INTERACTION OF LYMPHOCYTES WITH ALIEN TISSUES

A THESIS

submitted for the degree of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

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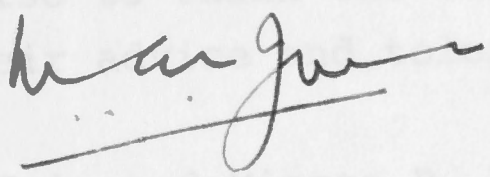
January 1968

Statement

The experiments described in Chapters 3, 4 and 5 were carried out in association with Dr. K.J. Lafferty. Mr. R. Hill cut and stained the histological sections required. With these exceptions, the experimental work described in this thesis was carried out by myself.



Candidate's Signature

Acknowledgements

The work reported in this thesis was carried out in the Department of Experimental Pathology, John Curtin School of Medical Research, Australian National University, from January 1965 to January 1968, during the tenure of an Australian National University Research Scholarship. I am indebted to Professor F.C. Courtice for the opportunity to undertake this work in his department.

My best thanks are due to my supervisor, Dr. K.J.Lafferty for suggesting the topic, for his guidance during the work and for his constructive criticism during the preparation of this manuscript.

Particular thanks are also due to Dr. B. Morris for his help with surgical operations and for the loan of sheep for experimental procedures. I wish also to thank the staff and other students in the School for their advice and tolerance towards me.

Finally, I thank Mrs. G.J. Cole and Misses P. Nangle and P. Smith for their assistance in the preparation of the manuscript.

Table of Contents

CHAPTER 1

INTRODUCTION

Chapter 1 - Introduction

The capacity to destroy transplated tissues had been shown to reside in the recipients lymphoid tissues by Mitchison (1953) and Billingham (1954). Gowans et al. (1963) drew attention to the interaction of lymphocytes with alien tissues when he demonstrated that the fate of transplanted tissues was determined by the activities of lymphocytes or their descendants. It follows from these findings that if allogeneic lymphoid cells are injected into an immunologically unreactive host then the grafted cells should react against the foreign tissues of the recipient. This idea was put forward by Simonsen et al. (1953) and called by them 'The graft versus host reaction'.

The Graft Versus Host Reaction (GVH)

The GVH reaction was first demonstrated by injecting adult chicken blood into 18 day old chicken embryos (Simonsen 1957). At the same time Billingham and Brent (1957) were attempting to induce tolerance in new born mice of the F_1 generation of two inbred parental strains by the injection of parental spleen cells. They found that the young mice developed a wasting disease, later called 'Runt disease'. Although the pathological changes reported by these two groups of workers had been previously described by Murphy (1914), Simonsen (1957) was the first to interpret the changes as a GVH reaction. It was concluded from these observations that the grafted cells attacked the antigens of the hosts which were incapable of defending themselves. A description of the GVH reaction can be simplified by breaking it down into a number of separate topics. These are the conditions under which the GVH reaction occurs, the lymphoid cell type that produces the reaction, the stimulus of this cell type and subsequent responses by the recipient's lymphoid tissue.

The Conditions Under Which the Graft Versus Host Reaction Occurs

Simonsen (1965) has summarized the three situations in which the host fails to keep the grafted lymphoid cells in check, so that a GVH reaction can take place. These are :-

1. Immaturity of the host so that it is unable to respond immunologically and protect itself from the attack mediated by the grafted cells. This occurs following the implantation of the lymphoid cells from adult donors into embryos and new born animals.
2. A depression of the host's response following exposure to ionizing radiation or thymectomy in neonatal life. An alternative way by which the production of a specific depression of the host response can be achieved is by the induction of tolerance to donor antigens.
3. An experimental design which prevents the reaction of a completely normal competent host. This is thought to occur if lymphocytes are injected into a localized area where they can temporarily establish themselves for long enough to initiate a reaction against the host (Brent and Medawar, 1964).

The one common feature of the three circumstances under which GVH reaction can occur is that the hosts' ability to reject the donor cells must be impaired in some way or other.

The Donor Cells Responsible for Inducing GVH Reactions

The initial demonstrations of GVH reactions by Simonsen (1957) and Billingham et al. (1959) established that cells capable of producing these reactions could be found in the blood and lymphoid tissue. Terasaki (1959) revealed that although injections of adult chicken lymphocytes regularly produced GVH reactions in chicken embryos, similar injections of monocytes had no detectable effect. Confirmation of lympho-

cytes as the effecter cells leading to a GVH reaction was produced by a number of workers who reproduced the reaction in new born rats with cells from the thoracic duct of allogeneic adults (Billingham et al., 1960; Gowans et al., 1961; Gowans, 1962). Thoracic duct lymph contains both large and small lymphocytes. At first it was thought that the large lymphocyte was responsible for producing GVH reactions (Cole and Garver, 1961), but subsequent experiments did not support this (Porter and Cooper, 1962), and Gowans (1962) found that the ability of an inoculum of thoracic duct lymphocytes to produce GVH reactions was independent of the number of large lymphocytes it contained, but rather that small lymphocytes were the cells responsible for initiating GVH reactions.

The Nature of the Factor That Stimulates Allogeneic Lymphocytes to Produce a Graft Versus Host Reaction

It has been assumed that the antigens that are responsible for graft rejection are also involved in the production of GVH reactions (Gowans and McGregor, 1965). When reviewing the evidence then available concerning the nature of GVH reactions, Simonsen (1962) cited the following points in support of the assumption that these reactions represented immunological events :-

1. For GVH reactions to occur there must exist a genetic and hence antigenic difference between donor and recipient.
2. The direction of the 'antigenic' stimulus must come from the host to the grafted cells.
3. Spleen cells from a donor that has been rendered tolerant of the recipient will not produce GVH reactions in that host.
4. Immunization of the donor against the recipient should result in a more violent GVH reaction than that produced by cells taken from a non-immune donor.

This last prediction was simply the application of the phenomenon of second set rejection of allografts to GVH reactions. Early workers concluded that lymphocytes from pre-immunized donors provoked a stronger GVH reaction than those from normal ones (Billingham and Brent, 1959; Gorer and Boyse, 1959). Simonsen (1962) reinvestigated the effect of immunization of the donor upon the GVH reaction using 4 inbred strains of mice. These 4 inbred strains of mice differed from a fifth (C3H) mouse strain to varying degrees, as judged by the survival time of skin grafts exchanged between it and the other 4 strains. It was considered, the more rapidly a skin graft was rejected, the greater was the antigenic difference between donor and recipient strains. Simonsen, then immunized individuals of the C3H strain with spleen cells from the F_1 hybrids from matings between the C3H strain and each of the other 4 strains. When the ability of spleen cells obtained from immunized donors to produce GVH reactions in the F_1 hybrid against which they were immunized, was compared with those from non-immune mice it was found that the greater the 'antigenic' difference the smaller the effect of immunization. In fact no detectable effect could be shown where there was a very strong 'antigenic' difference between donor and recipient. These observations were confirmed in inbred chickens using the local GVH reaction of pock formation or the chorioallantoic membrane as a measure of GVH reaction (Lind and Szenberg, 1961; Warner and Szenberg, 1964).

Since pre-immunization of the donor against the recipient did not always result in an increased GVH reaction, doubt was cast upon the suggestion that all the splenomegaly produced during a GVH reaction resulted from donor cell proliferation. It is obvious that if the splenomegaly resulted from the proliferation of sensitive donor cells reacting against the antigens of the host then an increase in the number

of sensitive cells in the inoculum, as would be produced by immunization, should bring about larger spleen weights following their proliferation. An alternative explanation of the failure of immunization to increase the GVH reaction would be that the 'clones' of reactive cells to certain 'strong antigens' are fully expanded. This would constitute an atypical situation since animals usually respond vigorously to stimulation by strong antigens. This anomaly led to an investigation of the host's participation in the GVH reaction.

Responses by the Hosts' Lymphoid Cells During the GVH Reaction

A rapid depletion of host lymphoid tissue would be a reasonable prediction from the assumption that a GVH reaction results from an attack by the grafted lymphocytes on the cells bearing transplantation antigen. This ought to occur because of the susceptibility of lymphocytes to cytotoxic antibodies and also because lymphoid cells are such a rich source of transplantation antigen (Palm and Manson, 1966). Indeed, extreme atrophy of the lymph nodes was one of the cardinal signs of advanced 'Runt disease' (Simonsen, 1962). However, early in GVH reactions another prominent pathological change was splenomegaly, which reached a peak in mice, about 8 to 10 days after the injection of the donor lymphocytes and then declined (Simonsen and Jensen, 1959). Histologically the splenic changes were seen to be due to the accumulation of large numbers of large pyroninophilic cells and an apparent mass destruction of myeloid cells which was though to be caused by proliferating donor cells (Simonsen, 1957).

On looking for the origin of the proliferating cells in the enlarged spleen of an animal undergoing a GVH reaction by means of a cytological marker, Davies and Doak (1960) found that the cells in mitosis were predominantly of host origin. Howard et al., (1961) and Fox (1962) confirmed these findings and suggested that this cell proliferation in the host's spleen

was a response by the recipient to damage produced by the attacking donor cells. An attempt to assess the separate contributions made by the host and donor lymphoid cells to the splenomegaly observed during GVH reactions in the chicken embryo was made by Seto and Albright (1965). They found that X-irradiation of either the grafted cells or the host embryo markedly reduced the splenomegaly, and that donor cells accounted for less than 25 per cent of the total number of proliferating cells. These workers explained the host cell proliferation as a low grade immunological response by the recipient.

It is unlikely that the proliferation of host lymphoid cells seen during GVH reactions is due to a low grade immunological response by the host, since the use of the T6 chromosomal marker in recipient F_1 generation mice has shown that host lymphoid cell proliferation occurs during GVH reactions produced by parental cells (Fox, 1962). These observations are quite the opposite of those that would be predicted with antigen as the stimulus for the proliferation of lymphoid cells during a GVH reaction. The F_1 hybrid recipients are known to be incapable of mounting an attack against the parental antigens, therefore, the host cell proliferation observed when parental cells are injected into F_1 hybrid animals cannot be accounted for in terms of an immunological response on the part of the host. It is possible that this proliferation during a GVH reaction may be in response to some unknown factor that is closely related to histocompatibility differences. Further evidence against antigen being the stimulus for the proliferation of lymphoid cells occurring during a GVH reaction has been obtained by increasing the antigen disparity by grafting xenogeneic lymphoid cells. Early work had shown that when pigeon and turkey blood leucocytes were inoculated into the chicken embryo, they did not produce splenomegaly

(Simonsen, 1957) although later turkey leucocytes were shown to produce some splenomegaly in chicken embryos (Mitchison, 1958). Payne and Jaffe (1962) have examined the ability of leucocytes from large numbers of xenogeneic avian donors to produce splenomegaly in chicken embryos. Two points emerged from this survey. Firstly, that in general the greatest splenic changes were produced by lymphoid cells from birds of the order Galliformes. Secondly, the leucocytes from the most reactive xenogeneic avian donors were much less potent than allogeneic leucocytes in producing splenomegaly. Thus despite an increase in antigenic disparity between donor and recipient, the GVH reactions were reduced and not increased.

Local Graft Versus Host Reactions

Some insight into what stimulus allogeneic lymphocytes react to during a GVH reaction has been obtained by experiments with local GVH reactions. Two responses produced in the recipient by the local inoculation of allogeneic lymphocytes have been interpreted as local GVH reactions. These are the pocks produced on the chorio allantoic membrane (CAM) in the chicken embryo (Murphy, 1916; Boyer, 1960) and the normal lymphocyte transfer reaction (Brent and Medawar, 1963).

Chorioallantoic Membrane Pocks in the Chicken Embryo, or "The Simonsen Phenomenon".

When Murphy (1916) inoculated small pieces of adult chicken spleen and bone marrow on to the CAM of 7 day old chicken embryos, small grey translucent nodules were produced on the embryonic membranes. Similar lesions appeared when cell suspensions prepared from adult allogeneic chicken leucocytes or spleen were inoculated onto the membranes of 10 to 14 day old chicken embryos (Boyer, 1960). Histologically, these nodules contained large pyroninophilic and small mononuclear cells similar to those seen in the spleen of the chick embryo during the graft versus host reaction.

The 'Simonsen phenomenon' is considered to be a GVH reaction on the results of two independent experiments. These showed that a single gene difference between two inbred lines of chickens was sufficient genetic disparity to allow lesions to be produced when leucocytes obtained from adult chickens of one inbred strain were placed on the CAM of the other. (Burnet and Burnet, 1961; Schierman and Nordskog, 1963). Similarly, leucocytes from inbred parental birds were able to produce pocks on the chorioallantoic membrane of the chicken embryos of the F_1 generation, while blood leucocytes from adult F_1 generation were unable to react against embryos of the parental strains. These observations are explained by the tolerance of the F_1 hybrid cells of parental antigens. A similar tolerance was shown to be produced by the inoculation of 15 day old chicken embryos with 400 thousand embryonic spleen cells from another inbred strain. Leucocytes from hatched chickens treated in this way did not produce pocks until 3 weeks after their hatching, when there was a rapid rise in their pock producing ability (Hilgard et al., 1962). On the other hand, the effect of pre-immunization of the donor on the GVH reaction was the same as seen earlier in the mouse. It was found that no increased pock count occurred following immunization of the donor against the recipient except when donor and recipients were separated by a weak "histocompatibility" difference, there was then a significant increase in pock counts (Warner and Szenberg, 1964).

The Normal Lymphocyte Transfer Reaction (NLT)

The normal lymphocyte transfer reaction was the name given to an inflammatory response seen in the skin of guinea pigs following the intradermal inoculation of 5 million allogeneic lymph node cells (Brent and Medawar, 1963). The rationale behind such an experiment was that the grafted allogeneic small lymphocytes react against transplantation antigens

of the host in a GVH reaction. In normal animals such a reaction is terminated by a reciprocal attack on the grafted cells by the recipient (Brent and Medawar, 1964). In their earlier work, Brent and Medawar (1963) were able to correlate the size of the dermal inflammatory response produced during the NLT reaction with the length of time, skin allografts exchanged between lymphocyte donor and recipient survived.

Confirmation of these observations has been produced by a number of workers (Warner, 1964; Ramseier and Billingham, 1966) who described similar reactions in chickens and hamsters respectively. But on the other hand Mannick and Egdahl (1962) were unable to produce consistent NLT reactions in rabbits, which showed that the reactions were not identical in all species.

The NLT reaction appears to consist of two major components. These are, an initial reaction by the donor lymphocytes against the host, with a subsequent response by the recipient against antigens present on the grafted cells. Brent and Medawar (1966) using X-irradiated guinea pigs further divided the reaction into 3 parts. An initial inflammatory episode, which lasted for 3 days, followed by a flare-up period and finally the fade out. They attributed the inflammatory episode and flare-up period to the reactions of the donor cells and the fade out to those of the host. Various attempts have been made to dissect out the parts played by donor and recipient cells respectively in the production of the NLT reaction and the results are summarized below.

The donor component of the NLT reaction - The assumption that the donor lymphocytes play an important part in NLT reactions was shown to be correct by the failure of dead donor lymphocytes to produce a reaction (Brent et al., 1962). Thus, in the absence of an initial reaction by the injected lymphocytes, no NLT reaction occurs. It would be logical under

such circumstances to investigate the contribution of donor cells, by attempting to produce NLT reactions in recipients unable to respond immunologically to the lymphocyte donor, thus eliminating a host response. Recipients unable to respond to allogeneic lymphocytes have been obtained either by treating the recipient with X-irradiation or by using F_1 hybrid animals that are naturally tolerant of the antigens of the parental donor. It would be predicted in such immunologically incompetent or naturally tolerant hosts the NLT reactions would be larger and persist much longer, since the host would be unable to inactivate the attacking cells.

The NLT reactions produced by the inoculation of lymphocytes from inbred parental donors into their F_1 generation has been investigated in guinea pigs (Brent and Medawar, 1964; 1966) and in hamsters (Ramseier and Billingham, 1966) and were found to be no bigger than the reactions produced between unrelated normal donors and that the reactions regressed after 5 to 6 days. As it had been argued that the injected lymphocytes could have become exhausted or tolerant after 6 days, thus allowing the reaction to subside, Elkins (1966) investigated this proposition by transferring to other susceptible rats, pleiomorphic mononuclear cells obtained from local GVH reactions produced in the kidney of F_1 generation rats by parental cells. He ascertained that the original parental lymphocytes survived in the primary host for at least 8 days and that whilst this sojourn in the primary host greatly reduced the ability of the grafted lymphocytes to react against tissues other than those identical to the primary host their capacity to attack the F_1 generation was unimpaired. Thus even in naturally tolerant hosts some process must be operating to curtail local GVH reactions, otherwise, in theory, the injected allogeneic lymphocytes should continue to proliferate until a full systemic GVH reaction occurs. This deduction was

supported by the observations made in recipients rendered immunologically incompetent experimentally.

Brent and Medawar (1964) found that doses of X-irradiation of 500 to 1500 rads to host guinea pigs allowed NLT reaction to become 'huge purple lesions' after 6 to 7 days. Ramseier and Billingham (1966) repeated these experiments but with doses of 1500 to 2000 rads and found that they produced leucocytopaenia in both the guinea pig and hamster and abolished the ability of the host to express NLT reactions. These findings were supported by observations in rats by Elkins (1966) who showed that doses of irradiation increasing from 200 to 500 rads greatly reduced the manifestation of local graft versus host reactions, whilst doses in excess of 1000 rads completely abolished them. Since it is unlikely that the antigenicity of the tissues was affected by the doses of X-irradiation used in the above experiments (McCormick and Egdahl, 1966) these observations lead to the conclusion that injected lymphocytes must be reacting against radiosensitive material or cells.

The stimulus to which donor lymphocytes react during the NLT reaction - It has been suggested that the NLT reaction is a local GVH reaction produced by the interaction of the injected lymphocytes with the antigens of the host. This suggestion was based upon the observations that while lymphocytes from inbred strains of parental guinea pigs were able to produce NLT reactions in their F_1 generation progeny, lymphocytes from the progeny were unreactive or produced weak reactions in their parents (Brent and Medawar, 1964; 1966; Ramseier and Billingham, 1966).

At the same time the finding that the NLT reaction can be inhibited by giving the recipient animal sufficient irradiation to render it leukopaenic suggested that the injected lymphocytes were not reacting against the antigens of the

host skin but were being stimulated by circulating leucocytes. This supposition was supported by grafting skin from MHA strain hamsters to MHA x CB hamsters, where such grafts were tolerated. When however MHA lymphocytes were injected into the tolerated MHA graft, a NLT reaction resulted. This discovery by Ramseier and Billingham (1966), repeatable in other hamster hybrids, clearly indicates that the stimulus for the production of a GVH reaction is carried on the circulating cells. It is possible that the circulating cells responsible were lymphocytes, a possibility made more likely by the capacity of mixtures of either allogeneic or human lymphocytes to produce NLT reactions in X-irradiated hamsters (Ramseier and Streilein, 1965; Ramseier and Billingham, 1966).

These observations indicate that the injected lymphocytes may be able to interact with allogeneic leucocytes in the tissues of the recipient to produce local GVH reaction. Such an interaction between allogeneic lymphocytes has been demonstrated *in vitro* by Bain *et al.* (1964).

The Mixed Lymphocyte Reaction (MLR)

The demonstration of the mixed lymphocyte reaction by Bain *et al.* (1964) followed an observation by Schrek and Donnelly (1961) that a cultured mixture of two allogeneic human lymphocytes contained a small number of large abnormal cells and a few cells in mitosis. Bain *et al.* found that when peripheral blood leucocytes from two unrelated donors were mixed together and cultured for 5 days, a mutual proliferative response occurred with the appearance of a number of large cells with basophilic cytoplasm. These cells had a fine evenly distributed chromatin, prominent nucleolar material and would incorporate ^{3}H-thymidine. Autoradiographic studies showed a rise in the proportion of cells incorporating ^{3}H-thymidine from 0.9 per cent in the controls to 5 per cent in the mixtures. Incorporation of ^{3}H-thymidine was not

observed if plasma or erythrocytes were substituted for the leucocytes from one animal, nor when the leucocytes came from monozygotic twins. On the other hand, mixtures of leucocytes from dizygotic twins gave a response identical to that produced when leucocytes from allogeneic humans were mixed. These findings by Bain and her colleagues were confirmed by Hirschhorn et al. (1963) and Schrek (1964). Subsequently, other workers have extended these findings to mixtures of spleen cells from mice (Dutton, 1966) and leucocytes from rats (Schwarz, 1966). The capacity to stimulate blastogenesis in lymphocytes is shared by allogeneic macrophages (Marshall et al., 1966), but the effect upon lymphocytes by cells from tissues other than reticular tissue is not known.

It has been tacitly assumed that the lymphocytes were responding to transplantation antigens present on the other lymphocytes in the mixture. Support for this assumption was provided by Bach and Hirschhorn (1964) who claimed that when allogeneic lymphocytes, killed by freezing and thawing were substituted for one of the members of the mixture, transformation of the viable lymphocytes into blast-type cells occurred. This claim could not be substantiated by several workers (Gordon and Maclean, 1965; Marshall et al., 1966). Transformation of lymphocytes has also been claimed to occur following treatment in vitro with a ribonuclease sensitive microsomal fraction from allogeneic lymphocytes (Hashem and Rosen, 1964). But this finding remains to be confirmed, although Dutton (1966) was able to stimulate DNA synthesis in rabbit spleen cells suspensions with a soluble extract from allogeneic rabbit spleen cells.

When the uptake of ^{3}H-thymidine by lymphocytes stimulated with allogeneic lymphocytes in vitro is compared with that induced in unsensitized lymphocytes by antigenic materials, it is found to be much larger (Dutton, 1966). In fact, the

amount of ^{3}H-thymidine taken up by mixtures of lymphocytes is comparable with that recorded to occur when sensitized lymphocytes stimulated by the specific antigen used to sensitize the donor animal (Pearmain *et al*., 1963; Dutton, 1966).

The evidence presented so far is not incompatible with the notion that the lymphocytes in mixtures were responding to allogeneic transplantation antigens albeit in an aberrant form to the way the same cells would respond to other antigens. However, if this were the case, then it would be predicted from the work of Pearmain *et al*. (1963) that pre-immunization of the lymphocyte donors against each other should greatly increase the rate of ^{3}H-thymidine incorporation. Moynihan *et al*. (1964) and Bain *et al*. (1965) claimed to have shown enhancement of the MLR following graft rejection by one of the donors, yet in both cases the experiments lacked the essential control where the activity of the lymphocytes from the grafted recipients against allogeneic cells other than the graft donors were tested. Dutton *et al*. (1966b) were unable to confirm enhancement of the MLR in mice following pre-immunization of the donors under carefully controlled conditions. Thus no effect of immunization on the MLR has been conclusively shown at present.

If the MLR is of an immunological nature, the size of the response by lymphocytes from unsensitized donors indicates an unusually intense primary response to transplantation antigens many times greater than the lymphocyte response to other antigens. Alternatively, the MLR may be a manifestation of a pre-existing state of immunization of the donor against transplantation antigens. But the existence of such a natural sensitivity to allogeneic tissue extracts is denied by the failure to demonstrate delayed type hypersensitivity to transplantation antigens in the normal animal, although this can be shown in an animal that has rejected a graft (Brent *et al*.,

1962). Finally an alternative explanation is that the MLR may not be an immunological response but one resulting from an unknown stimulus to which all lymphocytes could be potentially able to respond.

Synopsis

The accumulated evidence from studies on the GVH reaction shows that not all the changes that occur following the inoculation of allogeneic lymphoid cells can be attributed to an attack by the grafted cells against the antigens of the host. The proliferative response of the host's reticular tissue takes place under circumstances where antigenic stimulation of the host tissues is not possible, yet the proliferative response only occurs when the injected lymphoid cells are potentially capable of reacting against the antigens of the host. These observations therefore suggest that the proliferation of host cells occurs in response to some factor present in lymphoid cells obtained from a host genetically different to the recipient. The experimental work in this study was aimed at determining the nature of the stimulus which induces the manifestations of the GVH reaction and relating this to the general physiology of the lymphoid system.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2 - Materials and Methods

Experimental Animals

Sheep - Randomly bred Merino, South Suffolk and Corriedale sheep, 1½ to 4 years of age were used in the experimental work to be described. The sheep were obtained from local flocks on the Southern Tablelands of New South Wales and the Australian Capital Territory.

