>No. ill</td>
<td>No. killed</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

* Male and female (DA x H)F₁ hybrid rats of 60-90 grams body weight. The animals, previously immunized against Lewis spleen cells, were exposed to 455 rad irradiation on the day of injection. Animals were observed for 60 days after the intravenous transfer of DA parental cells for weight loss due to GvH disease.

Thus, unless the number of parental lymphocytes in the thoracic duct lymph of animals with chronic GvH disease were greater than 20%, it would be difficult to detect reactivity using (DA x H)F₁ recipients at the dosage levels of chronic GvH TDL used in these experiments.

These experiments have shown that the (Lewis x DA)F₁ thoracic duct lymphocytes from animals with chronic GvH syndrome retain some GvH activity themselves. They also indicate that the parental strain DA lymphocytes present in the thoracic duct lymph of animals with chronic GvH syndrome form less than 20% of the cell population. It was not possible to obtain an estimate of the relative activity of parental DA cells in the thoracic duct lymph of (Lewis x DA)F₁ hybrids, against Lewis and Hooded strain tissues.

5 THE RESPONSE OF RATS WITH CHRONIC GvH DISEASE TO SHEEP RED CELLS

A reduced responsiveness to various antigens on the part of animals in the acute phase of the graft-versus-host syndrome has been described (Howard and Woodruff 1961, Lawrence and Simonsen 1967, Blanden 1969,
Davis, Cole and Schaffer 1970, Möeller 1971). In order to ascertain whether antibody responses were also depressed in the chronic phase of the disease, rats suffering from chronic GvH syndrome of 5 weeks duration were immunized intravenously with $10^8$ sheep red blood cells. The haemolysin responses of 5 hybrid rats with a chronic GvH syndrome are shown in figure 9.18. Control titres were obtained by immunization of 4 normal (Lewis x DA)$F_1$ hybrids of similar age. In none of the animals with chronic GvH syndrome was the peak haemolysin titre as high as the lowest peak titre in the control animals. Furthermore the peak haemolysin titres were delayed in the group of animals suffering from chronic GvH disease.

It is possible that the poor response to SRBC of animals with chronic GvH syndrome is a consequence of a quantitative reduction of the recirculating lymphocyte pool, and the disorganization of the lymphoid tissue previously demonstrated. Alternatively the recirculating population itself may be qualitatively deficient in some specific cell population necessary for the response.

To distinguish between these two possibilities the response to sheep erythrocytes of thoracic duct lymphocytes from animals with chronic GvH syndrome was compared with that of an equivalent number of normal (Lewis x DA)$F_1$ thoracic duct lymphocytes, using an adoptive transfer system. In all 6 cases examined, the lymphocytes from animals with chronic GvH syndrome gave a greatly reduced haemolysin response after transfer to irradiated recipients as compared to lymphocytes from normal $F_1$ donors (Fig. 9.19).

It was concluded that the recirculating lymphocyte pool of animals with chronic GvH syndrome has been depleted of a lymphocyte population which is necessary for a normal response to sheep erythrocytes.
5 male (Lewis x DA)F₁ hybrids were injected intravenously with $10^8$ neuraminidase treated DA thoracic duct lymphocytes at the age of 4 to 5 weeks. 5 weeks later these animals were immunized with $10^8$ sheep red blood cells intravenously, and bled on alternate days for determination of serum haemolysin.

Individual responses of 5 hybrids with chronic GVH disease.

Mean response of 4 normal (Lewis x DA)F₁ hybrids of similar age.
10^8 (Lewis x DA)F_1 thoracic duct lymphocytes from normal adult animals, or animals with chronic GvH disease, were injected intravenously into irradiated (730 rad) adult (Lewis x DA)F_1 recipients in the first 24 hours after irradiation. 10^8 sheep red blood cells were administered with the lymphocytes. The recipients were bled on alternate days for determination of serum haemolysin.

- Normal thoracic duct lymphocytes.