At night, sheep were housed in covered pens and during the day allowed to graze in small paddocks of ryegrass and clover. When insufficient grazing was available and during skin testing, the sheep remained in the pens and were fed lucerne chaff and oats with free access to water. A few days before cannulation, the sheep were placed in mobile metabolism cages (Fig. 2.1) to become accustomed to the experimental conditions. All food and water were withheld for the 24 hours before the sheep were anaesthetized.

Goats - Adult goats were bought from a local flock. They were housed in indoor pens and fed lucerne chaff and oats with free access to water. They were not allowed to graze during the day. Treatment before operations was the same as that for sheep.

Rats - The rats used for cannulation of the thoracic duct were supplied by the Animal Breeding Establishment of the John Curtin School of Medical Research. They were outbred albino Wistar female rats, weighing 250-260 gms. Whilst in the Bollman restraining cages they were fed animal pellets and water containing 0.4 per cent sodium chloride *ad libitum*.

Guinea Pigs - Outbred albino female guinea pigs, weighing 200 to 400 gms. were supplied by the Animal Breeding Establishment of the John Curtin School of Medical Research. Groups of up to 10 guinea pigs were kept in wire-bottomed cages, where they were fed small animal pellets and water *ad libitum*. This diet was supplemented daily with green feed

Figure 2.1

A merino sheep with cannulated efferent popliteal lymph duct in a metabolism cage. The sling prevents the sheep lying down and dislodging the canula.

in the form of clover, lucerne or cabbage.

Ducks - Adult outbred Peking ducks were kept in indoor pens and fed oats and commercial "growers" pellets with free access to water until used.

Geese - The adult outbred geese used in this study were housed and fed in the same manner as the ducks.

Pigeons - Adult outbred homing pigeons were obtained from the Microbiology Department of the John Curtin School of Medical Research.

Domestic chickens - All the chickens used in the experiments were white leghorns. A number of different strains were used. They were :-

Outbred. Adult outbred white leghorn female chickens were used when possible.

Inbred. Adult inbred male and female chickens from the inbred line AA were obtained from the C.S.I.R.O., Animal Genetics Section, West Ryde, N.S.W. After arrival the birds were housed in single bird battery units and fed "layer pellets" and water ad libitum.

Fertile domestic chicken eggs - A regular supply of these was available from two sources :-

a. Inbred. Unincubated fertile hens' eggs of the inbred line AA were obtained from the C.S.I.R.O., Animal Genetics Sections, West Ryde, N.S.W.

b. Outbred. 6 to 8 days incubated fertile outbred eggs were air freighted from B. Talbot, Merada, Victoria, each week.

Eggs from both sources were incubated at 38.5°C in humidified air incubators.

Fertile duck eggs - These were obtained from Strike Bros., Matraville, N.S.W., and incubated in the same manner as the chicken eggs.

Other Materials Used

Chemicals - All chemicals used were analytical reagent

grade unless otherwise stated.

Sodium chloride solution (physiological saline, normal saline) - A 0.9 per cent solution of sodium chloride in distilled water was used as physiological saline.

Dulbecco's phosphate buffer saline (PBS) - This was prepared in three separate solutions, that were sterilized by autoclaving at 15 lbs per square inch for 20 minutes. The solutions were mixed immediately before use (Dulbecco and Vogt, 1954).

Hank's balanced salt solution - This was prepared as follows :-

Sodium chloride	8.0 g	Sodium hydrogen phosphate	0.06 g
Potassium chloride	0.4 g	Potassium dihydrogen phosphate	0.06 g
Magnesium sulphate	0.1 g	Glucose	1.0 g
Magnesium chloride	0.1 g	Deionized water to 1 litre	
Calcium chloride	0.14 g		

5 mls. of 0.4 per cent phenol was added to each litre of salt solution. The solutions were sterilized by seitz filtering.

Hank's Tris lactalbumin - This was prepared by the Microbiology Department, John Curtin School of Medical Research as follows :-

Hank's balanced salt solution	10 litres
Tris (hydroxymethyl) amino-methane	12.10 g
Lactalbumin hydrolysate	50.0 g

The pH was adjusted to between 7.6 and 7.8 by the addition of 0.1N hydrochloric acid and the solutions sterilized by autoclaving for 15 minutes at 10 lbs pressure.

Pucks' saline "A" - The following chemicals were mixed in the proportions shown :-

Phenol Red	0.02 g
Sodium chloride	8.0 g
Potassium chloride	0.4 g
Glucose	1.0 g

Sodium bicarbonate	0.35 g
Deionized water to 1 litre	

Solutions were sterilized by autoclaving at 15 lbs per square inch for 20 minutes.

Trysin solution - This solution was prepared according to the following recipe :-

Sodium chloride	8.5 g
Sodium bicarbonate	1.1 g
Trypsin (Difco, Detroit)	2.5 g
Penicillin	0.1 g
Neomycin	0.25 g
Sulphaquanidine	0.015 g
0.1 per cent phenol red	10.0 mls
Deionized water to 1 litre	

The solutions were sterilized by seitz filtration and stored frozen at -25°C until used.

Eagle's basal medium (EBM) - The method of preparation of EBM was that used by the Microbiology Department of the John Curtin School of Medical Research, Australian National University. EBM was prepared as two solutions which were stored frozen after sterilization by seitz filtering. The recipe was a slight modification of one described by Eagle (1959) and is given below.

Solution I

This was prepared as follows. Preparations 1 and 2 were made and then mixed together. The solid materials in Preparation 3 were dissolved by boiling in water and the preparation, after cooling, added to the above mixture. Finally preparation 4 was made and added before the volume was made up to 2 litres with water. The whole was sterilized by seitz filtration and the solution held at 4°C until used.

Preparation 1

sodium chloride	136.0 g	Magnesium chloride	1.6 g
Potassium chloride	8.0 g	Glucose	20.0 g

Sodium hydrogen phosphate	3.2 g	Water to 500 mls.	
Calcium chloride	2.0 g		

Preparation 2

L-arginine	420 mgs	L-phenylalanine	330 mgs
L-histidine	210 mgs	L-threonine	476 mgs
L-isoleucine	524 mgs	L-valine	468 mgs
L-leucine	524 mgs	L-tryptophan	82 mgs
L-lysine	730 mgs	Water to 500 mls.	
L-methionine	150 mgs		

Preparation 3

L-tyrosine	360 mgs
L-cystine	240 mgs
Water to 200 mls	
Concentration hydrochloric acid	1 ml.

Preparation 4

Biotin	20 mgs	Thiamine	20 mgs
Choline	20 mgs	Riboflavin	2 mgs
Folic acid	20 mgs	Phenol red	400 mgs
Pantothenic acid	20 mgs	Penicillin	500 mgs
Pyridoxal	20 mgs	Streptomycin sulphate	500 mgs
Nicotinamide	20 mgs	Water to 500 mls	

Solution II

5.84 gms of L-glutamine were dissolved in 400 mls of water and then the solution was sterilized by filtration through seitz filters. It was stored frozen until used. Preparations of Eagle's basal medium for use was by combining solutions I and II and adding 880 mls of sterile water. The pH was adjusted to 7.4 by the addition of 30 mls of 1.4 per cent sterile sodium bicarbonate solution.

Anticoagulant solution - This solution was used to prevent the coagulation of lymph during collection. 100 I.U. heparin ('Pularin', heparin injection B.P. Evans) were added to each millilitre of 0.9 per cent sterile sodium chloride solution together with 1,000 I.U. crystalline penicillin.

Antibiotics - The following antibiotics were used to control bacterial growth in culture media, media in which lymph was collected and to dust surgical wounds that could have been contaminated by bacteria.

Penicillin. 'Crystapen'. Crystalline sodium penicillin G. (Glaxo-Allenburys).

'Triplopen'. Benethamine, procaine and sodium penicillins for aqueous injections (Glaxo-Allenburys).

Streptomycin. Streptomycin sulphate injection B.P. (Glaxo-Allenburys).

Neomycin. Neomycin sulphate (Andrew Laboratories, Sydney)

Stains - Trypan blue. Prepared as 0.5 per cent aqueous trypan blue in phosphate buffered saline, which was diluted 1 in 10 in the cell suspension to be stained. The stained cell suspension was examined in a haemocytometer 3 to 4 minutes after mixing and the proportion of stained (dead) cells determined.

White cell counting fluid. 0.01 per cent methyl violet was added to 1.5 per cent acetic acid. To stain the white blood cells and haemolyse any contaminating red blood cells 0.1 mls of the cell suspension to be counted was added to 0.9 mls of white cell counting fluid. After 1 to 2 minutes the stained white cells were counted in a haemocytometer.

Preparation of bentonite - Bentonite (Laboratory grade, BDH) was prepared according to the method described by Fraenkal-Conrat et al. (1961).

Tritrated thymidine - Thymidine that had had one of the hydrogen atoms in the methyl group exchanged for a tritium was obtained from "The Radioactive Centre", Amersham, England. It had a specific activity of 16,100 mc/mM and was prepared as a sterile solution. It was diluted in Eagle's basal medium to 20 µc per ml.

Liquid scintillation fluids -

Scintillation fluid to accommodate non-aqueous solvents

P.P.O.	[2,5-Diphenyloxazole (Scintillation grade)] Packard Inst. Co., Illinois, U.S.A.	4.0 gms
PoPoP	[1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene]	0.2 gms
Toluene		1000 mls

Scintillation fluid to accommodate small quantities of aqueous solvents

Dioxane	880 mls
Methyl alcohol	100 mls
Ethylene glycol	20 mls
P.P.O. (2,5-Diphenyloxazole)	4.0 gms
PoPoP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene]	0.2 gms
Naphthalene	60 gms

General Methods

Surgical Procedures

Cannulation of the efferent popliteal lymph duct of sheep - The technique of cannulation of the popliteal lymph duct was essentially that described by Hall and Morris (1962) except for two differences. Firstly, an alternative surgical approach between the two heads of the biceps femoris muscle was used. Secondly, the cells were not collected in a cooled receptacle.

Cannulation of the thoracic duct in rats - The thoracic duct was cannulated in the abdomen under ether anaesthesia using Gowan's (1959) modification of the method of Bollman et al. (1948). After cannulation, the rats were placed in restraining cages as designed by Bollman et al. (1948) where they remained unanaesthetized, during the period of lymph collection.

Intradermal injection of allogeneic lymphocytes and measurement of the subsequent reaction - Lymph was collected for 24 hours from the chronic lymphatic fistulae into 5 mls of anticoagulant solution. After washing in Eagle's basal medium, the lymphocytes contained in the above lymph, were resuspended at a concentration of 50 million per ml in Eagle's basal medium to which 5 per cent inactivated calf serum had been added. The lymphocytes in 0.1 mls of media were injected into the wool free, medial aspect of the thighs of sheep, after any caked mud or wool grease on the injection area had been removed by washing with warm water and soap. The injections were made with a tuberculin syringe fitted with a 28 gauge needle.

Daily measurements of the site of the reaction, before and after the injection, were made with 'Schnelltaster' skin calipers. A fold of skin, to include the site of the reaction was measured, so that always two thicknesses of skin were between the jaws of the calipers. The calipers were closed with a firm and what was judged to be a constant pressure. When two or more injections were made into each leg, the sites were marked with an ink pencil and this marking persisted for at least 4 to 5 days by which time the reactions themselves were apparent.

Preparation of single cell suspensions from sheep liver and kidney - The sheep, from which the kidney and liver were to be removed asceptically, was killed by exsanguination. After

their removal from the abdominal cavity, the kidneys and liver were placed in Hank's balanced salt solution. Each organ was then finely minced with scissors, placed in 0.25 per cent trypsin in Puck's saline and stirred with a magnetic stirrer at 20°C until a single cell suspension was obtained. The remaining large pieces of tissue were removed by slow centrifugation and the single cells remaining in the supernatant were washed twice in Hank's balanced salt solution to remove the trypsin. After the second wash the cells were resuspended in Eagle's basal media containing 5 per cent inactivated sheep serum at the required concentration.

Preparation of single cell suspensions from spleens and lymph nodes - The donors of sheep spleens and lymph nodes were killed by exsanguination and the chicken donors by dislocation of the neck. The spleen or mesenteric lymph nodes were removed asceptically and placed in Hank's balanced salt solution. These organs were then finely minced with scissors in a small volume of Hank's balanced salt solution and the pieces rubbed through a fine stainless steel mesh. The resultant single cells were washed twice in Hank's balanced salt solution and resuspended at the required final concentration in Eagle's basal medium containing 5 per cent inactivated sheep serum.

Immunization of Animals

Guinea pigs - The guinea pigs to be used in delayed hypersensitivity tests were immunized by the injection into each hind foot of 0.25 mls of Freund's complete adjuvant which had been emulsified with 100 million sheep lymphocytes or chicken spleen cells. Skin tests for delayed hypersensitivity were carried out by the injection of 5 million sheep lymphocytes or chicken spleen cells contained in 0.1 mls of Eagle's basal medium into the clipped flanks of the immunized guinea pigs.

Immunization of adult outbred chickens with sheep lymphocytes - These birds received an intraperitoneal injection of 0.5 mls of Freund's complete adjuvant to which 200 million sheep lymphocytes had been added. Subsequent intramuscular injections of 200 million sheep lymphocytes in Eagle's basal medium were made 4, 7, 10, 30, 37, 43, 57 and 71 days after the first injection respectively.

Immunization of adult chickens with pigeon spleen cells - The chickens in this group received an initial intraperitoneal injection of 1 ml of Freund's complete adjuvant in which 500 million pigeon spleen cells had been emulsified. Each bird received two subsequent monthly intramuscular injections of the same number of pigeon spleen cells contained in 1 ml of Eagle's basal medium.

Geese and Ducks - Adult ducks and geese were immunized by the intraperitoneal injection of 1 ml of Freund's complete adjuvant containing 500 million chicken spleen cells followed by a second similar injection a month later.

Inoculation of chicken embryos - Chicken embryos were injected intravenously according to the method of Beveridge and Burnet (1946).

Transplantation of Tissues to the Chorioallantoic Membrane (CAM) of the Chicken Embryo

Fertile chicken eggs after 10 days incubation were candled and the position of the air sac marked with a soft pencil. A mark was also made on the side of the eggs at a position over the CAM, care being taken to avoid any large blood vessels that might be damaged when a hole is drilled in the egg shell. The air sac was opened by drilling a hole through both the shell and the underlying shell membrane. A small hole was then drilled through the egg shell on the side of the egg. A drop of gelatin saline (3% gelatin in 0.9% sodium chloride solution) was placed over this latter

hole and the CAM was separated from the shell membrane by breaking the shell membrane with a sterile needle and allowing the gelatin saline to flow in between the two membranes. In some cases the separation of the CAM was encouraged by applying slight suction over the hole drilled in the air sac. Free cellular grafts were introduced onto the CAM by means of a Pasteur pipette. Solid grafts were placed on the membrane either by means of a wide bore pipette or by using a pair of fine forceps. After the graft had been placed on the CAM the hole in the shell was sealed with elastic tape and the eggs were incubated at 38°C in a humidified incubator.

Preparation of Tissue for Transplantation

The bones used for transplantation were the long bones of the leg and on some occasions wing bones were also transplanted. The limb was removed from the donor embryo and the muscles surrounding the bone were carefully removed with the aid of forceps and a dissecting microscope. Chicken embryo bones were taken from 10 or 11 day old embryos. Duck embryo bones were obtained from 14 day old embryos. In the case of the pigeon embryos it was not possible to accurately estimate their age. Pigeon embryos were sacrificed at what was judged to be the middle of their developmental period by size of the embryo and the spread of the CAM over the inside of the shell.

Transplants of chicken embryo heart were first perfused with Hank's balanced salt solution to remove any blood cells from the coronary circulation. This process was carried out by placing a fine plastic canula in the aorta and tying it off at a position close to the junction of the aorta and the heart. The other vessels leaving the heart were then tied off and the heart was removed from the embryo. The coronary circulation was perfused with about 2 to 3 ml of Hank's salt solution which escaped from an opening that had been made in the left auricle of the heart. The perfused heart was then cut

into small pieces about 2 mm^3 which were washed in Hank's solution and then transplanted onto the CAM of recipient embryos.

The transplantation of embryonic chicken spleen was accomplished by placing whole spleens removed from 10 or 11 day old chicken embryos on the CAM of the recipient embryo. Grafts of other tissues were cut into pieces 2 mm^3 before transplantation to the CAM.

Chromosome Analysis

The cells under examination were cultured in Eagle's medium containing 10 per cent sheep serum and colchicine at a concentration of 1 part in 40,000 for a period of 4 hrs. in an atmosphere of 5% CO_2 and air. At the completion of the incubation period the cell suspension was made hypotonic by the addition of two parts of distilled water. After standing for 10 minutes in the hypotonic solution, the cells were sedimented by centrifugation and fixed as a pellet for a period of 10 minutes with a solution of 9 parts of glacial acetic acid and 1 part of 1 N hydrochloric acid. The cells were stained by resuspending the pellet in 2 per cent aceto orcein. Chromosome spreads were then prepared by squashing the cells suspended in 2 per cent aceto orcein between a coverslip and glass slide. Permanent preparations were obtained by sealing the edges of the coverslip with wax.

Tissue Culture Procedures

Determination of suitable conditions for the culture of sheep lymphocytes in vitro - The following experiment was designed to investigate the factors that affect the survival of sheep lymphocytes in vitro. Lymphocytes were obtained from chronic fistulae placed in the efferent lymphatic ducts of sheep. The lymphocytes were washed in Hank's balanced salt solution and resuspended at a concentration of either 1 million or 10 million cells per ml in the different media

TABLE 2.1

The effect of different culture conditions upon the survival of lymphocytes in vitro

Period of culture (hours)	Media	Percentage of viable cells after different period of culture											
		Concentration of cells/ml and percentage of homologous serum added to media											
		10% homologous serum						40% homologous serum					
		$1x10^6$ cells/ml		$1x10^7$ cells/ml		$1x10^6$ cells/ml Heparin		$1x10^6$ cells/ml		$1x10^7$ cells/ml		$1x10^7$ cells/ml Heparin	
3	EBM	97.4	98.6	99.4	99.2	96.2	>99	>99	99.3	99.4	99.6	>99.9	98.9
	199	>99	>99	98.4	98.0	>99	98.75	>99	98.6	>99	99.8	>99.9	99.9
	Lact.Alb.*	>99	>99	99.8	99.8	98.1	98.1	99.5	>99	99.8	99.8	>99.9	99.4
	Lymph.	ND	ND	ND	ND	ND	ND	ND	ND	99.8	99.5	ND	ND
22	EBM	76.0	63.0	93.9	91.7	90.4	80.0	97.1	83.5	96.2	96.2	95.6	93.2
	199	84.5	86.0	82.4	81.5	90.8	86.0	96.6	93.7	92.8	92.4	97.7	94.3
	Lact.Alb.	78	84	89.0	92.0	59.5	75	90.0	96.1	98.8	97.0	86.0	79.0
	Lymph.	ND	ND	ND	ND	ND	ND	ND	ND	97.0	94.1	ND	ND
48	EBM	85.5	72	94.1	91.7	65.0	35	91.7	89.0	96.5	96.3	64	73
	199	83.5	88.5	92.3	93.9	85	75	90.8	88.0	92.8	95.4	76	76
	Lact.Alb.	67.0	77.0	97.0	94.8	0	0	92.0	85.0	94.4	97.8	68	62.5
	Lymph.	ND	ND	ND	ND	ND	ND	ND	ND	85.2	91.8	ND	ND
72	EBM	93.7	69.0	89.1	89.2	81.2	11.8	81.2	66.7	92.1	90.1	0	0
	199	93.0	90.9	88.6	80.0	46.7	52	84.2	80.0	90.5	89.4	0	0
	Lact.Alb.	5.0	0.0	91.0	92.3	0.0	0.0	64.0	71.4	94.0	82.2	0	0
	Lymph.	ND	ND	ND	ND	ND	ND	ND	ND	61.9	60.2	ND	ND
96	EBM	85.7	50	81.8	85.2	0	0	0	0	79.3	71.9	0	0
	199	88.2	83.2	85.0	86.7	0	5.0	0	0	78.7	66.7	0	0
	Lact.Alb.	0	0	79.0	69.9	0	0	0	0	69.9	53.1	0	0
	Lymph.	ND	ND	ND	ND	ND	ND	ND	ND	0	0	ND	
120	EBM	54.6	50.0	69.3	59.4	0	0	0	0	87.1	89.4	0	0
	199	63.8	69.8	65.6	75.7	0	0	0	0	80.5	71.3	0	0
	Lact.Alb.	0	0	53.0	0	0	0	0	0	96.0	80.0	0	0
	Lymph.	ND	ND	ND	ND	ND	ND	ND	ND	0	0	ND	ND
144	EBM	0	12.5	0	0	0	0	0	0	74.4	0	0	0
	199	73.3	61.3	0	0	0	0	0	0	85.9	70.6	0	0
	Lact.Alb.	0	0	0	0	0	0	0	0	88.2	0	0	0
	Lymph.	ND	ND	ND	ND	ND	ND	ND	ND	0	0	ND	ND

* Lactalbumin hydrolysate

that were being examined. Three different media were used. These were, Eagle's basal medium, medium 199 (Commonwealth Serum Laboratories, Melbourne) and 0.5 per cent lactalbumin hydrolysate. These three media were each supplemented with 10 and 40 per cent inactivated homologous serum respectively in separate tests. The mean survival of viable lymphocytes was assessed by a trypan blue exclusion test. The results of this experiment are presented in Table 2.1. When the percentage of viable cells in each medium was compared after 4 days cultivation, it was apparent that there was no significant difference between Eagle's basal medium and medium 199, both of which were superior to 0.5 per cent lactalbumin hydrolysate. In addition, at the lower cell concentration, survival was greater at the reduced (10 per cent) serum levels. It was noted that the presence of heparin was distinctly detrimental to the cells. From these results it was decided to culture sheep lymphocytes at a concentration of greater than 1 million per ml in Eagle's basal medium, to which 10 per cent serum had been added. Subsequent experience allowed the level of serum to be reduced to 5 per cent with no detectable effects. Table 2.2 records the effect on the survival of lymphocytes in Eagle's basal medium to when isologous, homologous and heterlogous sera were added at 10 to 40 per cent concentrations. It can be seen that more lymphocytes survived for longer at the lower concentration of homologous serum.

Preparation of Tissues for Histological Examination

Cells in efferent lymph - Cells contained in efferent lymph were deposited by centrifugation, the cell free lymph decanted and the sides of the centrifuge tube dried. An equal volume of sheep serum was added and the cells resuspended by shaking. A drop of these resuspended cells was placed on a slide and smeared with the end of another slide held at an angle of 45° to the first. The smear, which usually covered about half the slide, was air dried. 1 ml of Leishman's

TABLE 2.2

The affect on the survival of sheep lymphocytes cultured in media containing different concentrations of sera from various sources

Time of cultivation (hours)	Viable cells as a percentage of 24 hour sample								
	Concentration of serum added to Englis basal medium								
	10% Isologous (sheep)	10% Homologous (sheep)	10% Heterologous (calf)	20% Isologous (sheep)	20% Homologous (sheep)	20% Heterologous (calf)	40% Isologous (sheep)	40% Homologous (sheep)	40% Heterologous (calf)
24	100 100	100 100	100 100	100 100	100 100	100 100	100	100 100	100 100
48	87 ND	124 89	84 63	89 75	66 66	79 84	40	57 96	67 61
72	81 ND	94 85	66 57	ND	51 48	67 46	28	39 37	41 43
96	77	90 88	57 54	ND	71 47	63 ND	18	32 36	44 49
120	77	88 68	43 52	ND	45 38	45 ND	16	25 43	34 44

stain was placed on the smear for 5 minutes and then diluted with 2.5 mls of distilled water. After a further 15 minutes, the diluted stain was washed off with a stream of distilled water. The smear was then gently blotted before being allowed to dry in the air.