- Thoracic duct lymphocytes from animals with chronic GvH disease. The duration of the GvH syndrome (weeks) prior to collection of thoracic duct lymphocytes is recorded beside the symbols.
DISCUSSION

Two main points arise for discussion from the experiments described above. The first is the effect of neuraminidase treatment of parental lymphocytes in reducing the severity of the GvH syndrome produced by these cells after intravenous injection, while the ability of these cells to cause a local GvH reaction is unimpaired. The second is the nature of the chronic GvH syndrome produced in animals given neuraminidase treated cells intravenously, and the light that study of this syndrome throws on GvH reactions in general.

The reduction in severity of the systemic GvH syndrome

The ability of neuraminidase treated parental lymphocytes to engage in GvH activity is equivalent to that of normal parental lymphocytes. This was established by the response of the regional node in the F₁ host after injection of parental lymphocytes into the retroperitoneal fat or foot pad. Proliferation and transformation of parental lymphocytes after local injection into F₁ hybrid tissues has been described as an essential feature of the local GvH reaction by Elkins (1970). Neuraminidase treatment of lymphocytes does not seem to inhibit this process, as indicated by the appearance of blast cells at the site of the injection of neuraminidase treated cells into the retroperitoneal fat. This has been confirmed in vitro, by demonstration of the transformation of neuraminidase treated thoracic duct lymphocytes to blast cells when cultured on monolayers from rat embryos of a different genetic strain (chapter 7). The ability of neuraminidase treated parental lymphocytes to abrogate tolerance to sheep red blood cells (chapter 8), a process which requires allogeneic interaction, suggests that neuraminidase treated cells are capable of participating in GvH activity. The allogeneic interaction responsible for initiating the abrogation of sheep red cell tolerance is substantially completed within an hour after injection.
of the parental cells (McCullagh 1972), at a time before the neuraminidase treated lymphocytes which have migrated to the spleen regain normal surface properties (chapter 5). This is also in accord with the appearance of transformed cells in the splenic white pulp noted soon after intravenous injection of neuraminidase treated parental cells.

Recipients of neuraminidase treated thoracic duct cells demonstrated reduced runting and less marked symptoms, together with a lower mortality, than did recipients of an equivalent number of control lymphocytes. There are several possible explanations for the reduction in severity of the acute stages of GvH disease following the intravenous injection of neuraminidase treated lymphocytes.

Loss of viability of the neuraminidase treated population as a result of the incubation procedure can be excluded. The ability of neuraminidase treated lymphocytes to restore an antibody response to sheep red cells in irradiated recipients (chapter 8) and to engage in local GvH reactions is the same as that of untreated lymphocytes.

Other possible reasons to be considered are, "unmasking" of lymphocyte surface antigens determined by recessive parental genes, increased "allogeneic inhibition" of the parental lymphocytes, or coating of the parental cells with host material, which might occur to a greater extent with the neuraminidase treated lymphocytes than with the controls. However all these suggestions do not explain why these effects were not also operative in local GvH reactions.

The possibility that neuraminidase treatment of lymphocytes promotes their opsonization and phagocytosis by the host, thereby reducing the number of cells available to take part in the GvH reaction, should be considered. Neuraminidase treatment of heterologous red blood cells prior to opsonization, has been reported to increase their susceptibility to phagocytosis.
by mouse peritoneal macrophages (Lee 1968). Treatment of lymphoid cells with anti-lymphocyte serum prior to transfer results in a considerable increase in their localization in the liver after intravenous injection (Martin and Miller 1967). This increase appears to be a result of phagocytosis of lymphocytes by the reticuloendothelial cells of the liver (Martin 1969). However there is some evidence against the idea that neuraminidase treated cells suffer the same fate. Both the splenic plaque-forming cell response and the haemolysin response of irradiated recipients of neuraminidase treated lymphocytes are similar to those of recipients of untreated lymphocytes. In an autoradiographic study, neuraminidase treated lymphocytes have been identified within the liver sinusoids in similar locations to untreated cells (Gesner et al. 1969). The amount of $^{51}$Cr label in the liver tends to decrease, while that in the nodes increases during the first 24 hours after injection of labelled neuraminidase treated lymphocytes (chapter 5). The reverse is the case when labelled anti-lymphocyte serum treated lymphocytes are injected (Martin and Miller 1967). Opsonization and phagocytosis would also be expected to diminish the GvH response in lymph nodes draining a local site of injection of neuraminidase treated cells, unless it were postulated that cells recovered their normal surface properties before migrating to the lymph node.

The most likely explanation of the reduction in severity of GvH disease produced by neuraminidase treated cells is altered cell migration after injection into the $F_1$ hybrids. The most dramatic changes in GvH disease are seen in the lymphoid tissues, and changes observed in host lymphoid tissue such as the spleen correlated to some extent with the dose of parental cells injected, and the severity of disease (Simonsen 1962). Neuraminidase treatment of lymphocytes, by diminishing their initial localization in lymphoid tissues may reduce the severity of the runting syndrome subsequently observed.
The source of parental cell stimulation in GvH reactions appears to be the host leucocytes rather than tissue antigens. This 'passenger cell' concept has been reviewed by Billingham (1971) who concluded that the host leucocytes constitute the principal and indispensable source of antigen in practically all forms of local and systemic graft-versus-host reactivity. Parental lymphocytes localizing in lymphoid tissue will be in an optimal environment for maximal stimulation, with an abundant supply of host lymphocytes and a favourable environment for stimulation. Conditions pertaining elsewhere in the host (e.g. liver) may not be so favourable for stimulation of the parental cells, and hence the severity of attack on the host is diminished. An alternative explanation to be considered is that parental lymphocytes are stimulated to an equivalent degree wherever they localize, but the subsequent severity of runting observed depends on the damage sustained by the host tissues in which this initial stimulation is taking place. That lymphoid cells can engage in GvH activity in sites where they would not normally localize is shown by the reactions produced under the kidney capsule (Elkins 1964), and in the retroperitoneal fat after transfer of parental cells to F₁ recipients. If this alternative explanation is so it would indicate that the severity of the wasting produced in the acute phase of GvH disease is related to the degree of damage which is sustained by the lymphoid tissues in the early phase of the disease.

It is also possible that lymphocytes migrating to the liver undergo some form of immunosuppression. Picryl chloride (Chase 1966), dinitrochlorobenzene (Cantor and Dumont 1967), and histocompatibility antigens (Mayer et al. 1965) all evoke a lesser immune response if administered so that antigen is delivered to the liver prior to reaching the lymphatic system. a₂ glycoproteins, known to be synthesized by liver, have been shown to suppress responsiveness to antigens (Mowbray 1963a, Mowbray and Hargrave 1966) and allograft rejection (Mowbray 1963b, Mannick and Schmid 1967) in vivo, and
antigen or PHA induced lymphocyte transformation in vitro (Cooperband et al., 1968). Lymphocytes situated in the liver could be subjected to a similar form of immunosuppression, and as nearly 50% of neuraminidase treated lymphocytes localize in the liver at one hour after transfusion as compared to only 10% of the untreated lymphocytes, differences in the severity of the GvH disease could result.

Experiments described previously (Chapter 5) demonstrated that neuraminidase treated lymphocytes regained normal migratory properties by 24 hours after transfer, and that they could then colonize the spleen of an irradiated host and undertake an immune response in an equivalent manner to untreated cells (Chapter 8). Thus it might have been expected that after this recovery, F₁ recipients of neuraminidase treated parental cells would have developed acute GvH disease of equal severity to that produced by normal lymphocytes. The fact that this did not happen indicates that the initial site of exposure of the parental lymphocytes to the F₁ tissues is crucial in determining the course of the GvH disease. Evidence for the importance of early events in the development of the GvH syndrome has also been obtained by Silvers and Billingham (1969). They found that neonatal recipients of allogeneic lymphocytes could be protected against the development of acute GvH disease by administration of syngeneic lymphocytes from immunocompetent donors. It was necessary that the syngeneic lymphocytes were administered at the same time as the attacking allogeneic cells. Delay in administration by as little as 4 hours greatly reduced the protection afforded by the syngeneic lymphocytes. Further experiments by Streilein and Billingham (1970), on the epidermolytic manifestation of acute GvH disease in hamsters, have shown that the site of the initial exposure of parental cells to the F₁ tissues can determine the reactivity of the parental cell population. Parental lymphocytes injected into F₁ hybrid recipients by the intracutaneous route produced an acute form of GvH disease characterized
by severe epidermolysis, and this could be transferred to secondary $F_1$ hybrid hosts by lymph node cells administered by either the intravenous or intracutaneous routes. However, if the attacking parental cells had been administered to the primary host by the intravenous route, lymph node cells from these animals could not produce epidermolysis in a secondary host when given by either the intravenous or intracutaneous route. In a similar manner, neuraminidase treated parental lymphocytes, exposed initially to the $F_1$ host within the liver may exert a different form of reactivity on the lymphoid tissues when they eventually migrate there, to those lymphocytes which are initially presented with $F_1$ tissues within the nodes.