Solid tissues - These were fixed in 10 per cent formol saline for at least 48 hours. After fixing the solid tissues were dehydrated in ascending grades of ethyl alcohol before being cleared in chloroform. The cleared blocks of tissue were then embedded in paraffin wax for section cutting. After cutting the tissue sections were placed in xylol to remove the wax before being passed through descending grades of alcohol to water. Re-hydrated sections were stained with haematoxylin and eosin or methyl green and pyronin according to the methods described by Pearce (1961). The staining of reticulin in sections of skin was by the technique of Gomori (1937).

Chemical Manipulations

The extraction of ribonucleic acid from mammalian tissues - Ribonucleic acid (RNA) was extracted from animal tissues by a modification of the method described by Fenwick (1963). Briefly, the method was to homogenize the tissue from which RNA was to be extracted, at a temperature of 4°C, with equal volumes of PBS and freshly distilled, water saturated phenol. Solid tissues were homogenized in a Waring blendor and a microid shaker was used for the extraction of cell suspensions. After homogenization any containing large particles were sedimented by slow centrifugation. The homogenate was then centrifuged at 4000 g for 10 minutes which broke the emulsion, producing a clear upper aqueous layer, a white viscous interfacial layer and a lower phenol portion. The aqueous layer was removed and re-extracted 3 times with fresh phenol. After the third extraction the aqueous phase was decanted and one tenth of its volume of 2 m sodium chloride

and 1 per cent bentonite were added, followed by twice its volume of ice cold absolute ethanol. After 1 hour, the resultant floccular precipitate was sedimented by centrifugation at 4000 g for 15 minutes. The supernatant was discarded and the precipitate redissolved in PBS. The reprecipitation of RNA with ethanol was repeated 4 times. The RNA was then dissolved in PBS and centrifuged at 10,000 g for 10 minutes to remove the bentonite.

Determination of amount of ribonucleic acid present in a solution - The concentration of RNA present in a solution was determined by the method described by Hotchkiss (1956). The optical density of the test solution is determined at the wavelength 260 mμ for a 1 cm light path. The amount of RNA contained in the solution is calculated from the observation that a solution containing 40 γ RNA per ml has an optical density of 1.0 under the above conditions.

Methods for measuring uptake of ^{3}H-thymidine by lymphocytes

Two different methods were used to measure the incorporation of thymidine into lymphocytes.

Measurement of the uptake of ^{3}H-thymidine by lymphocytes - The uptake of thymidine by cultured lymphocytes was determined by the addition of 2 μc ^{3}H-thymidine (specific activity 16,000 mc/mM) to each 3 mls of culture. Four hours after the addition of the ^{3}H-thymidine to the lymphocyte culture, the lymphocytes were sedimented by centrifugation and carefully washed 4 times with cold normal saline. After washing, the lymphocytes were resuspended in normal saline and 1 million washed lymphocytes were added to 10 mls of dioxane based scintillation fluid and the tritium content determined in a Beckman Liquid Scintillation Spectrometer.

Measurement of incorporation of ^{3}H-thymidine into acid precipitable constituents of lymphocytes - The method of measuring the rate of DNA synthesis in mixed lymphocyte cul-

tures was essentially that described by Dutton and Page (1964). Lymphocyte cultures after 72 hours incubation were inoculated with 1 µc ^{3}H-thymidine (specific activity 16,100 mc/mM) to each 3 mls of culture. 24 Hours later the 10 million lymphocytes contained in the inoculated cultures were sedimented by centrifugation and the culture media discarded. After washing with normal saline, the acid-precipitable constituents of the lymphocytes were precipitated with 5 per cent trichloroacetic acid. The precipitate was sedimented by centrifugation, washed again with 5 per cent trichloroacetic acid and then twice with methyl alcohol. All washings were carried out at 4°C. The washed precipitate was allowed to dissolve in 1 ml of "hyamine 10x" for 24 hours at 4°C. The "hyamine 10x" was then added to 10 mls of toluene based scintillation fluid and the tritium content determined in a Beckman Liquid Scintillation Spectrometer.

Titrometric Assay for Ribonuclease

The assay for ribonuclease makes use of the fact that ribonuclease can act on the molecule of relatively small molecular weight cyclic cytodylic acid. The method used was a modification of that described by Cinadar and Lafferty (1964). Assays using a solution of 0.05 M cyclic cytidylic acid in 0.12 N KCl as substrate were carried out with a Radiometer TTT-1a pH -Stat. A water-jacketed vessel maintained the temperature of the 1.5 ml reaction mixture at 37°C. The velocity of the enzyme reaction was estimated by measuring the rate at which 0.0005 M NaOH had to be added to keep the pH constant at 7.5.

Estimation of Amount of Protein Present in Solution of Ribonucleic Acid by the Microbiuret Test

The amount of protein present in the solutions of RNA extracted from sheep lymphocytes was ascertained by means of the microbiuret test as described by Zamenhof and Chargaff

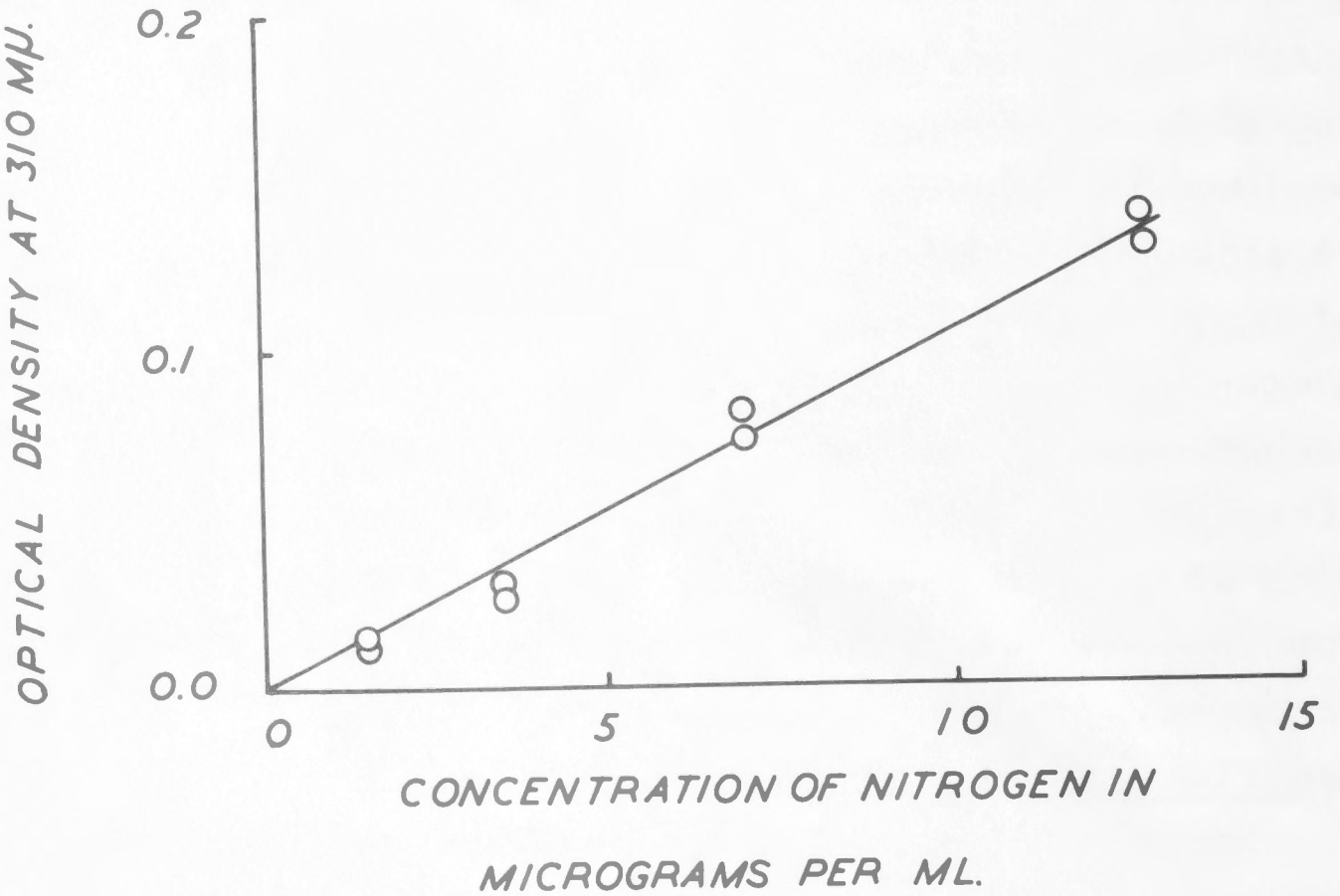


Figure 2.2

The effect of increasing concentration of nitrogen on the optical density at 310 mμ 1 cm of the micro-biuret reagent.

(1957). The relationship between the optical density of the microbiuret reagent at 310 mμ and the weight of nitrogen per ml was determined by the use of solutions of ovalalbumin of known concentration (Fig. 2.2). The determinations of the nitrogen concentration in the solutions of ovalbumin were made by the Kjeldahl method.

Kjeldahl Method of Nitrogen Determination

The method described by Grant (1957) was used for all determinations of the nitrogen content of protein solution.

CHAPTER 3

THE BEHAVIOUR OF XENOGENEIC LYMPHOID CELLS IN THE CHICKEN EMBRYO

Chapter 3 - The Behaviour of Xenogeneic Lymphoid Cells in the Chicken Embryo

Introduction

When adult allogeneic lymphoid cells are injected into chicken embryos, they produce certain pathological changes. Simonsen (1957) has used the term graft versus host (GVH) reaction to describe these events. The ability of injected lymphoid cells to react against the host may be judged by measuring changes in spleen weight of the recipient embryo or by counting the number of pocks produced on the CAM (Simonsen, 1962; Boyer, 1960; Coppleson and Michie, 1965).

A comparison of GVH reactions produced by lymphoid cells from different donors revealed a large variation in the capacity of the donated cells to induce splenomegaly in recipient embryo. Several workers (Cock and Simonsen, 1958; Burnet, 1963) found that providing there was a genetic difference between donor and recipient and that the donor was not naturally tolerant of the host, lymphoid cells from the same species produced a GVH reaction in the chicken embryo. On the other hand, xenogeneic lymphoid cells produced either a much reduced GVH reaction or no signs of one in the recipient chicken embryos (Payne and Jaffe, 1962). There is no apparent reason for this, at best, weak GVH reaction and a lack of antigenic stimulus is an unlikely explanation of the poor capacity or incapacity of the xenogeneic cells. Simonsen (1962) has suggested that xenogeneic cells suffer generalized immunological depression from the encounter with too many strong antigens in the injected embryo, which would leave few immunologically competent cells. However, the finding of Zlotnick (1963) that rabbit lymph node cells injected into the hamster cheek pouch were able to survive and produce antibody in this foreign environment makes the validity of such an explanation doubtful.

In an attempt to understand more clearly the failure or reduced capacity of xenogeneic lymphoid cells to produce GVH reactions in chicken embryos a study of the behaviour and fate of the injected lymphocytes was undertaken.

Experimental Results

The Behaviour of Allogeneic and Xenogeneic Lymphoid Cells in Chicken Embryos

Grafts of dissociated lymphoid tissues - The capacity of lymphoid cells to produce a graft versus host reaction in the chicken embryo can be followed by measuring the changes in the spleen weight or by counting the pocks formed on the CAM following the intravenous or CAM route of administration. Using both of these methods, an experiment was conducted in which leucocytes from normal adult chickens, pigeons, ducks and geese, lymphocytes from efferent popliteal lymph of sheep and spleen cells from guinea pigs were inoculated into 11 day old chicken embryos. 6 to 10 embryos were inoculated with cells from each species. The spleen weights of the inoculated embryos were determined 6 days after the intravenous inoculation with donor lymphoid cells. Any pocks produced following the inoculation of lymphoid cells directly onto the CAM were counted either 4 or 6 days later.

The results of the experiment are recorded in Table 3.1. It can be seen that while allogeneic lymphoid cells produced marked increases in the spleen weights of recipient chicken embryos, xenogeneic cells did not produce any such significant changes. Nevertheless it would not be valid to maintain that all xenogeneic lymphoid cells fail to induce splenic enlargement in the chicken embryo. In an extensive survey Payne and Jaffe (1962) found that the greatest increases in spleen weights occurred when the xenogeneic lymphoid cell donors were from the order Galliformes. This order which has the domestic chicken as the type species contains such birds as the

TABLE 3.1

The production of pocks on the chorioallantoic membrane and the changes in the spleen weights of chicken embryos injected with alien lymphoid cells

Cell Donor	Number of cells injected $x10^6$	Number of embryos injected	Pocks produced on the CAM with		Mean spleen weight in mg
			Adult Leucocytes	Medium	
chicken	1	5	+++	-	132.4
Duck	10	6	+	-	7.8
Goose	10	6	-	-	9.4
Pigeon	10	6	-	-	8.4
Sheep	10	6	-	-	10.1
Guinea Pig	10	5	-	-	9.3
EBM (control)	0	49	-	-	10.6*

* The standard deviation of the population was $\pm$ 3.8 mgs

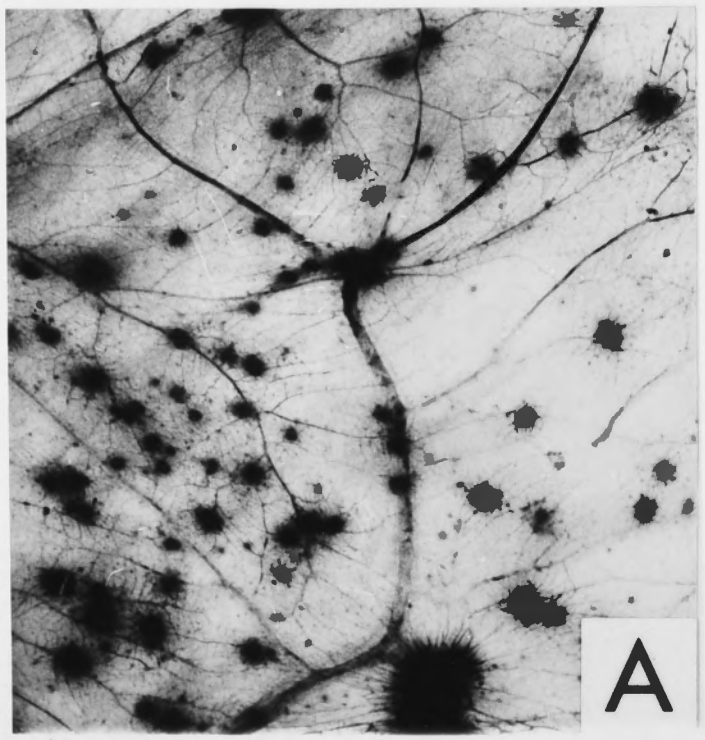
Red jungle fowl, silver and golden pheasants, pea fowl and turkey. Lymphoid cells from all these species produced significant splenomegaly when they were inoculated into chicken embryos. It seems likely from these observations that the degree of GVH reactivity decreases, as the phylogenetic separation of donor and recipient increases. This matter was clearly seen when xenogeneic lymphoid cells from different species are compared on the basis of their ability to produce pocks on the CAM of chicken embryos. It will be seen from Table 3.1 that duck leucocytes, while not producing any significant change in the weight of the spleen of recipient embryos will sometimes cause the formation of a few pocks on the CAM. In this experiment, however, only 2 out of 5 membranes inoculated with 10 million duck cells bore any pocks. Figure 3.1 is a comparison of membranes that have been inoculated with blood leucocytes of chicken, duck or goose origin. It can be seen also that, while allogeneic chicken leucocytes produced large number of pocks on each membrane inoculated, 10 times as many duck leucocytes produced a single pock on 2 membranes only out of the 5 inoculated. The lymphoid cells of geese, pigeons, guinea pigs and sheep, when inoculated onto the CAM of chicken embryos failed to produce any pocks at all (Table 3.1).

The failure of injected lymphoid cells obtained from species of birds unrelated or only distantly related to domestic chickens to produce a GVH reaction in chicken embryos has been reported by a number of workers (Simonsen, 1957; Payne and Jaffe, 1962). These workers have suggested that the environment in the chicken embryo may be unsuitable for the survival of the xenogeneic lymphoid cells.

Determination of the survival of xenogeneic lymphoid cells in the chicken embryo - One way to determine the survival of grafted cells in the chicken embryo would be to recover the

Figure 3.1

A comparison of pock formation on the CAM of the chicken embryo by chicken (A), duck (B), and goose (C) blood leucocytes. The arrow points to the pock produced by the duck cells. Most membranes show a single small non-specific lesion at the site where the hole was drilled in the egg shell.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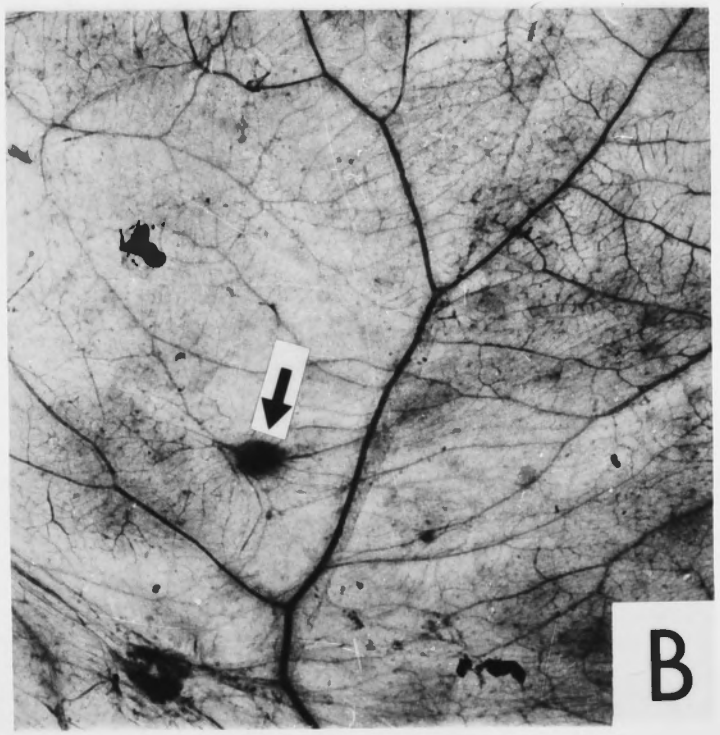
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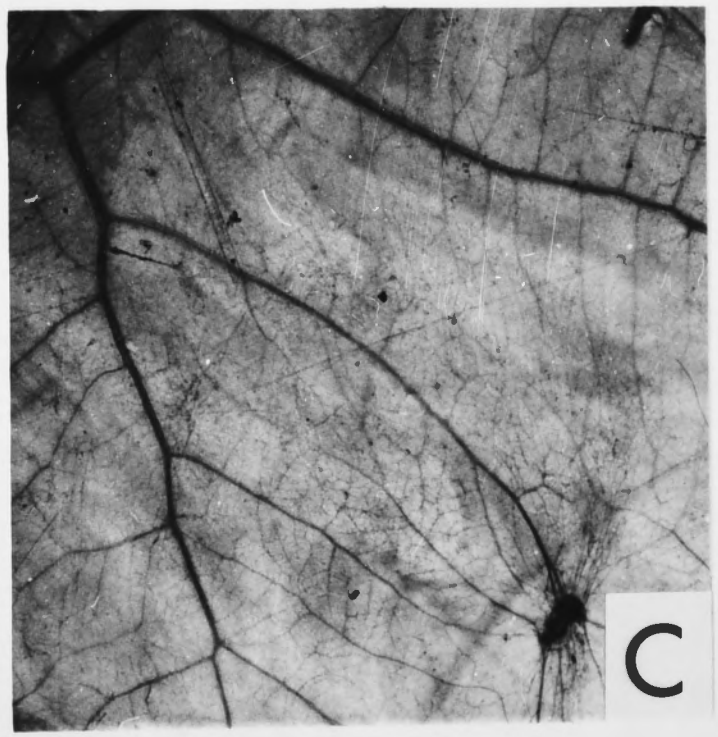

C

cells after grafting and thus ensure that they were then still viable. It proved to be difficult to recover dissociated cell grafts from the chicken embryo chorioallantoic membrane and it was necessary to graft solid pieces of lymphoid tissue which could be easily found again. Solid grafts of lymphoid tissue from mammalian and avian origin were successfully grafted on the CAM of 11 day old chicken embryos.

The method used was to remove the adult avian spleen or sheep lymph node, under sterile conditions. After being placed in Hank's balanced salt solution, the spleen or lymph node was finely minced with scissors into pieces 1 to 2 MMs in size. These pieces were then placed on the CAM of 11 day old chicken embryos. When the membranes were examined microscopically 6 to 7 days later, lymphoid tissue grafts from pigeons, ducks and 10 per cent of those from young lambs were seen to have been incorporated into the mesodermal layers of the chicken embryos. The embedded grafts were found not to have retained much of their normal architecture but consisted mainly of large number of lymphocytes (Fig. 3.2). Many of the cells in successful grafts from all donors showed a considerable amount of proliferative activity and many were pyroninophilic.

Although the histological evidence demonstrated that the grafted xenogeneic lymphoid tissue contained viable cells, it did not exclude the possibility that these cells may have been invading lymphoid elements from the embryo that had replaced the original donor cells. By examining the chromosomes of the cells in mitosis it can be shown that this was not the case for the grafted sheep lymph node. The mitotic figures of cells from the graft donor and recipient embryo can readily be distinguished from each other since the chicken has a large number of minute chromosomes not present in sheep cells (Fig. 3.3). Chromosome spreads were prepared by first removing the

Figure 3.2

A comparison of the histological appearances of sheep popliteal lymph node and pigeon spleen 6 days after grafting onto the CAM of 11 day old chicken embryos.

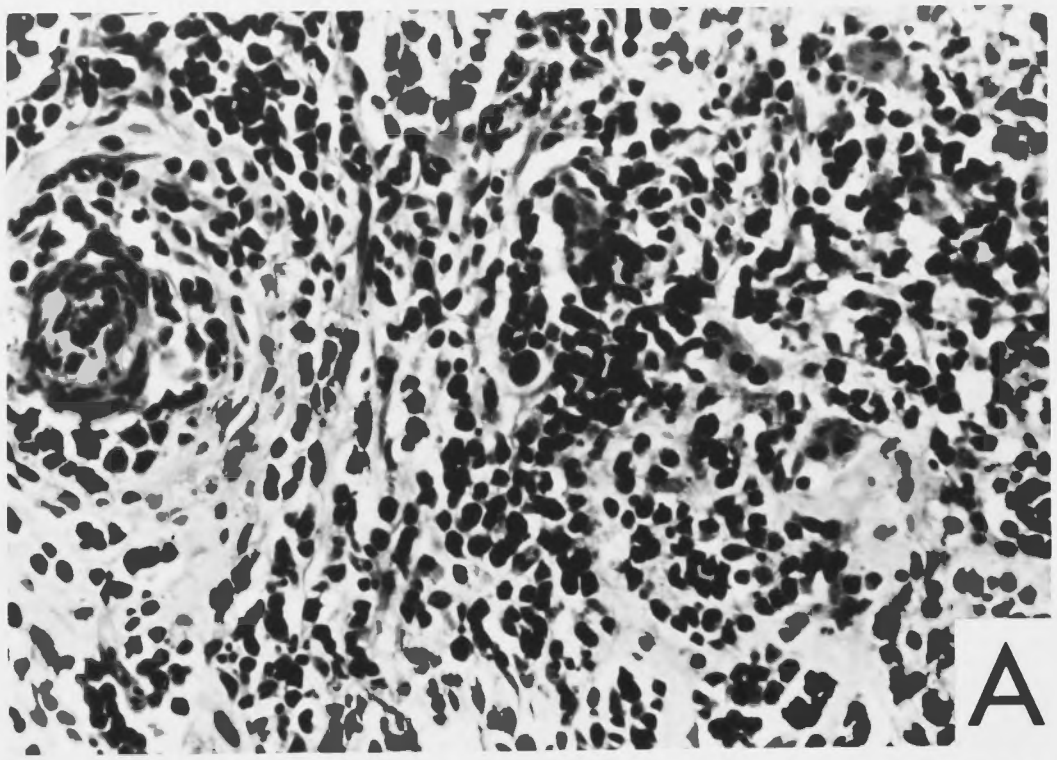
A Sheep lymph node, magnification x160

B Sheep lymph node, magnification x26

C Pigeon spleen, magnification x160

D Pigeon spleen, magnification x 26

Stain, haematoxylin and eosin.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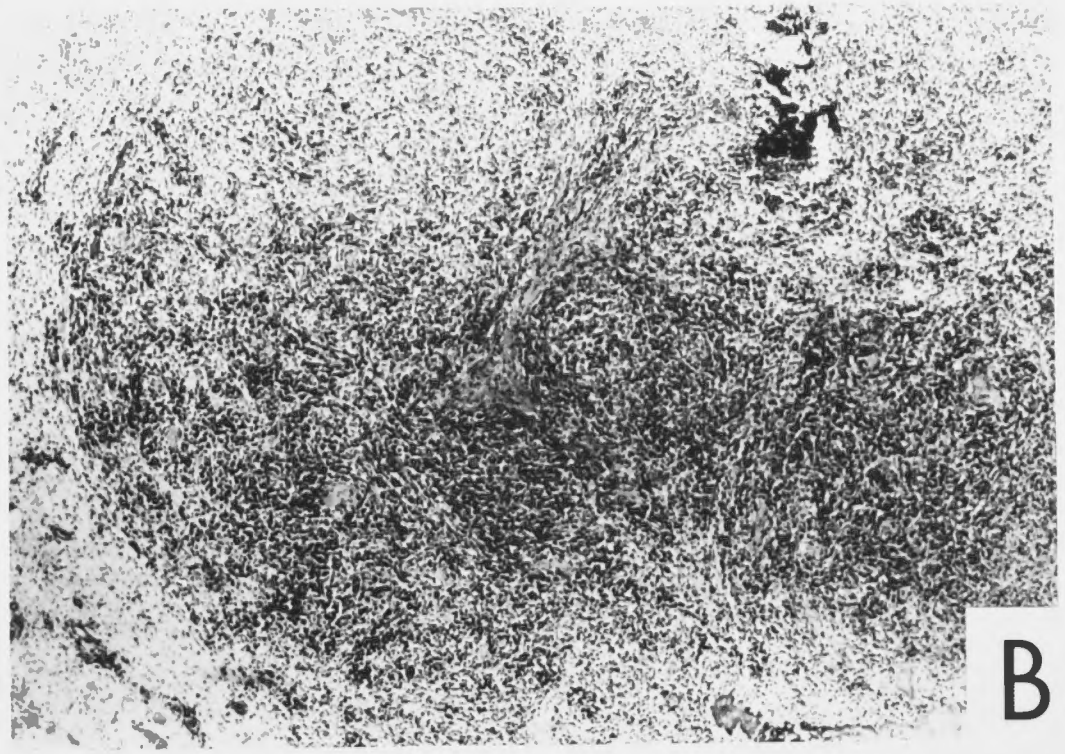
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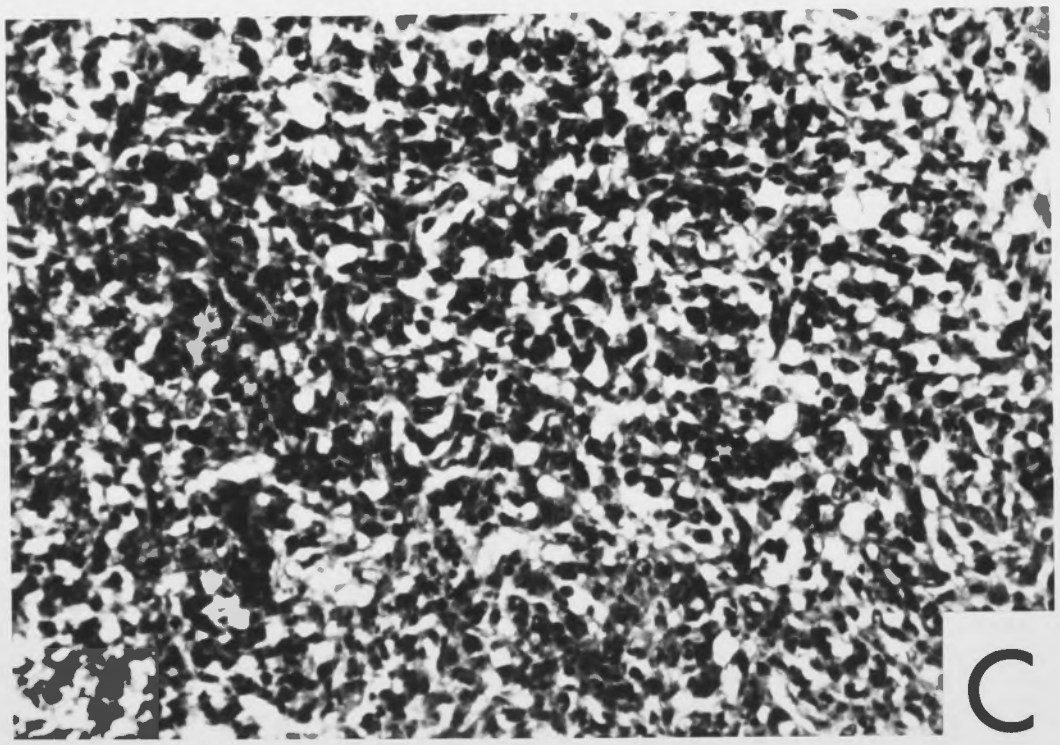
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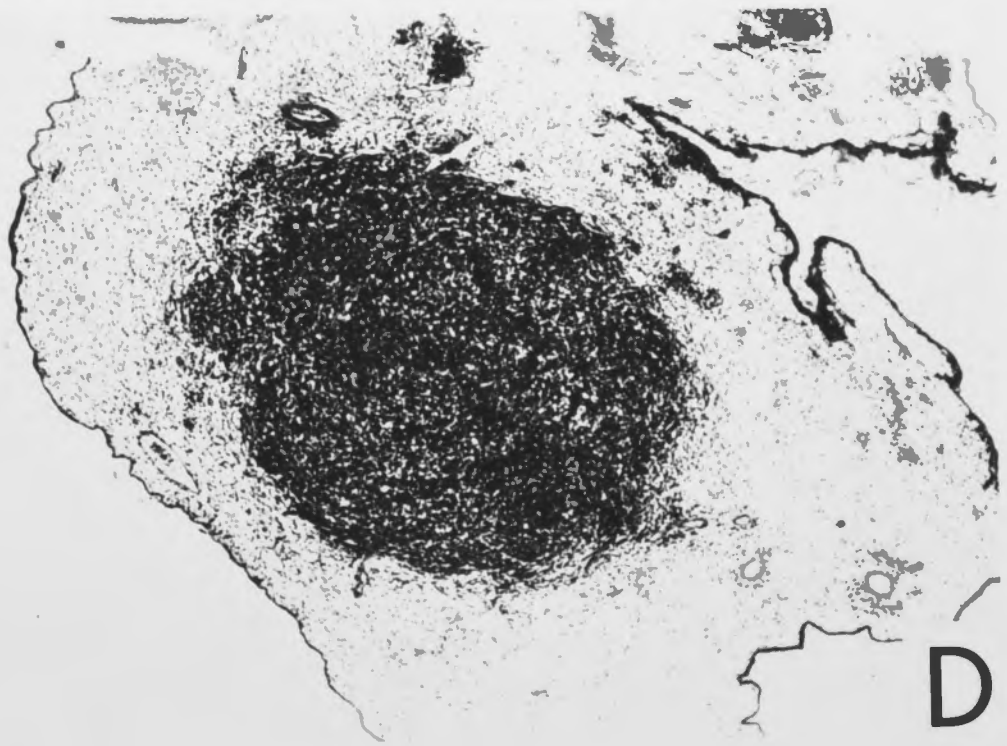
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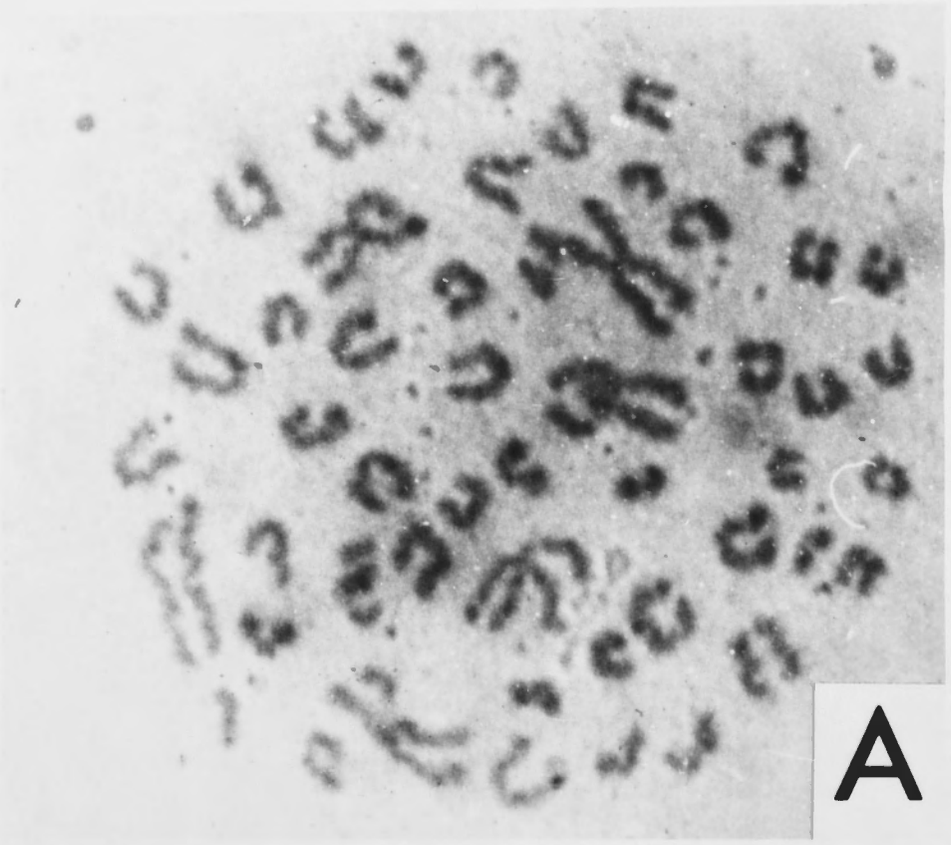
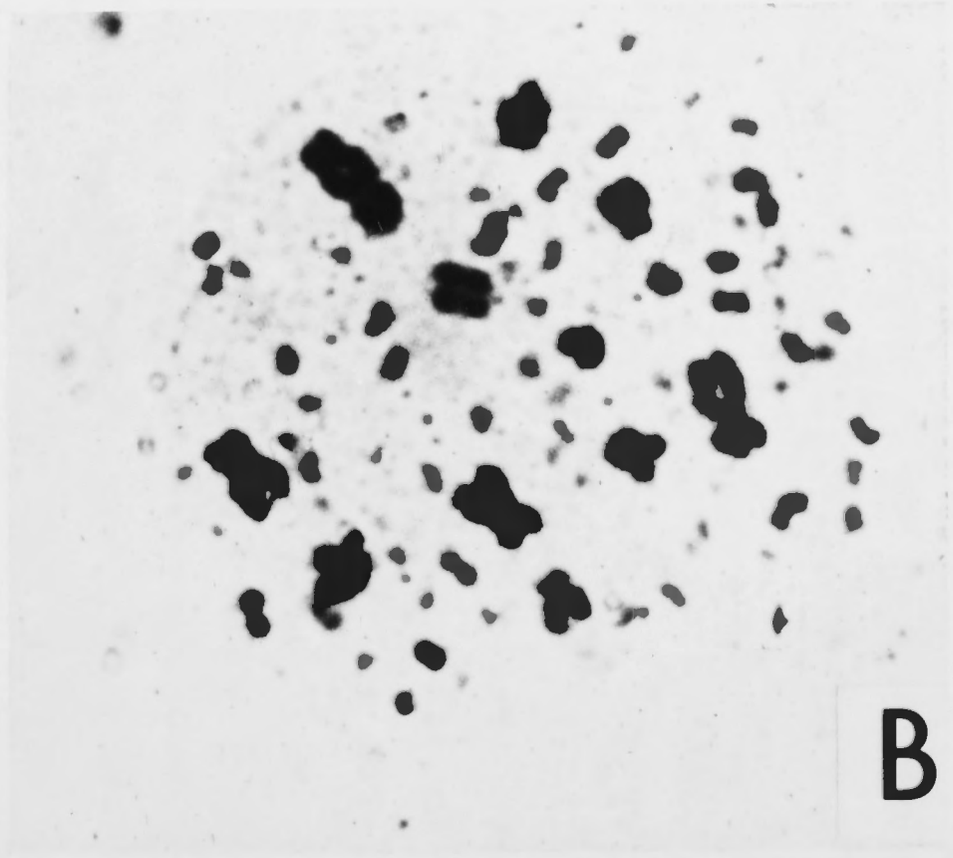
Figure 3.3

A comparison of chromosome spreads from dividing sheep cells (A) and chicken cells (B). Magnification x2000.



A



B

embedded grafts of sheep lymph node from the membrane and then releasing the cells by rubbing it against a fine stainless steel wire mesh. The single cell suspensions obtained in this manner were cultured in Eagle's basal medium, containing 10 per cent sheep serum and 1 part in 40,000 of colchicine. Four hours later chromosome preparations were made from the cultured cells. A total of 20 chromosome preparations were examined and all were found to be of sheep origin.

The experiment indicated that the grafted xenogeneic lymphoid cells were able to survive and proliferate in the chicken embryo, although they did not produce any significant increase in the spleen weight of the recipients compared with the control embryos that had received allogeneic chicken embryo heart grafts (see Table 3.2). In marked contrast, pieces of adult allogeneic chicken spleen grafted into the same situation produced a marked enlargement of the recipients' spleens (Murphy, 1916).

It does not seem likely that the inability of xenogeneic lymphoid cells to induce splenomegaly in the chicken embryo can be ascribed to their non-survival. This being the case, there are two possible explanations of the failure of a graft versus host reaction to result from the grafting of xenogeneic lymphoid cells into a chicken embryo. Firstly, the grafted lymphocytes may not have been stimulated by the host's antigens, or secondly, although stimulated they were unable to function sufficiently well in the unfavourable environment of the chicken embryo to produce the graft versus host reaction.

The following experiments show that under appropriate conditions, xenogeneic cells can produce a strong graft versus host reaction.

<u>The effect of preimmunization of the lymphoid cell donor on the production of the graft versus host reaction in xenogeneic hosts</u> - In an attempt to determine whether the

TABLE 3.2

Effect of lymphoid tissue grafts to the CAM on spleen weight of the recipient chicken embryo

Tissue Grafted	Mean spleen weight (mgm) 6 days after grafting. The bracketed figure is the number of embryos in the group
Duck spleen	12.2 (6)
Pigeon spleen	7.9 (6)
Sheep lymph node	10.5 (5)
Embryonic chicken heart (control)	12.5 (24)*

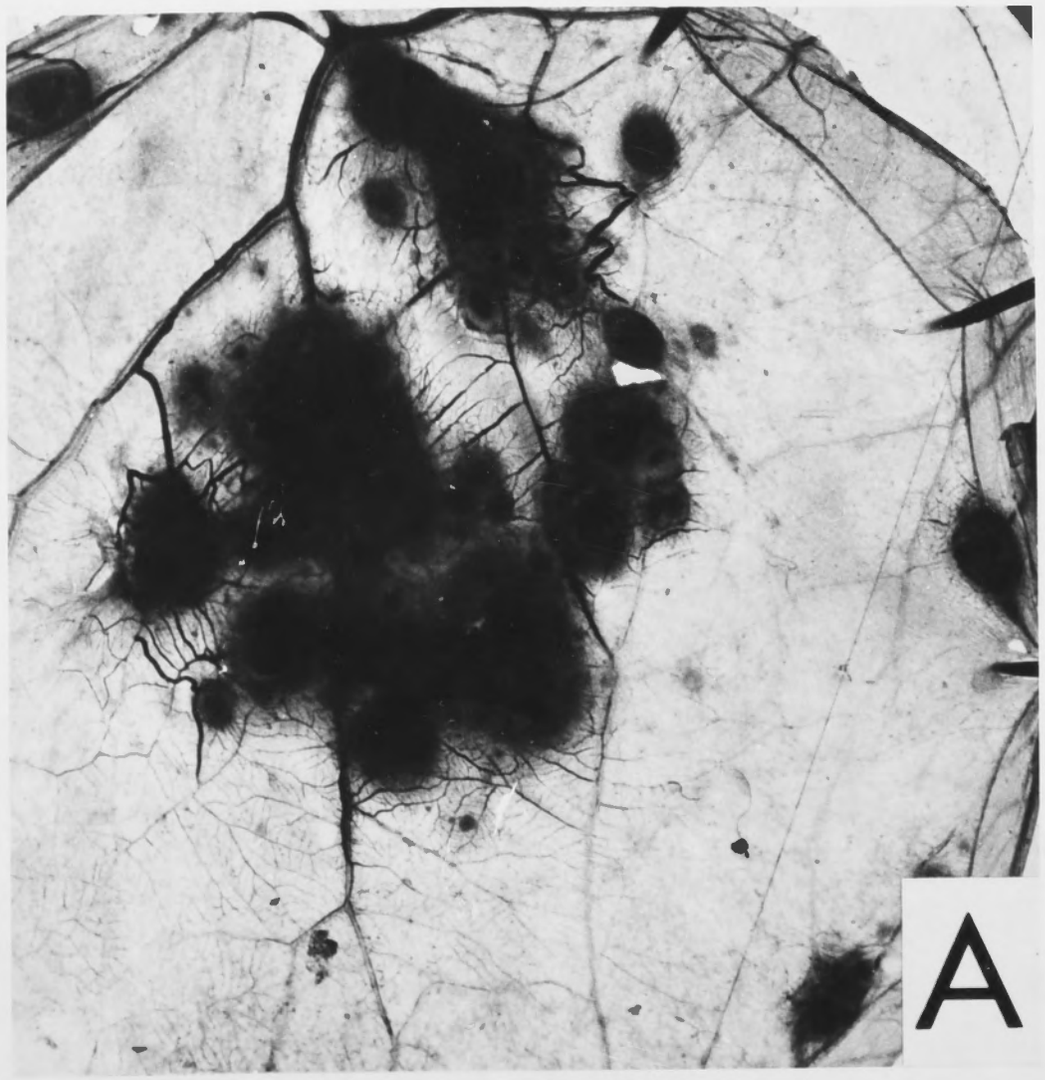
* The standard deviation of the mean was $\pm$ 3.8 mgm

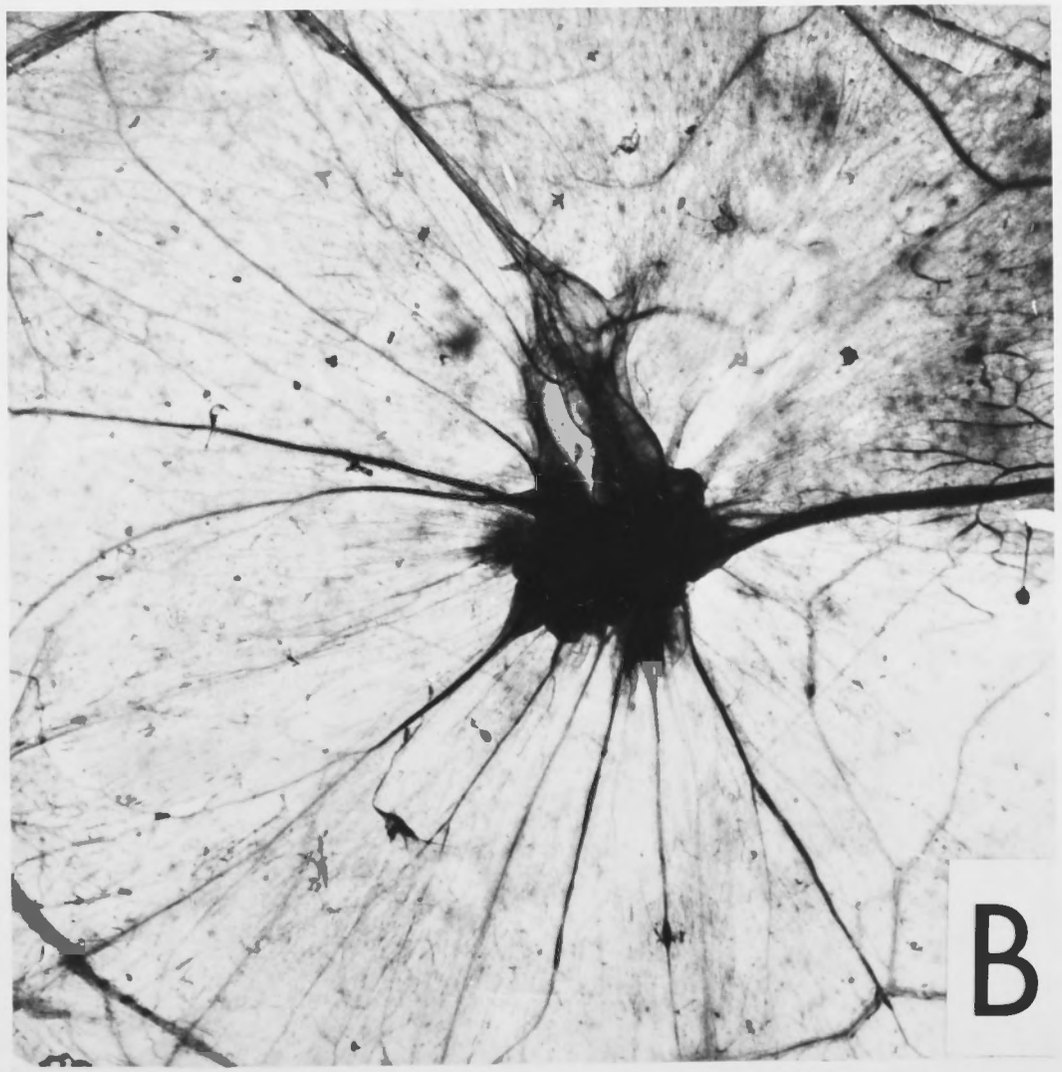
chicken embryo environment was suitable for xenogeneic lymphocytes to respond to antigens, lymphoid cell donors were immunized against chicken antigens as described in Chapter 2. Blood leucocytes were obtained from pigeons, ducks and geese, 3 to 4 weeks after they had received the initial immunizing dose of chicken embryo spleen cells. 10 million blood leucocytes from each donor were inoculated onto the CAM of each 11 day old chicken embryo. When the membranes were examined 6 days later, it was found that cells from the immunized duck produced multiple pocks, but that a single large central lesion, presumably at the site of inoculation was produced by cells from the other xenogeneic donors (Fig. 3.4). Reactive blood cells were detected in avian donors, when tested 10 days after immunization and again a month later. The capacity of blood leucocytes from immunized donors to produce lesions on the CAM was destroyed following freezing and thawing of the donors cells before inoculation.