Whatever the actual mechanism of the decreased severity of the acute stages of the GvH disease observed after transfer of neuraminidase treated lymphocytes, these experiments have provided evidence that the severity is dependent on the initial site at which immunocompetent cells are presented to the host tissues.

Experimental manipulations of lymphocytes are frequently interpreted in terms of an alteration in the individual reactivity of the cells. The experiments described above emphasise the importance of the possible modification of other functions, such as cell migration, when modified cells are tested in vivo. Such an effect may explain the reduction in severity of GvH disease after incubation of immunocompetent parental cells with liver cell suspensions (Bortin et al. 1969), which appeared to be mediated by a warm enzyme system. An enzymic alteration of the lymphoid cell surface, with altered cell localization after transfer, was not considered as a possible explanation but appears quite feasible in the light of the present observations. Similarly, alterations in GvH activity after incubation of cells with anti-immunoglobulin sera could be interpreted to be a consequence of a different migratory pattern rather than the blocking of a specific cell receptor (Mason and Warner 1970).
The nature of the chronic GvH syndrome produced by neuraminidase treated lymphocytes

There are many similarities between the chronic GvH syndrome described above and the long term effects reported after thymectomy or prolonged anti-lymphocyte serum treatment. Thymectomy of young rats reduced the circulating lymphocyte population to 60% of the control level, 8 weeks later (Bierring 1960), a similar reduction to that noted in the present syndrome. Neonatal thymectomy resulted in depletion of small lymphocytes in the splenic white pulp, the paracortical areas of the lymph nodes and of the mantle surrounding the germinal centres in rats (Waksman et al. 1962). Daily injections of antilymphocyte globulin in adult rats produced lymphopenia, depletion of lymphocytes in splenic white pulp, cortex of the lymph nodes and cortical areas of the thymus and a reduction in the number of germinal centres in the lymph nodes. Other features were the appearance of plasma cells in the thymus and extramedullary haemopoiesis in the spleen (Denman and Frenkel 1967). All of these features were present in the chronic GvH syndrome described in the present experiments.

It is likely that the chronic GvH syndrome produced by neuraminidase treated parental cells is due to an attack by the parental cells on the thymus and thymus derived cells of the F1 hybrid host with subsequent reduction in their numbers. The thymus itself shows progressive atrophy. The recirculating lymphocyte pool, which is represented by the cells collected over the period of rapid fall in output after thoracic duct cannulation (Gowans and Knight 1964, Gowans 1970), is greatly depleted. However, the later output of cells from the thoracic duct is not so markedly diminished. The most marked depletion of lymphocytes occurs in areas of the lymphoid tissue occupied by the recirculating pool, i.e. the periarteriolar sheathes of the spleen and the paracortical areas of the lymph nodes.
Other experimental evidence suggests that GvH reactions in vivo, and the mixed lymphocyte reaction in vitro, occur between thymus derived lymphoid cells. Lymphocytes from thymectomized rats (Rieke 1966) and mice (Miller et al. 1967) or mice treated with anti-thymocyte serum (Nehlsen 1971) show reduced or absent GvH activity. Ninety per cent of the responding cells in a mixed lymphocyte reaction between blood lymphocytes of rats were thymus derived (Johnston and Wilson 1970). The stimulating cell in the GvH reaction also appears to be a thymus derived lymphocyte (Maclaurin 1971). Many of the observed features of the chronic GvH syndrome which is produced in rats receiving neuraminidase treated parental lymphocytes may develop because of an attack by these parental lymphocytes on the thymus and thymus derived recirculating lymphocyte population of the host, with the elimination or suppression of this population.