A somewhat different situation was encountered when the production of pocks on the CAM of chicken embryos by lymph node cells from immunized guinea pigs was examined. The guinea pigs were immunized and tested for delayed-type hypersensitivity by injections of suspensions of chicken spleen cells (see Chapter 2). It was found that the immunized guinea pigs exhibited delayed-type hypersensitivity to an intradermal injection of chicken spleen cells 17 days after the initial immunizing injection into the foot pad. These animals were killed 17, 36, 43, 64 and 74 days after primary immunization and single cell suspensions made from their popliteal lymph nodes. When 10 million of these dispersed lymph node cells were inoculated on to the CAM of 11 day old chicken embryos, no lesions were seen 6 days later, with cells from guinea pigs immunized 17, 36 and 43 days before being killed. However, cells from guinea pigs whose immunization had begun

Figure 3.4

A comparison of the pocks produced on the chorioallantoic membranes by blood leucocytes from an immune duck (A) and from an immune goose (B).


A


B

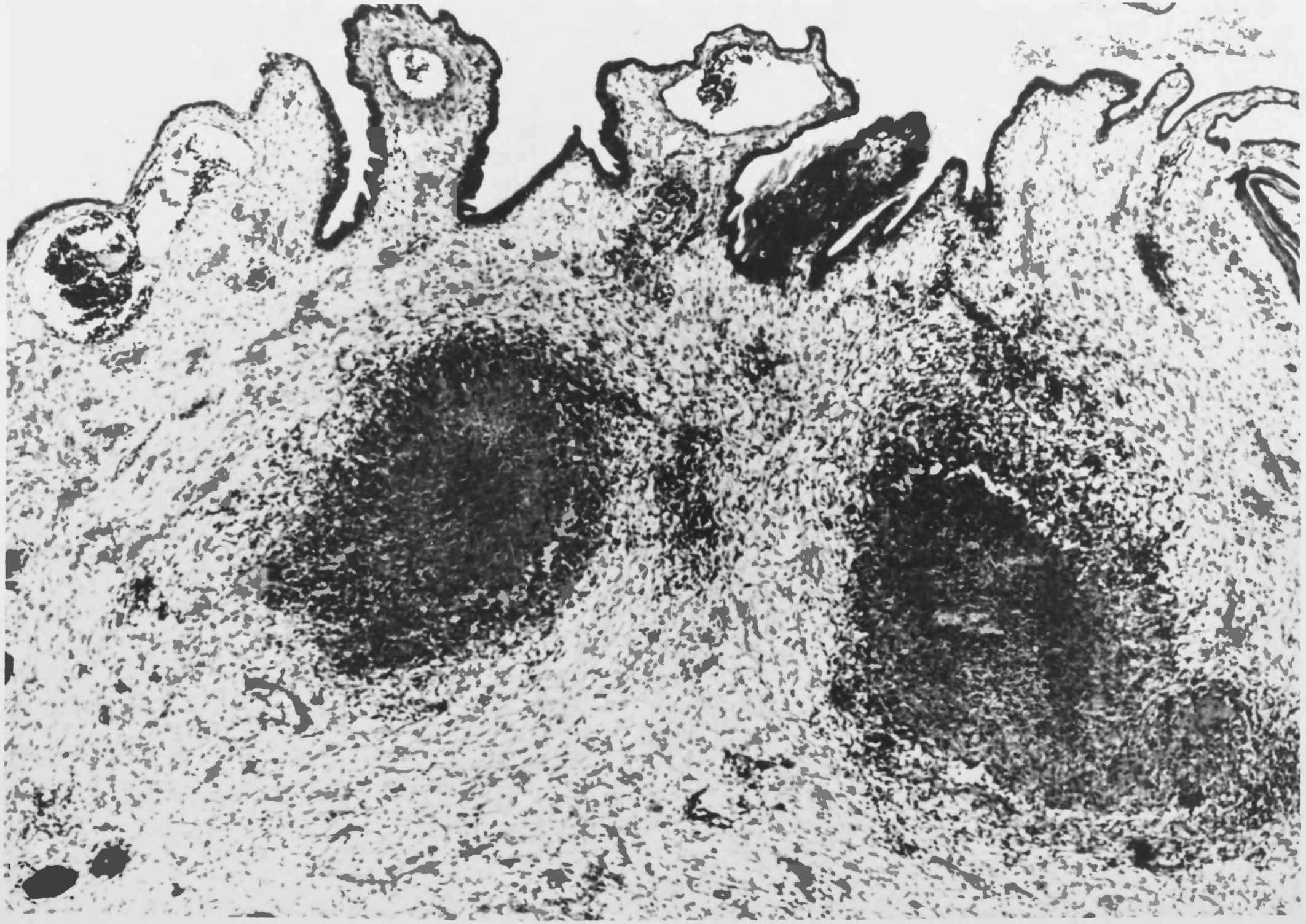
64 and 74 days before being killed, produced single large central pocks. Pocks produced by immune guinea pig cells were similar in appearance to those produced by immune pigeon and goose lymphoid cells. Figure 3.5 shows the histological appearance of a pock produced by immune guinea pig cells 6 days after their inoculation onto the CAM of a chicken embryo. It can be seen there has been marked proliferation of the mesodermal and ectodermal layers of the CAM. These lesions had a tumour-like appearance and often contained one or more necrotic foci within the proliferating lymphoid cells in the mesodermal layer.

Discussion

It has generally been accepted that the GVH reaction results from an attack by the grafted lymphocytes on the antigens of the recipient (Simonsen, 1962; Burnet, 1963). Such a concept of the GVH reaction is based mainly on the specificity of reactions of this type. Thus, Cock and Simonsen (1958) showed that little splenomegaly resulted when adult chicken spleen cells, obtained from an inbred strain of chickens were injected into embryos of the same inbred stock. Moreover, several workers have shown that the injection of parental strain lymphoid cells will produce a reaction in embryos or new born animals resulting from the mating of the two parental strains, whilst lymphocytes from such F_1 hybrid animals will not mount a GVH reaction against embryos or newly born animals derived from either of the parental stocks. The failure of adult lymphoid cells to react in embryos of the same inbred strain has been interpreted as a failure of the grafted cells to respond to "self" antigens. Similarly, in the case of the F_1 hybrid, parental strain combination, it is assumed that the hybrid animal is tolerant of antigens of both parents, but that the parental cells can mount a reaction

Figure 3.5

The histological appearance of a lesion produced on the chorioallantoic membrane of a chicken embryo 6 days after inoculation with 10^7 immune guinea pig lymph node cells. Magnification x128, haematoxylin and eosin.



against antigens derived from the other parent which are present in the F_1 hybrid. In the light of these conclusions, the failure of lymphoid cells to initiate a GVH reaction in a xenogeneic recipient has been dismissed by assuming that the conditions of the alien environment precluded the survival or normal functioning of the grafted cells or tissues (Simonsen, 1962). It is apparent from the experimental findings presented above that this is not the case. Grafts of xenogeneic lymphoid tissue, such as sheep lymph node or pigeon spleen survive and proliferate in the chicken embryo but do not produce any changes associated with a GVH reaction. It is unlikely that the cellular proliferation, which occurs in lymphoid tissue from sheep and pigeons grafted in to chicken embryos, represents a GVH reaction, since dissociated lymphoid cells from the same donors do not produce any detectable signs of GVH reaction when inoculated on to the CAM of chicken embyros. The proliferative response seen in such grafts probably represents the regeneration of the grafted tissue. This response is different to that seen when adult allogeneic spleen is grafted to the CAM of the chicken embryo. In this latter situation large areas of necrosis that are surrounded by multinucleate giant cells appear in the graft. Lymphoid cells obtained from donors that were unrelated, or only distantly related to the chicken were not able to mount a GVH reaction against the chicken embryo except when the donor animals had been previously immunized against chicken tissues. Lymphoid cells obtained from immune geese, pigeons or guinea pigs, produced a single lesion on the CAM of recipient chicken embryos. This lesion was much larger than those produced by normal allogeneic cells, and had the appearance one might expect if several pocks had been initiated in the one area and had coalesced as they grew larger. Such lesions often contained several necrotic foci.

On the basis of these results it seems likely that some xenogeneic lymphoid cells are unable to mount a GVH reaction against the chicken embryo although the same cells can survive and proliferate in the embryonic environment and are potentially capable of mounting a reaction against the antigens of the embryo. One must conclude, therefore, that the xenogeneic lymphoid cells cannot be primarily stimulated by the antigens they encounter in the chicken embryo to produce changes associated with GVH reactions. Alternatively, xenogeneic lymphocytes may be stimulated by the antigens of the host embryo, but the conditions these cells encounter in the embryonic environment may prevent the grafted cells reaching a fully reactive state.

Summary

The evidence presented in this chapter shows that lymphoid cells obtained from normal animals that are unrelated to, or only distantly related to the chicken, fail to produce an increase in spleen weight or pocks on the chorioallantoic membrane when inoculated into chicken embryos. Xenogeneic lymphoid cells such as those of sheep or pigeon origin are able to survive in the environment afforded by the chicken embryo. Xenogeneic lymphoid cells that are unable to initiate a primary GVH reaction against the chicken embryo will produce a reaction if the lymphoid cells are obtained from donors after their immunization with chicken tissues. It was concluded from these results that some xenogeneic lymphoid cells fail to initiate a primary GVH reaction in the chicken embryo either because,

(a) these cells cannot be stimulated by the antigens they encounter in the embryo, or,

(b) such cells, although stimulated by the foreign antigens they contact, are unable to develop into fully active cells in the embryonic environment.

CHAPTER 4

THE ADOPTIVE TRANSFER OF TRANSPLANTATION IMMUNITY TO THE CHICKEN EMBRYO

Chapter 4 - The Adoptive Transfer of Transplantation Immunity to the Chicken Embryo

Introduction

The results presented in the previous chapter indicate that the failure of some xenogeneic lymphoid cells to produce a GVH reaction, when introduced into the chicken embryo, might result either from a failure of the grafted cells to be stimulated by the antigens they contact, or may be due to the environment not being suitable for the normal development of such cells once they have been stimulated. This possible complication, by the environment in which the potentially active lymphoid cells are confined, will be eliminated if the interaction of lymphoid cells with alien tissues is examined in an environment that is syngeneic with respect to the lymphoid cells.

The use of inbred chickens and embryos would provide a suitable experimental system in which such investigations could be conducted. Both allogeneic and xenogeneic tissues can be grafted onto the CAM of the chicken embryo and since the chicken embryo is immunologically incompetent, these transplants survive and develop normally. The following experiments describe the behaviour of alien tissue transplants in embryos, of the highly inbred AA strain, that have been inoculated via the CAM with blood leucocytes obtained from adult birds of the same inbred strain. Embryos that have been inoculated with syngeneic adult leucocytes will subsequently be referred to as "treated embryos".

Experimental

Embryonic bone was selected for the following transplantation studies. Femurs and tibiae from 11 day old chicken embryos, 14 day old duck embryos and pigeon embryos (at about the same stage of incubation as judged by the development of

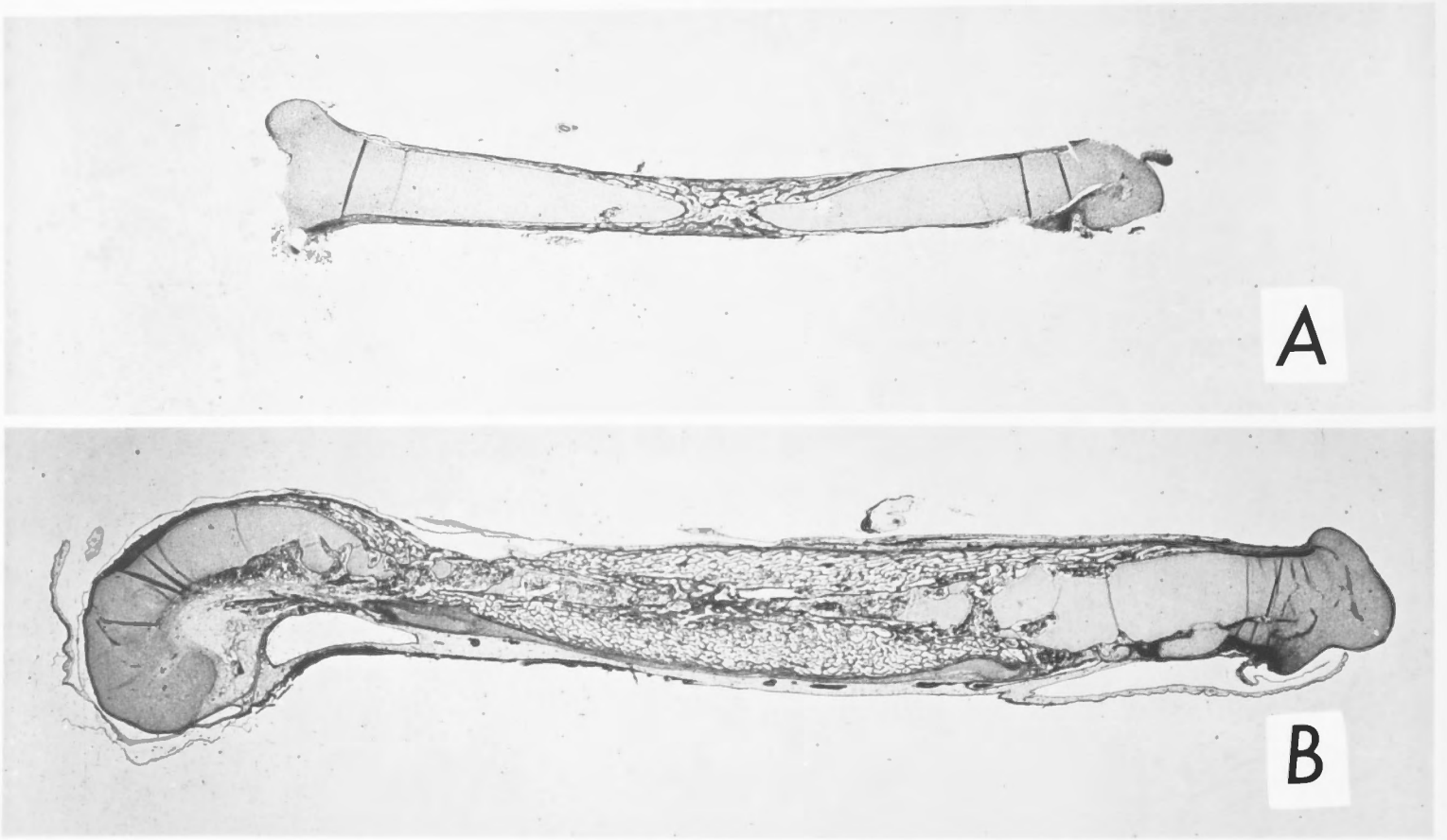


Figure 4.1

Comparison between a tibia bone graft from an 11 day old chick embryo before grafting A, and a similar bone after it had been grafted to the CAM of an 11 day old embryo 6 days previously. Magnification x 10. Haematoxylin and eosin.

the CAM) were denuded of their covering muscles and connective tissue. The bones after being washed in EBM were placed onto the CAM of 11 day old chicken embryos that had been prepared as described in Chapter 2. Bone grafts from both xenogeneic and allogeneic donors were readily incorporated into the CAM of the chicken embryo, where chicken bones increased in length by 50 per cent during the next 6 days incubation (Fig. 4.1 and 4.2).

Rejection of Allografts of Embryonic Bone from the Chicken Embryo by Adult Blood Leucocytes

The aim in these experiments was to confer on the recipient embryo the capacity to reject a bone allograft by transferring to it blood leucocytes from a syngeneic strain of adult chickens. In this way, the transferred leucocytes would not mount a GVH reaction against the recipient embryo themselves but would provide the host with adult lymphoid cells capable of rejecting tissue allografts. Initially, 10 million adult blood leucocytes were inoculated onto the CAM of 12 day old embryos 24 hours after the transplantation of foreign bones. In later experiments, it was found the same results could be obtained if the leucocytes were inoculated onto the CAM at the same time as the bones were transplanted. The grafts were removed and examined histologically, 6 days after the addition of the adult blood cells. Figure 4.3 shows the histological appearance of bone grafts in three different experimental situations. In the first group allogeneic bone was grafted into normal embryos that had not been treated with adult leucocytes but instead were inoculated with 0.1 ml of EBM. In the second, the grafted bone was allogeneic with respect to the embryo and the donated adult cells, and the third group consisted of grafts that were syngeneic (AA embryo bone) with respect to the other elements of the system. It is quite apparent that bones from embryos, treated with EBM only or with leucocytes from donors syngeneic

Figure 4.2

Histological appearance of a chicken embryo bone, 6 days after grafting to the CAM of a 11 day old chicken embryo.

Magnification x640 haematoxylin and eosin

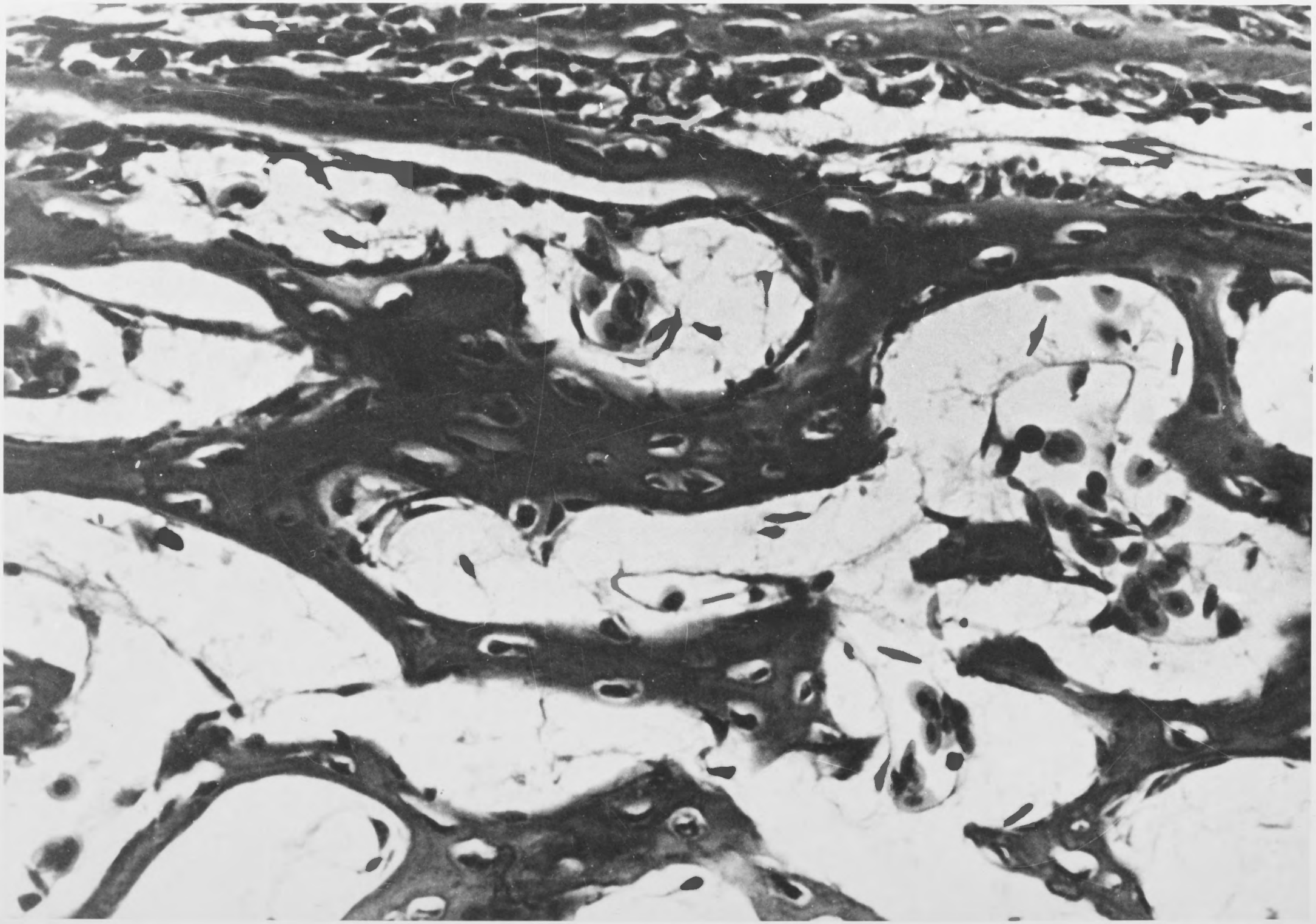


Figure 4.3

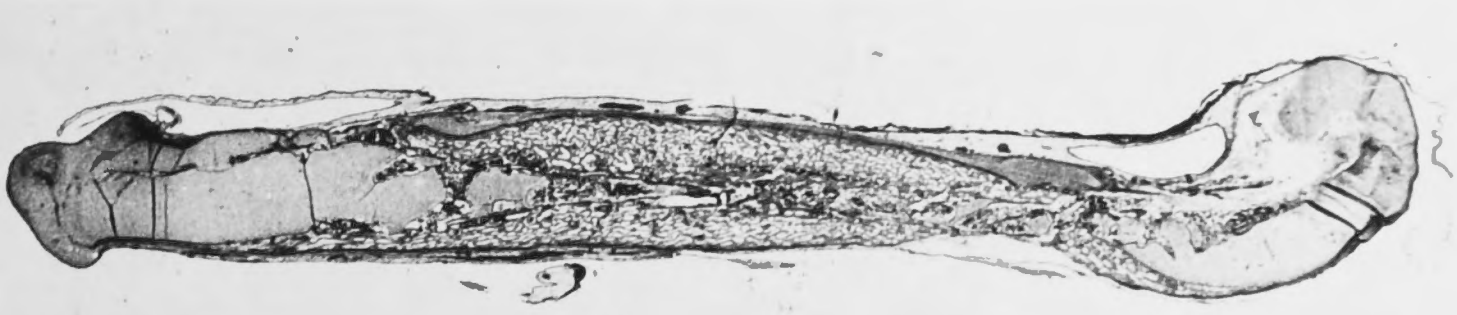
Embryonic bone 6 days after grafting on to the CAM of normal or "treated" embryos.

A Allogeneic bone grafted to normal embryos.

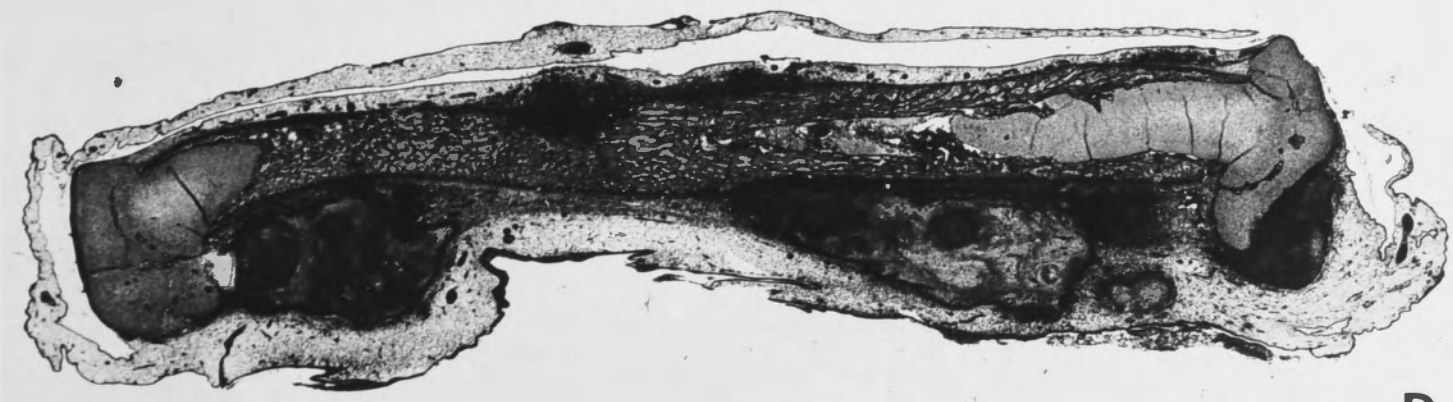
B and C Allogeneic bone grafted to "treated" embryos.

D Syngeneic bone grafted to "treated" embryos.