There has been much speculation on the fate of parental immunocompetent cells participating in GvH reactions. In general, attempts to transfer GvH activity from animals suffering from GvH disease have been unsuccessful, and have led to the idea that parental cells engaged in GvH activity against the host tissues either commit "allergic suicide" (Gorer and Boyse 1959), or lose reactivity specific to the host while retaining activity against a third party strain (Simonsen 1960, Dineen 1961).

In the present experiments parental strain cells reactive against F1 tissues could be obtained from the thoracic duct of F1 animals suffering from chronic GvH syndrome. The reactive parental cells were present in the thoracic duct lymph at all times examined from 1 to 9 months after their original inoculation. Such a finding is not consistent with notions of "allergic suicide" or the induction of specific unreactivity to the host strain as being the necessary end results of GvH activity of parental cells.
Most previous attempts at transferring GvH disease have utilized spleen cells from animals with GvH disease established in the neonatal period. Billingham et al. (1962) were able in a single case, to transfer activity from rats 15 days after neonatal induction of the disease, but could not repeat the experiment successfully. Siskind et al. (1960) were able to transfer runt disease from spleens of mice 8 weeks after the original injection of parental cells, at a time when the injected animals were obvious runts. Dineen (1961) had occasional success in the transfer of runting in mice 7 days after the induction of the disease. There are many differences between these and the present experiments. Factors such as the age of the animals at which GvH disease was induced, the population of cells transferred from these animals to the test system, and the sensitivity of the assay system could all be important in obtaining the present results.

There are other indications that specific inactivation or elimination is not the necessary fate of transferred parental cells in GvH reactions. Van Bekkum (1963) was able to demonstrate a rapidly induced, reversible tolerance of transferred semi-allogeneic mouse bone marrow cells to host tissue, but immunocompetent spleen cells with reactivity against the F₁ host could be recovered until 22 days after transfer. Heavy mortality of the recipients prevented further investigation of the possibility that this anti-host activity might change to specific tolerance. Occasional F₁ hybrid mice which had recovered from acute GvH disease underwent relapse months later. This has been observed spontaneously (Oliner et al., 1961) and in response to sublethal irradiation (Schwartz and Beldotti 1963). Streilein (1971) was able to induce a wasting syndrome in F₁ hamsters that had recovered from acute GvH disease by transfer of syngeneic F₁ marrow cells. In all these cases the suggested explanation was the activation of immunologically competent parental cells which had remained dormant in the host tissues. Armstrong et al. (1970) examined the spleens of F₁ hybrid mice with chronic GvH disease for the presence of parental cells with anti-host
activity. Although the majority of parental cells appeared to lose anti-host activity within 24 hours of transfer, there was some suggestion of the presence of parental cells with activity against $F_1$ hybrid host tissue at 1 month after transfer. The present experiments directly demonstrate parental cell activity which is present for at least 9 months after transfer.

Another approach to the problem of demonstrating immunologically reactive donor cells in the host animal has been to test the ability of the host to reject skin grafts compatible with the host but incompatible with the donor cells (Billingham and Silvers 1961, Stastny et al. 1963). The scaling contraction and scanty fur growth, which occurred with three of 6 Lewis grafts, observed in the present experiments resembled that described by Stastny et al. (1963). They described a similar process, prolonged over several months, in skin grafts of host type, when placed on adult animals rendered neonatally tolerant of allogeneic donor cells, and subsequently inoculated with these allogeneic cells to produce the GvH syndrome. In one of the present experiments, parental DA cell activity was demonstrated in a (Lewis x DA)$F_1$ hybrid which had been carrying DA and Lewis skin grafts for 4 months. These experiments did not investigate the cause of the prolonged survival of Lewis strain grafts in the face of reactive DA cells. However they do indicate that in such systems graft survival is not a sensitive index for the presence of parental cells.

The reduction of the antibody response to sheep red blood cells observed in animals with chronic GvH syndrome is of interest. The suppressive effect of GvH reactions on the response to antigens has been described in the acute phases of the illness. Mice undergoing GvH reactions have depressed antibody responses to sheep red blood cells (Lawrence and Simonsen 1967, Blanden 1969, Davis, Cole and Schaffer 1970, Möeller 1971), salmonella typhi (Howard and Woodruff 1961), $T_2$ bacteriophage (Blaese, Martinez and
Good 1964) and E coli lipopolysaccharide (Møller 1971). It has been suggested that the depression of response to antigens may be explained by antigenic competition for pluripotent antigen sensitive cells (Lawrence and Simonsen 1967) or suppression of immunocompetent cells of bone marrow origin by non-specific environmental factors (Møller 1971). The largest interval after induction of the GvH reaction at which immunization was attempted in any of these investigations was 19 days. In the present experiments, antibody responses were examined from 5-7 weeks after the start of the GvH syndrome.

A delayed induction, and a decreased total serum antibody response to sheep erythrocytes were found in animals suffering from chronic GvH syndrome. A reduction in the recirculating lymphocyte population and disorganization of lymphoid tissue may have contributed to the poor response, by inhibiting the efficiency of antigen processing and lymphocyte stimulation. However experiments in which thoracic duct lymphocytes from animals with chronic GvH disease were transferred to irradiated recipients indicated that there was a qualitative depletion from the recirculating lymphocyte pool of a cell type necessary for antibody production. This finding is in favour of the explanation that immunodepression as a result of GvH activity is due to elimination of a necessary reactive cell from the lymphocyte population, rather than being due to environmental factors within the affected animal. It also indicates that the parental cells can attack and eliminate immunocompetent lymphocytes in the F1 hybrid.

As the animals with chronic GvH syndrome show features suggestive of depletion of the thymus derived lymphocyte population, it might be suggested that the qualitative deficiency in the ability of thoracic duct lymphocytes from these animals to restore a normal haemolysin response, is due to absence of a necessary thymus derived lymphocyte. However the role of co-operation between thymus and bone marrow derived
lymphocytes in the response of rats to SRBC is not clear. Thus, neonatal thymectomy failed to diminish the subsequent immune response of rats to SRBC (Pinnas and Fitch 1966). McCullagh (1970a) was unable to demonstrate any significant synergism between thymocytes and bone marrow cells in the immune response to SRBC. Using thymectomized lethally irradiated rats which had been resuscitated with bone marrow cells, Johnston and Wilson (1970) were able to demonstrate augmentation of the response of these animals to sheep red blood cells by addition of thymus cells. However, the systems used were always semi-allogeneic to facilitate identification of the antibody forming cells, and GvH reactions between the two cell lines may have influenced the results. From the present experiments it can only be concluded that there is a depletion from the recirculating pool of a particular cell type needed for the response of thoracic duct lymphocytes to SRBC.

The evidence obtained from the experiments described in this chapter allows some deductions to be drawn about the pathogenesis of the GvH syndrome.

The severity of runting is dependent on the initial site of localization of the immunocompetent cells within the tissues of the host, and as is probably a consequence of an attack by the parental cells on the host lymphocytes or their precursors. In animals which survive the acute phase there is a continued attack, by the parental cells on the thymus, and thymus derived recirculating lymphocyte pool including immunocompetent antigen reactive cells, with elimination of these elements. The erythroid elements of the haemopoietic system tend to be spared relative to the leucocytes and their stem cell precursors. This process is not incompatible with long term survival and relatively normal growth patterns.
CHAPTER 10

GENERAL SUMMARY AND CONCLUSIONS

The experiments reported in this thesis are concerned with the effects of the removal of surface sialic acid on lymphocyte function. The population of lymphocytes belonging to the recirculating lymphocyte pool has been studied. Particular attention has been paid to the migratory properties of the cells and to their participation in immune reactions after experimental modification of the cell surface. By observing any alterations in lymphocyte function produced by removal of surface sialic acid, it was hoped to gain insight into the possible physiological significance of macromolecules containing sialic acid on the cell surface.