Magnification x10 haematoxylin and eosin



A



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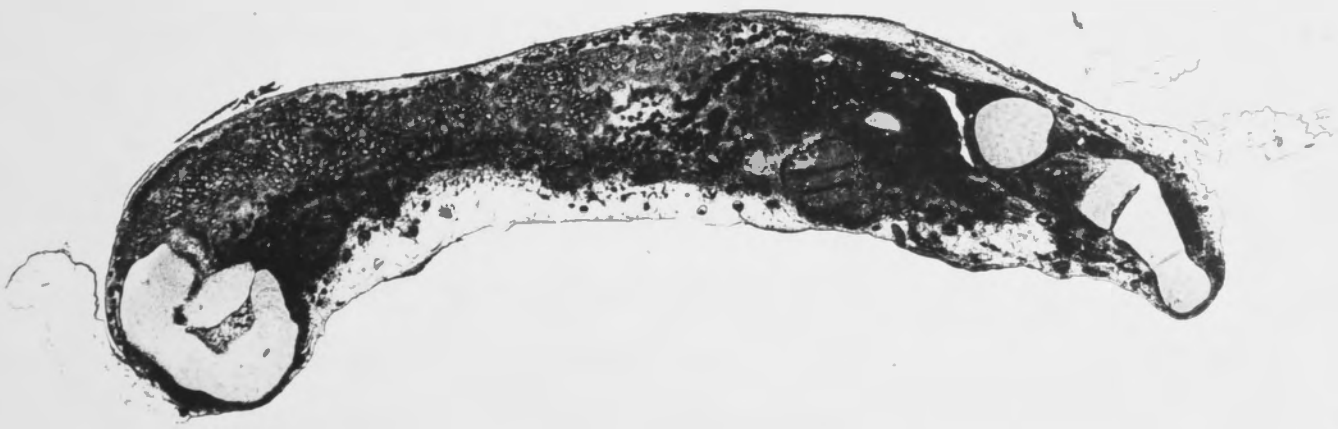

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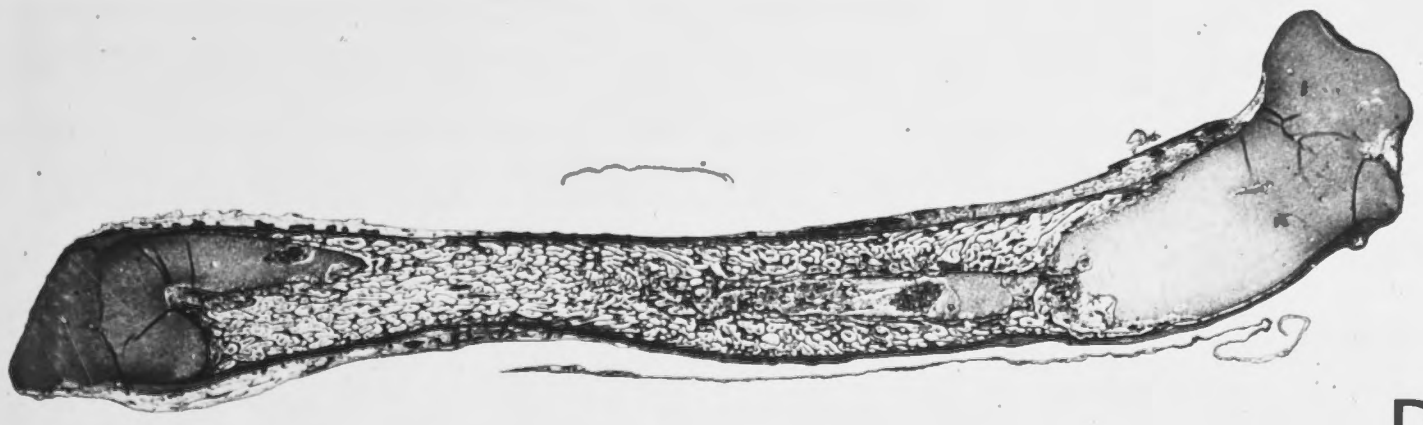


D

with the bone donors, show no signs of rejection. In marked contrast to this, bone grafts from donors allogeneic to donors of the adult blood leucocytes show a heavy nodular infiltration of mononuclear cells with a few granulocytes around the peripheries of the grafts. Often the centre of these areas of nodular infiltration show necrosis in a way similar to that seen in spleens from embryos undergoing a GVH reaction. In addition to a peripheral nodular infiltration of the bone allografts, there is a marked increase in the cellularity of the cortical bone tissue and the bone marrow in the medullary cavity (Fig. 4.4 and 4.5). In infiltrated allogeneic bones, dissolution of the bony tissue itself may occur following necrosis and, in a very vigorous reaction, the bone may be completely destroyed, leaving only the terminal cartilages (Fig. 4.3).

These observations show that an inoculation of adult blood leucocytes can confer on the recipient embryo the capacity to reject allografts of embryonic bone.

Survival of Xenografts in "Treated Embryos"

The transfer of 1 million adult blood leucocytes to syngeneic embryos, consistently results in the rejection of allogeneic bone grafts that have been transplanted to these embryos. However, embryos that have been treated in this manner do not invariably reject grafts of xenogeneic bone. When embryonic duck bone is transplanted into "treated embryos" the majority of the bone grafts are destroyed in a similar manner to grafts of chicken bone. However, about one third of the grafted duck bones show no histological evidence of damage (Figs. 4.6 and 4.7). The situation is even more striking when the donor of the grafts is a pigeon embryo. The great majority of pigeon bone grafts show absolutely no histological evidence of being rejected. In a small proportion of grafts (about 10 per cent) there was a very mild

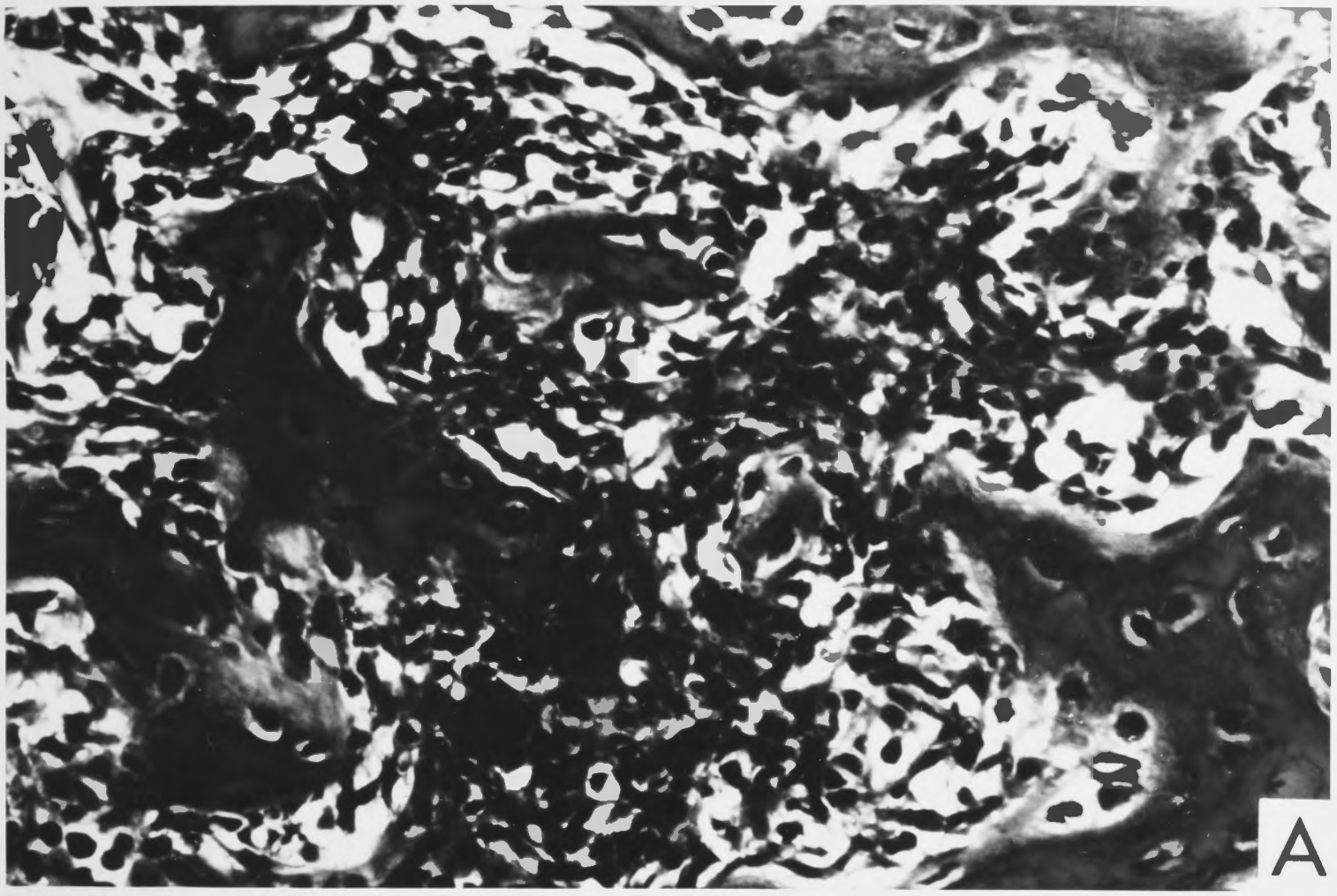
Figure 4.4

Destruction of allogeneic bone 6 days after grafting to the CAM of an 11 day old chicken embryo that had received syngeneic adult chicken cells.

A Proliferative response

B Frank necrosis

Magnification x320 haematoxylin and eosin


A

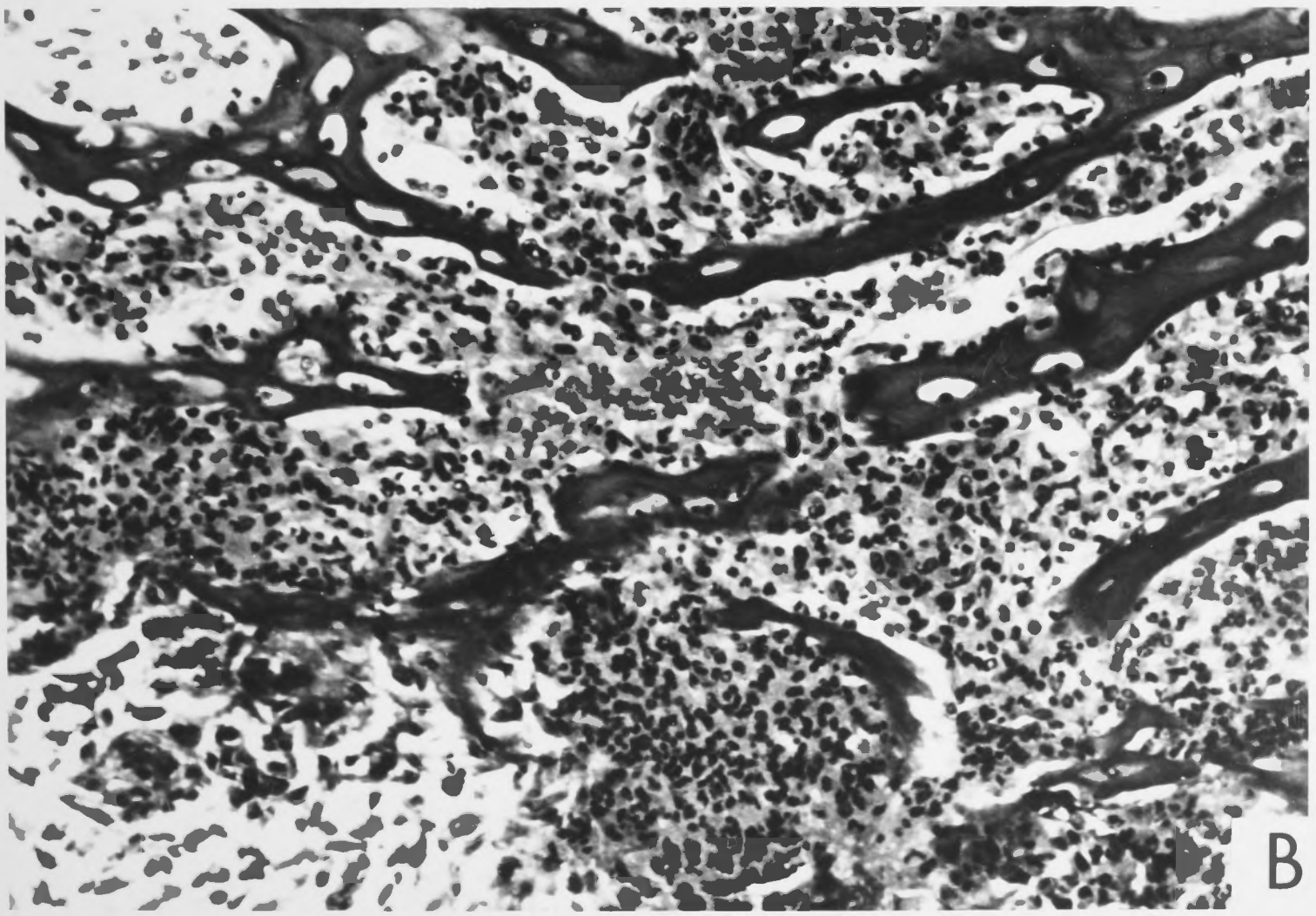
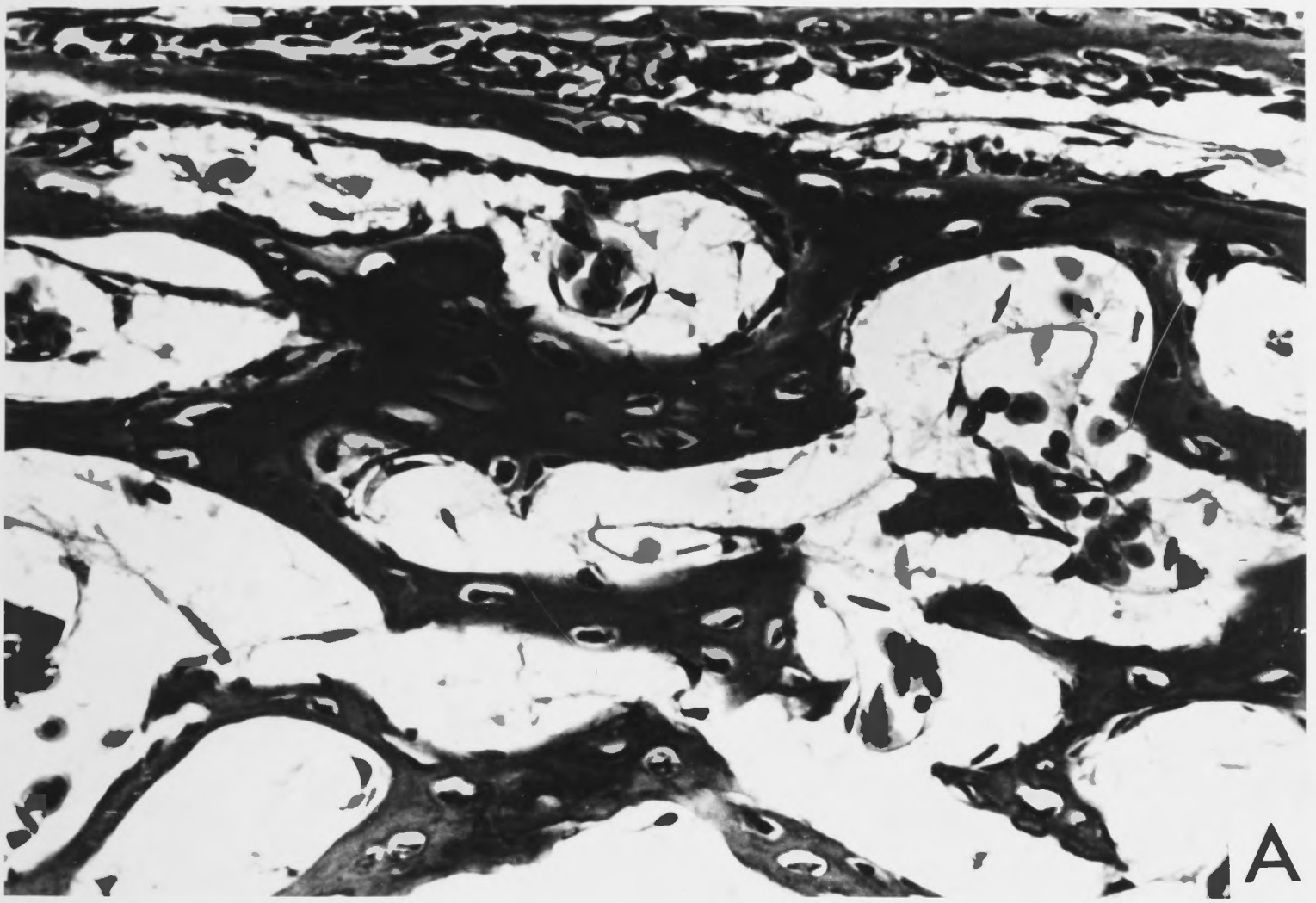

B

Figure 4.5

Histological appearances of allogeneic bone 6 days after grafting to the CAM of normal embryos (A) or syngeneic bone grafted to treated embryos (B).

Magnification x320 haematoxylin and eosin


A

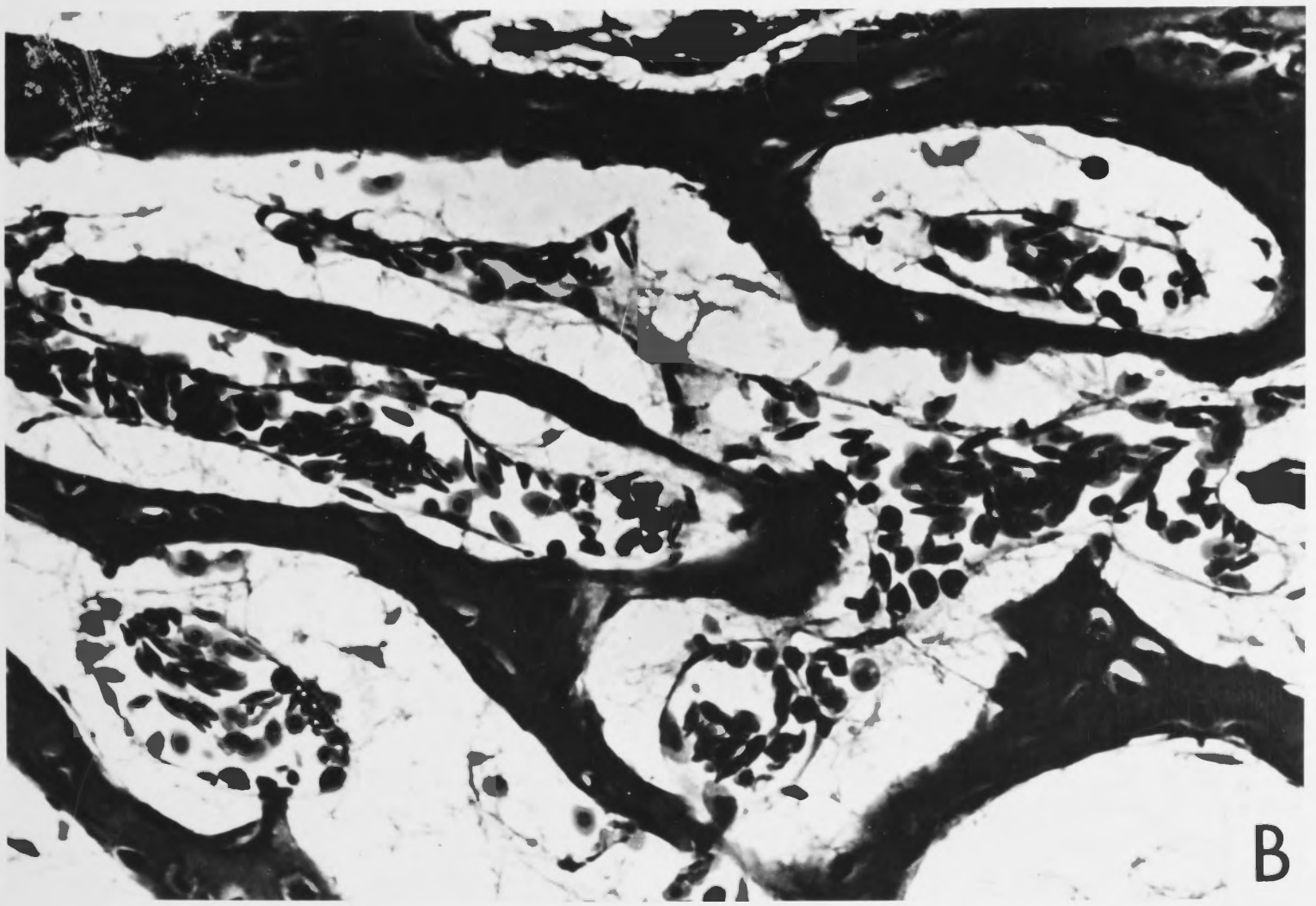

B

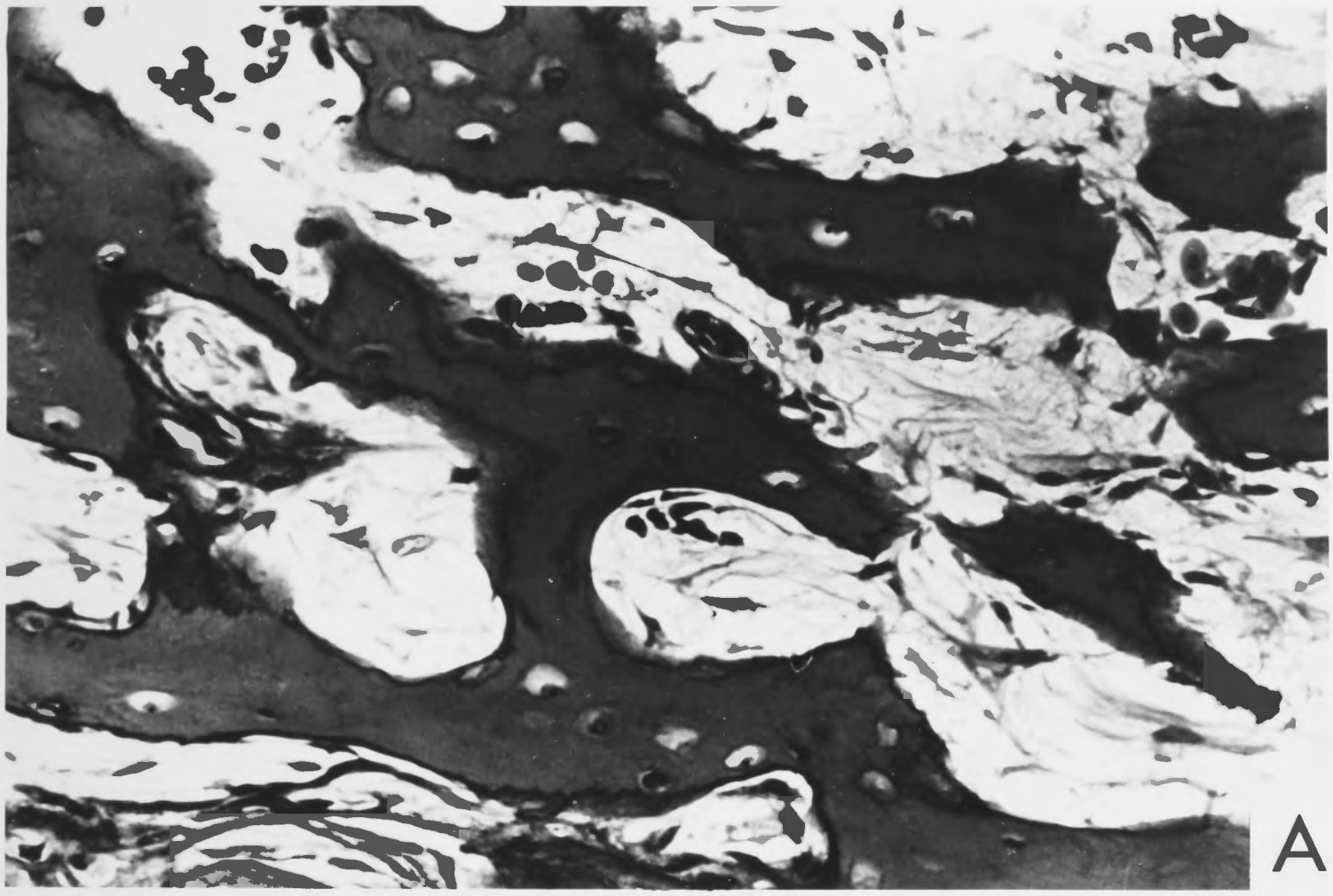
Figure 4.6

Comparison of the histological appearance of duck bones 6 days after grafting on to the CAM of 11 day old "treated" embryos.

A Graft appears unaffected

B Graft shows evidence of necrosis

Magnification x320 haematoxylin and eosin


A

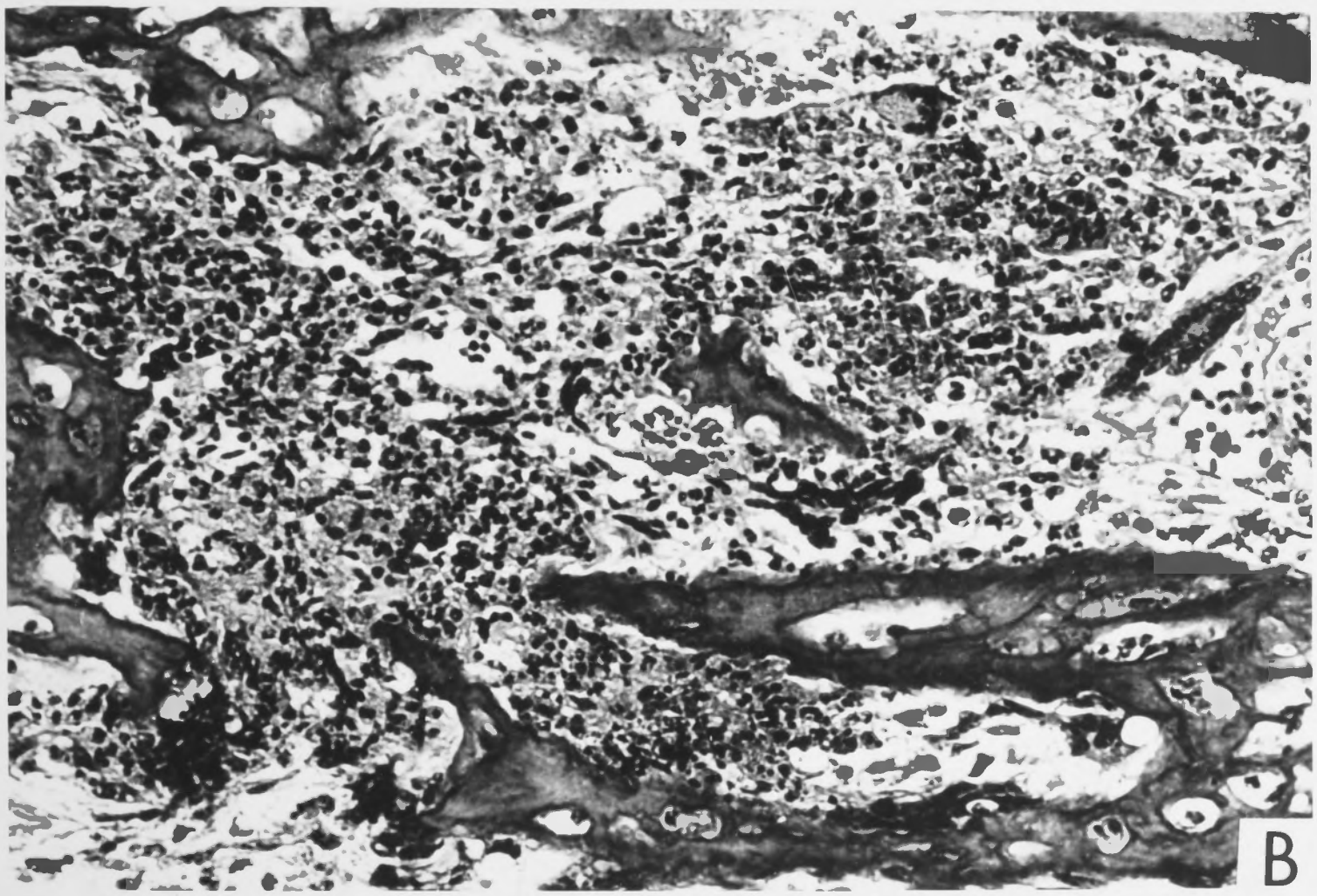
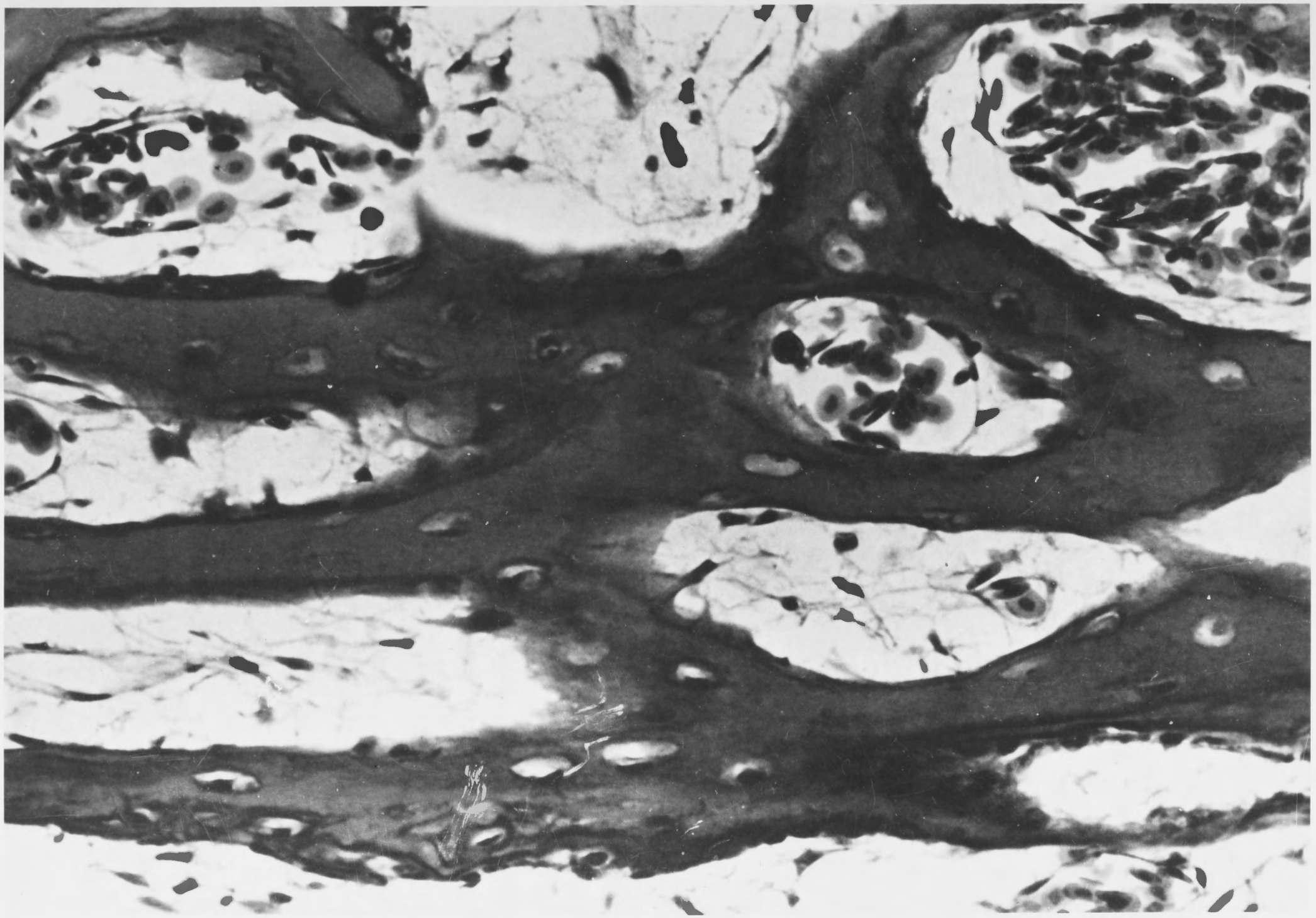

B

Figure 4.7

Normal duck bone graft 6 days after grafting to the CAM of an 11 day old chicken embryo.

Magnification x640 haematoxylin and eosin



cellular infiltration around the periphery of the bone but no cellular invasion of the cortex or medulla as seen in chicken bones being rejected by allogeneic cells (Fig. 4.8). The pigeon bone used in these experiments is rejected if the donated adult blood leucocytes are taken from a bird that has been immunized previously with pigeon spleen cells. In this experiment the blood leucocytes were obtained from the donor, one week after the completion of the immunization program, as described in Chapter 2. One million of these immune cells were inoculated on to the chorioallantoic membranes of 8 syngeneic embryos, bearing pigeon bone grafts. The grafts were examined 6 days later and all showed extensive signs of destruction and nodular infiltration. Figure 4.9 compares the grafts of pigeon bone removed from chicken embryos that had been treated with blood leucocytes obtained from an adult chicken before and after its immunization with pigeon spleen cells.

The Activity of Xenogeneic Lymphoid Cells in the Chicken Embryo

The evidence presented in the previous chapter suggested that the failure of xenogeneic lymphoid cells, such as pigeon cells, to produce a GVH reaction in recipient chicken embryos, was due to the grafted cells not being primarily stimulated by the antigens with which they came in contact. It has been shown that adult syngeneic chicken lymphoid cells can be primarily stimulated by grafted allogeneic tissues. To determine whether the chicken embryo provided a suitable environment for the primary stimulation of grafted xenogeneic lymphoid cells, embryonic pigeon bones were grafted onto the chorioallantoic membranes of 11 day old chicken embryos. One day later, 10 million adult pigeon spleen cells were inoculated onto the membranes bearing pigeon chicken embryonic bone grafts. In every case, the pigeon bone grafts were rejected and the chicken bones remained unaffected. Figure 4.10 compares the

Figure 4.8

Histological appearance of embryonic pigeon bones following grafting to the CAM of 11 day old chicken embryo 6 days previously.

Upper Bone grafted to normal chicken embryo

Lower Bone grafted to a "treated" embryo

Magnification x320 haematoxylin and eosin

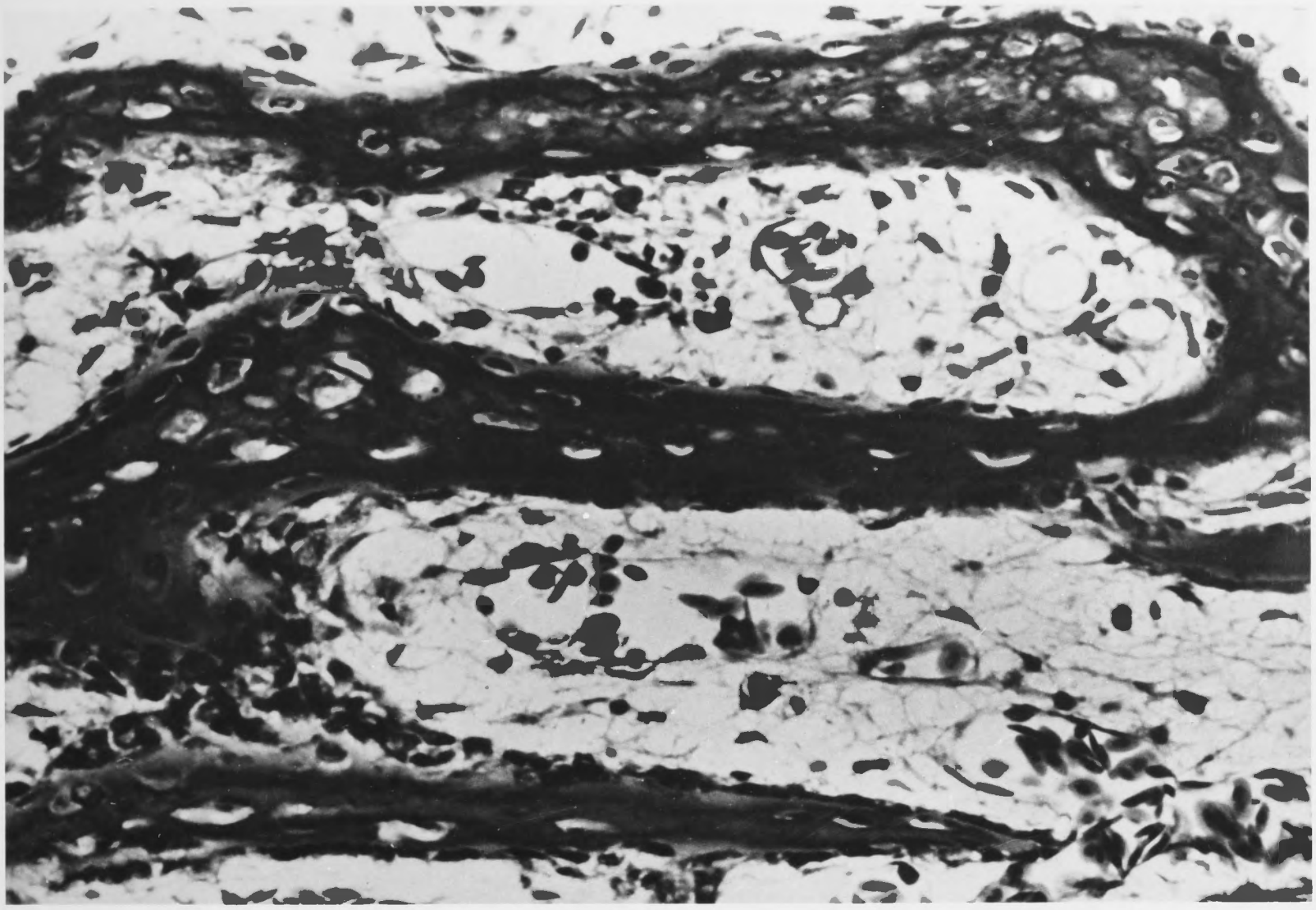


Figure 4.9

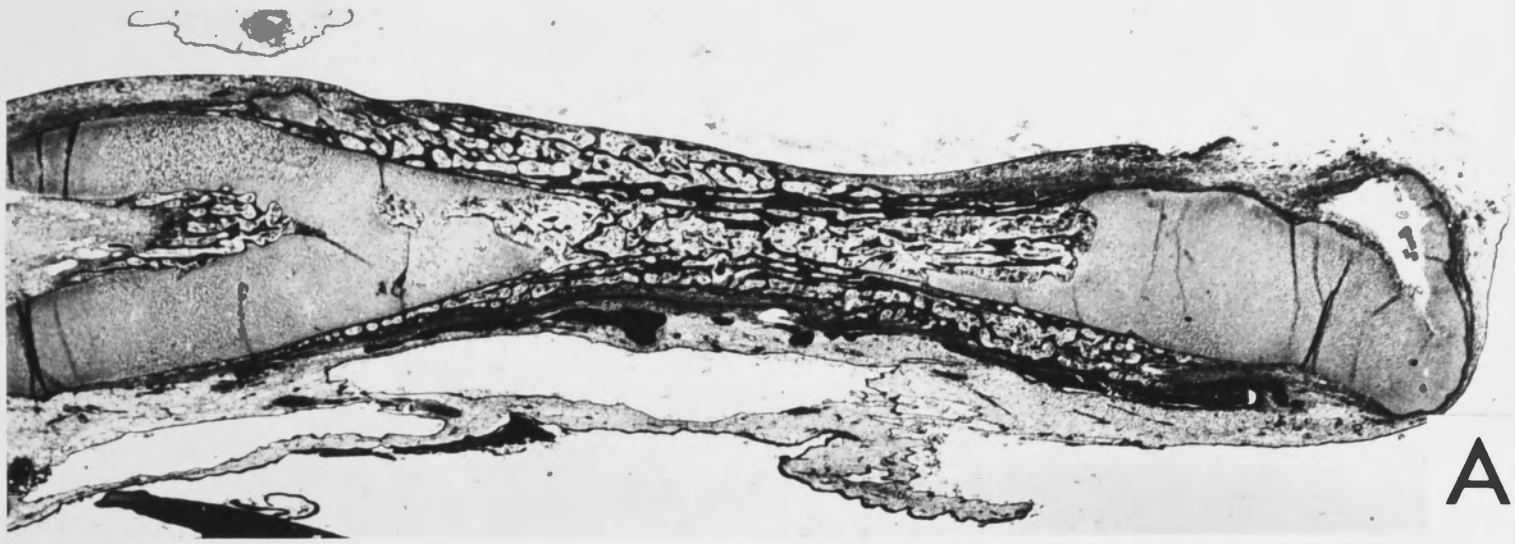
A comparison of the histological appearance of pigeon bone grafts from the CAM of chickens that have been treated with leucocytes from an adult chicken before and after immunization against the pigeon.

A Pigeon bone graft after 6 days, no treatment.

B Pigeon bone graft 6 days after treatment with non-immune cells.

C Pigeon bone graft 6 days after treatment with immune cells.

Magnification x13 haematoxylin and eosin



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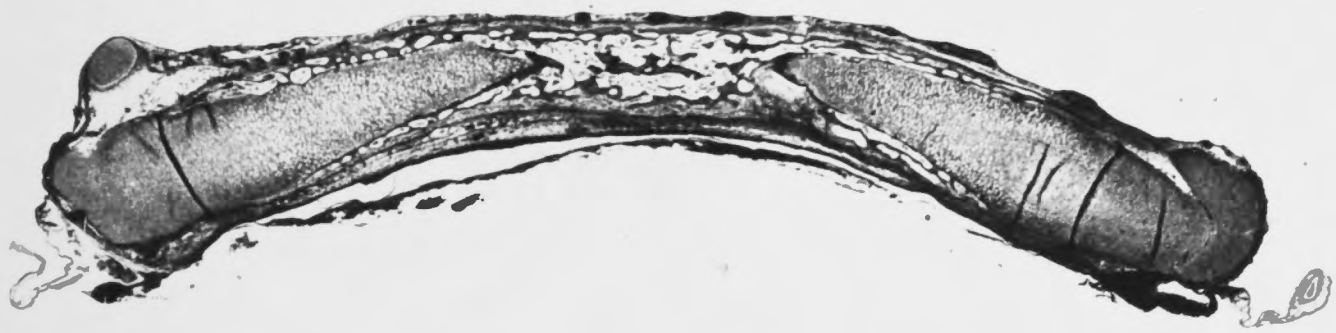

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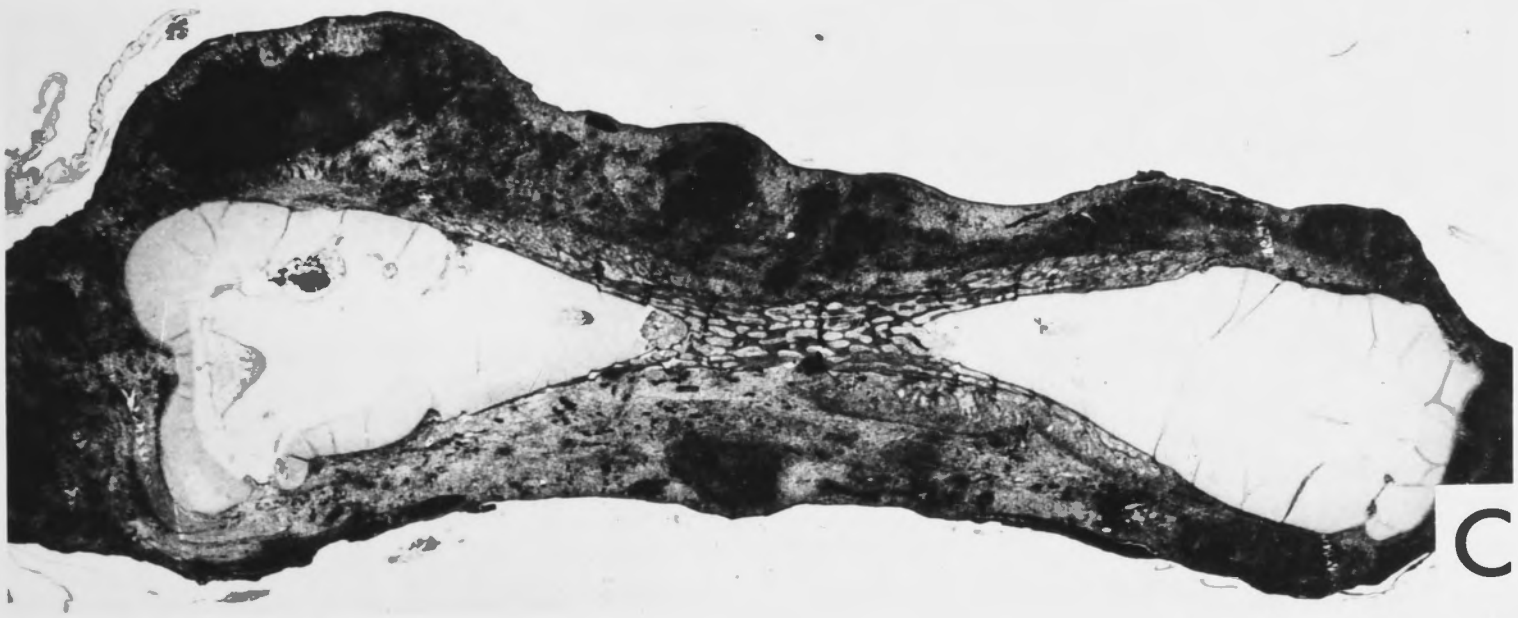


C

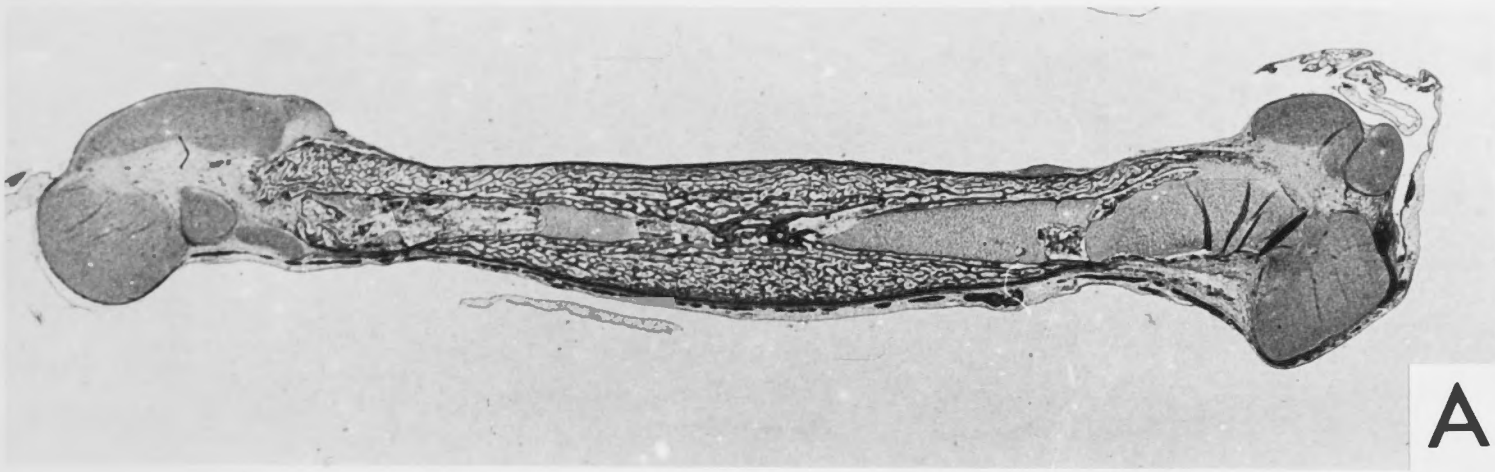
Figure 4.10

Comparison of the histological appearance of chicken and pigeon bone grafts removed from the CAM of 17 day old chicken embryos that had been treated with adult pigeon spleen cells 6 days previously.