Removal of surface sialic acid altered the migratory properties of lymphocytes as evidenced by subsequent transfer to syngeneic or semi-allogeneic recipients. An explanation was advanced for this observation, that is, with a specific recognition of the differences between the lymphocyte surface and the post-capsulary vessels towards the passage of neuraminidase-treated lymphocytes from the blood stream into the lymph nodes. The finding that some neuraminidase-treated lymphocytes were able to migrate into the thoracic duct lymph at a time when their surface was still altered did not accord with this idea. Another suggestion has been that the migratory changes noted are due to changes in cell metabolism. However, in the first 12 hours after cell transfer, enzymes created lymphocytes that had migrated out into the thoracic duct lymph of the recipients, and thus have selected for an ability to move from blood to lymph, still possessed the migratory pattern of the original neuraminidase-treated population. It was considered that the most likely explanation of the altered migratory pattern of the cells after neuraminidase treatment was that the surface alterations produced result in the cells being retained in the liver, and therefore unavailable to migrate elsewhere.
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Removal of surface sialic acid altered the migratory properties of lymphocytes as evidenced on subsequent transfer to syngeneic or semi-allogeneic recipients. An explanation commonly advanced for this observation is that interference with a specific recognition process which normally occurs between the lymphocyte surface and the postcapillary venules retards the passage of neuraminidase treated lymphocytes from the blood stream into the lymph nodes. The finding that some neuraminidase treated lymphocytes were able to migrate into the thoracic duct lymph at a time when their surface was still altered did not accord with this idea. Another suggestion has been that the migratory changes noted are due to changes in cell metabolism. However in the first 12 hours after cell transfer, enzyme treated lymphocytes that had migrated out into the thoracic duct lymph of the recipients, and thus been selected for an ability to move from blood to lymph, still possessed the migratory pattern of the original neuraminidase treated population. It was considered that the most likely explanation of the altered migratory pattern of the cells after neuraminidase treatment was that the surface alterations produced result in the cells being retained in the liver, and therefore unavailable to migrate elsewhere.
The question arises as to the function, if any, of cell surface sialic acid in the normal fate and distribution of lymphocytes. Demonstration that removal of this material can alter lymphocyte migration does not necessarily imply that this sugar has a function in the normal recirculation of lymphocytes. That cell surface sialic acid may have a function in vivo was suggested by the correlation observed between the migratory properties of two different lymphoid cell populations and the quantities of sialic acid that could be released from their surface. Lymphocytes obtained from the thymus possessed less surface sialic acid per cell than lymphocytes from the thoracic duct. The thymus cell population migrated in a fashion more reminiscent of thoracic duct lymphocytes from which the surface sialic acid had been removed, than of normal lymphocytes. Similar proportions of thymocytes and neuraminidase treated thoracic duct lymphocytes were found to localize in the spleen and lymph nodes at all times examined after cell transfer. It may be that the different migratory properties of thymocytes and thoracic duct lymphocytes are related to the different quantities of sialic acid-containing glycoproteins on the cell surface. Thymus cells were shown to contain a subpopulation which migrated into the thoracic duct lymph, and possessed the migratory properties of the population of thoracic duct lymphocytes. It is tempting to suggest that one change in thymus cells which occurs if they enter the recirculating lymphocyte pool is an increase in the amount of surface sialic acid.

Evidence for the reacquisition of surface sialic acid has been obtained with the demonstration that there is a return of normal migratory properties to enzyme treated cells after a period of 18 hours in vivo. Some lymphocytes themselves possess the capacity for regeneration of a normal cell surface, as partial recovery of the migratory properties of untreated cells could also be shown to occur in vitro. However, the ability to regenerate surface sialic acid-containing glycoproteins may be a property of a subpopulation of
thoracic duct lymphocytes. Despite evidence of a return of normal migratory properties, which could be reversed by retreatment with neuraminidase, the migratory pattern of neuraminidase treated lymphocytes after maintenance for 72 hours in vitro remained different from that exhibited by a similarly maintained population of normal cells. From a teleological point of view, the retention of the ability to resynthesize surface sialic acid would suggest that this function is utilized in vivo.

Identification of a specific instance is speculative, but the ability of the lymphocyte to modify its cell surface and by so doing influence mobility and migration through tissues in an immune response would clearly be of benefit.

The capacity of neuraminidase treated lymphocytes to participate in two immunological reactions was studied, namely, antibody formation to sheep erythrocytes and the graft-versus-host reaction. Removal of cell surface sialic acid did not compromise the ability of the lymphocyte population to participate in these reactions. In studies where the resultant immune response is measured some days after enzyme treatment of the cells, it is possible that the cell surface has been restored to a normal state prior to the participation of the cells in an immunological reaction. However, the abrogation of sheep erythrocyte tolerance in F1 hybrid animals by neuraminidase treated parental cells indicated that the lymphocytes can interact with allogeneic cells at a time when their cell surface is still in an altered state.

The ability to modify the migratory behaviour of lymphocytes while retaining full immunological reactivity provided a useful tool with which to examine the influence of lymphocyte migration on the evolution of the immune response. There was delay in the induction of the immune response in the spleen of irradiated rats restored with neuraminidase treated instead of control lymphocytes, if the antigen was administered prior to the lymphocytes. The 24 hour delay in induction
corresponded with the time taken for enzyme treated cells to recover normal migratory properties. This experiment provided an in vivo corroboration of the observations of Ford and Gowans (1967) in vitro, that the migration of lymphocytes through the spleen was an important factor in the immune response of that organ to sheep erythrocytes.

Study of the GvH reaction produced by neuraminidase treated lymphocytes allowed some conclusions to be drawn about the activity of enzyme treated cells, and the pathogenesis of the GvH syndrome. The GvH activity of lymphocytes was not affected by neuraminidase treatment as judged by the local graft-versus-host reaction or the ability to participate in such allogeneic interaction as was necessary to abrogate tolerance to sheep erythrocytes. Despite this there was a marked decrease in the severity of runting and death in the acute phase of the GvH syndrome, when neuraminidase treated instead of untreated thoracic duct lymphocytes were administered intravenously. This indicated that the initial site of localization of the attacking parental lymphocytes was of critical importance in determining the nature of the syndrome produced. As the localization of lymphocytes was reduced in the lymphoid tissues this suggested that the allogeneic interactions taking place within the host lymphoid tissue may be related to the severity of the acute runting which ensues. Acute runting did not ensue at a later stage even though it appeared that enzyme treated cells could regain normal migratory properties. It would seem that the site at which the parental lymphocyte population was confronted with F₁ hybrid host tissues determined the subsequent capabilities of that population of lymphocytes. This emphasizes that the cellular environment is an important factor in the immune response, to be considered along with the immunological capacity of the individual cells in determining the final result. The ability to "direct" immunocompetent cells so that they demonstrate a reduced acute GvH activity, and yet retain normal proliferative
and antibody forming capabilities, as evidenced by the repopulation of irradiated host spleens and haemolysin response to sheep erythrocytes, is a measure which may be useful in minimizing graft-versus-host disease in clinical situations.

F₁ hybrid animals transfused with enzyme treated parental cells developed a chronic GvH syndrome. Study of this syndrome showed that the parental lymphocytes maintained reactivity against F₁ tissues for up to 9 months after transfer. The manifestation of this activity in the F₁ host was a reduction in the recirculating lymphocyte population as suggested by a marked depletion of lymphocytes in the peripheral blood and in the paracortical areas of the lymph nodes. This was confirmed directly by quantitation of the lymphocyte output from the thoracic duct. Furthermore, a specific elimination of an immunocompetent cell type required for the immune response was demonstrated. This indicated that a type of recirculating lymphocyte attacked in the GvH reaction is one which also participates in the immune response to antigens. It was concluded that "allergic inactivation" or specific tolerance were not the necessary end results of parental cell activity in GvH reactions, but that parental cells could survive and enter the circulating lymphocyte pool of the hybrid host for many months after transfer, while retaining anti-host activity.

It can be concluded that removal of surface sialic acid from lymphocytes does not interfere with the immunological capacity of the cells. Any differences in the in vivo immune response produced by these modified cells appeared to be related to the altered migration of the lymphocytes. At a time when so much emphasis is placed on unravelling the complexities of the immune response of individual cells, it is important to bear in mind that other factors such as cell migration can determine the nature of the final response produced by a cell population.
REFERENCES


FORD, W.L. (1968b). Duration of the inductive effect of sheep erythrocytes on the recruitment of lymphocytes in the rat. Immunology 15, 609.


HOLM, G. and PERLMANN, P. (1967). Quantitative studies on phytohaemagglutinin-induced cytotoxicity by human lymphocytes against homologous cells in tissue culture. Immunology 12, 525.


LAWRENCE, H. and SIMONSEN, M. (1967). The property of "strength" of histocompatibility antigens and their ability to produce antigenic competition. Transplantation 5, 1304.