A Chicken embryo bone graft

B and C Pigeon embryo bone grafts

Magnification x13 haematoxylin and eosin



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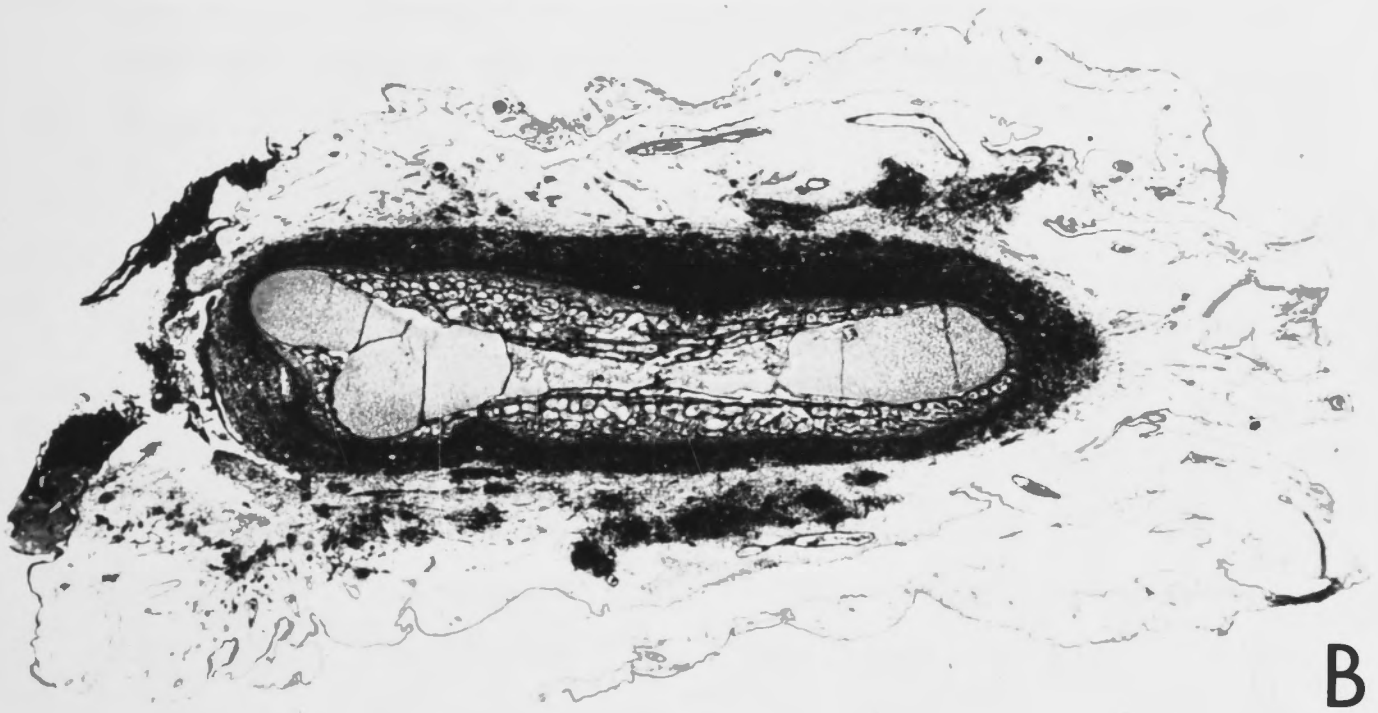


B

C

histological appearance of the chicken and pigeon bone grafts following treatment with pigeon lymphoid cells 6 days previously. It can be seen that one of the pigeon bones has been completely destroyed; the small amount of cartilage evident at each end of the rejected graft serves to locate the original position of the transplanted tissue.

These findings indicate that the environment of the chicken embryo is suitable for the stimulation of grafted xenogeneic lymphoid cells to react against foreign tissues, but primary stimulation of transplanted xenogeneic lymphoid cells is only elicited by tissues from donors allogeneic with respect to the grafted cells.

The observations presented so far in this chapter show that xenogeneic lymphoid cells can be primarily stimulated to react to foreign tissues in the chicken embryo, but with the important connotation that lymphoid cells appear capable of reacting only against allogeneic tissues. The question arising at this point concerns the component of the graft that stimulates allogeneic but not xenogeneic lymphoid cells. The findings of a number of workers (Ramseier and Streilein, 1965; Ramseier and Billingham, 1966; Elkins, 1966) have indicated that reticular tissue, as defined by Marshall (1956), present in a graft may be important in the stimulation of allogeneic lymphoid cells. Support for this suggestion was obtained from studies on the survival of allogeneic embryonic spleen, liver and heart grafts carried on the CAM of "treated embryos".

The Survival of Allogeneic Spleen, Liver and Heart Grafts on the Chorioallantoic Membrane

Spleen, liver and heart muscle obtained from allogeneic chicken embryos can be readily grafted onto the CAM of the 11 day old chicken embryos. The embryos bearing the grafts were treated with one million syngeneic adult blood leucocytes and the grafts examined 6 days later. It was found that the

splenic, and to a lesser extent, the hapatic transplants were enlarged and nodular in outline, indicating that they were in the process of being rejected. The histological examination revealed that the spleens contained tumour-like masses of reticulum cells and had prominent areas of necrosis. The liver grafts were heavily infiltrated with mononuclear cells, to such an extent in most instances, that little liver parenchyma was visible (Fig. 4.11).

The situation was markedly different in the case of cardiac muscle transplants. If the cornonary vessels of the embryonic heart were carefully perfused with Hank's balanced salt solution to remove as many residual blood cells as possible before grafting, few signs of damage were detectable (Fig. 4.11). If, however, instead of removing the blood leucocytes from the coronary vessels, more had been added by perfusion with embryonic spleen cells, then the heart graft was violently attacked when grafted into "treated embryos".

Figure 4.12 compares the histological appearances of embryonic hearts perfused with embryonic spleen cells before grafting onto the CAM of normal and "treated" chicken embryos. It can be seen that most of the heart muscle has been replaced by mononuclear cells in the graft placed in the "treated embryos".

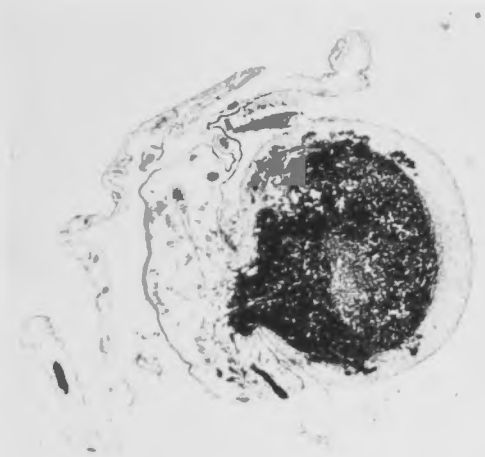
These experiments illustrate that transplants with a relatively high content of reticular tissue were damaged more extensively than those that contained little or no reticular elements. Further, if the amount of reticular tissue was experimentally increased in a tissue which naturally contains only a small amount, this tissue was vigorously attacked. In view of these findings, it would appear likely that the amount of reticular tissue in the graft determines to a large extent whether it will be attacked when transplanted into treated embryos.

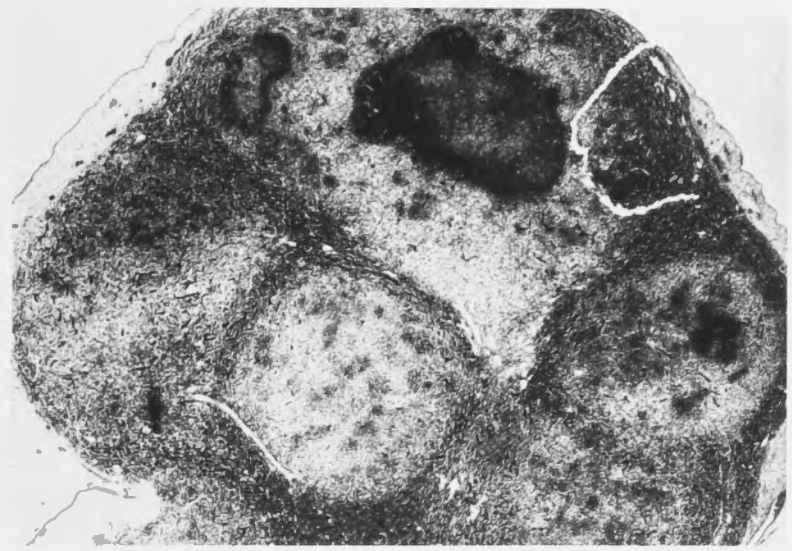
Figure 4.11

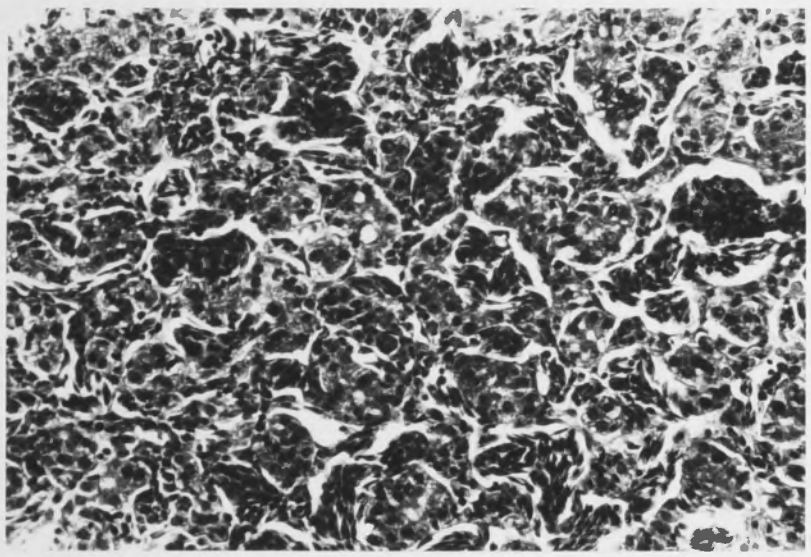
Histological appearances of allogeneic embryonic tissues 6 days after grafting to normal and "treated" 11 day old chicken embryos.

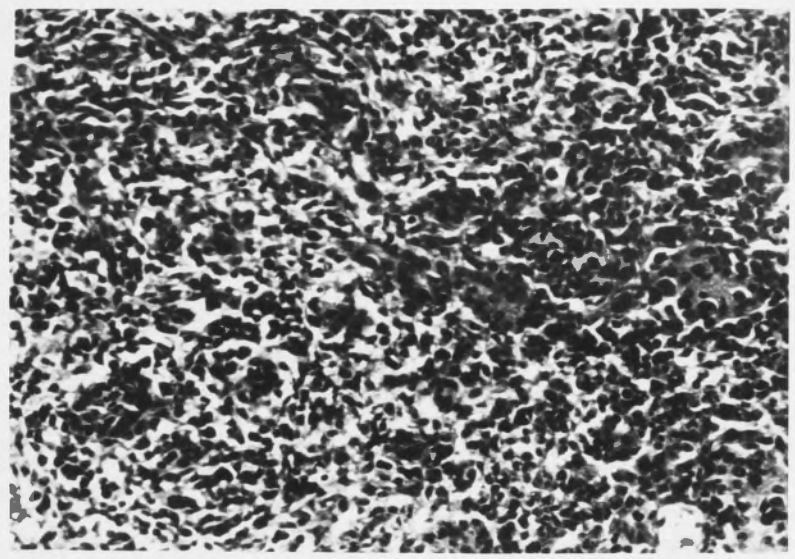
Upper left	Spleen into normal embryo
Middle left	Liver into normal embryo
Lower left	Heart into normal embryo
Upper right	Spleen into "treated" embryo
Middle right	Liver into "treated" embryo
Lower right	Heart into "treated" embryo

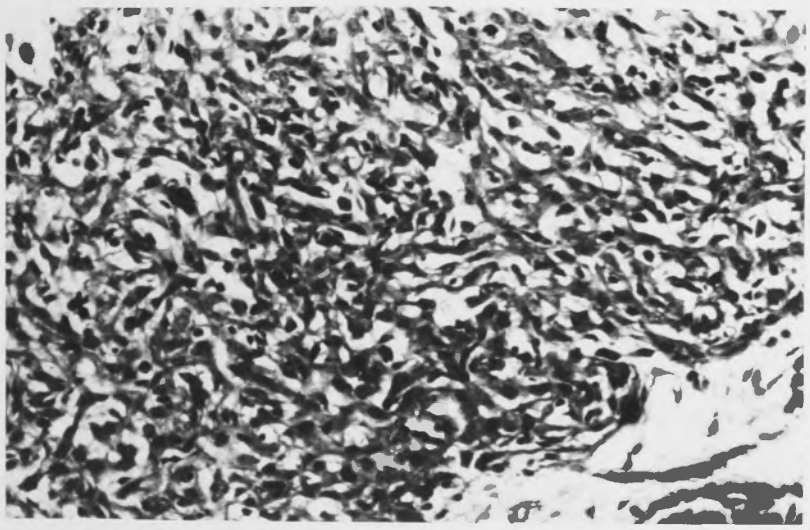
Magnification x220 haematoxylin and eosin











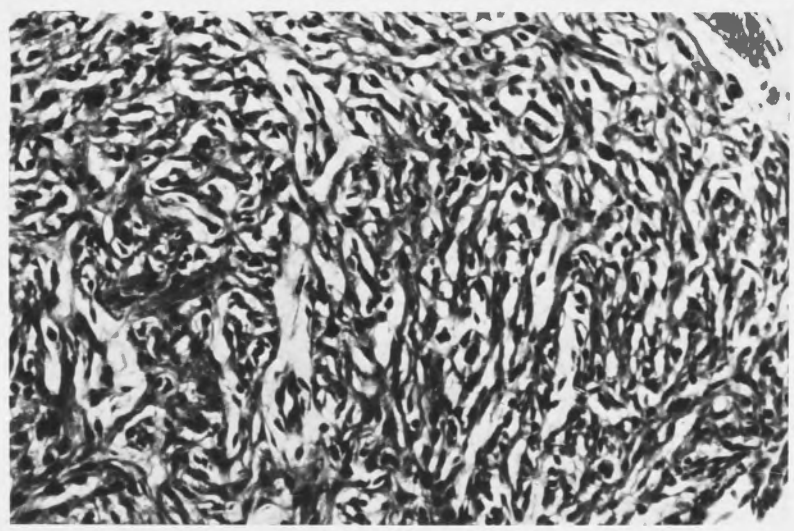


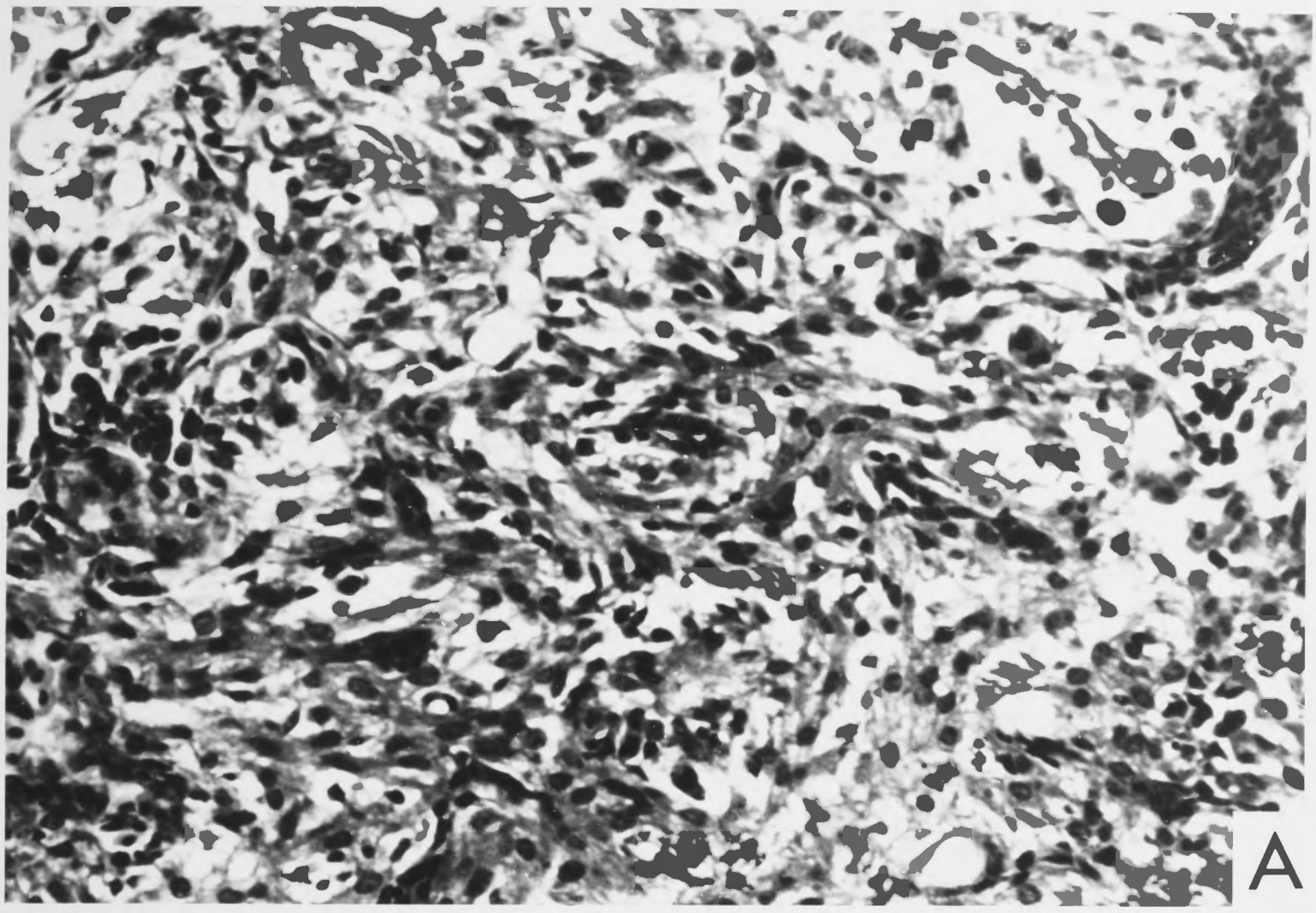
Figure 4.12

Comparison of embryonic chicken heart grafts 6 days after the addition of allogeneic cells.

A Portraits the histological appearance of a graft from which blood cells have been washed before grafting.

B Shows the appearance of a heart graft perfused with syngeneic spleen cells before grafting.

Magnification x320 haematoxylin and eosin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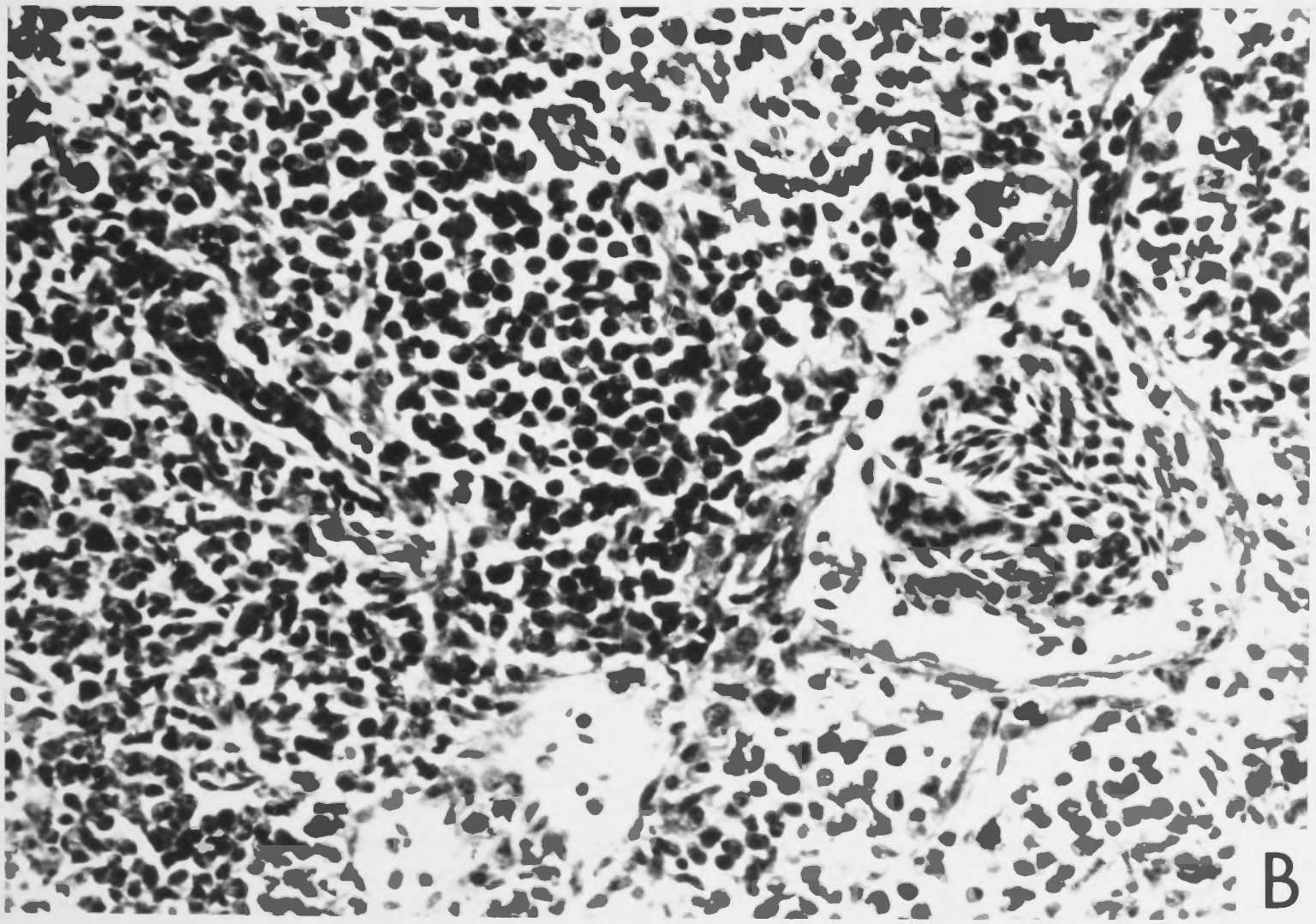

B

The Effect of Prior γ-ray Irradiation on the Survival of Allogeneic Grafts

If the content of reticular tissue determines the amount of damage allogeneic leucocytes will cause to a graft, it could be predicted that a reduction in the amount of this tissue would protect the transplant from destruction. It is possible to reduce the lymphoid and myeloid cell content of tissues by subjecting them to γ-irradiation from a cobalt-60 source.

Embryonic spleen and bone grafts were therefore subjected to increasing amount of γ-irradiation. It was found that doses of 2000 rads were sufficient to so damage splenic grafts that they were no longer viable after grafting on to the chorioallantoic membranes of recipient embryos. For this reason doses of irradiation given to spleen grafts were restricted to 1000 rads. Figure 4.13 compares the histological appearance of normal embryonic chicken spleen grafts with ones that had received 1000 rads of γ-irradiation prior to their grafting to the CAM of "treated embryos" 6 days previously.

It will be seen that the control group of normal unirradiated spleen grafts show the marked enlargement and necrosis described above. The irradiated spleens survive well in normal embryos and show little transplantation damage when transferred to the "treated embryos". In this experiment the irradiated spleens were grafted on to the CAM of allogeneic embryos 24 hours before the adult blood leucocytes were introduced into the system.

Embryonic chicken bone was found to be more resistant to damage by irradiation and it was shown that bones which had received up to 2000 rads of irradiation were still able to increase in length and had a normal histological appearance when grafted to the CAM of a normal chicken embryo.

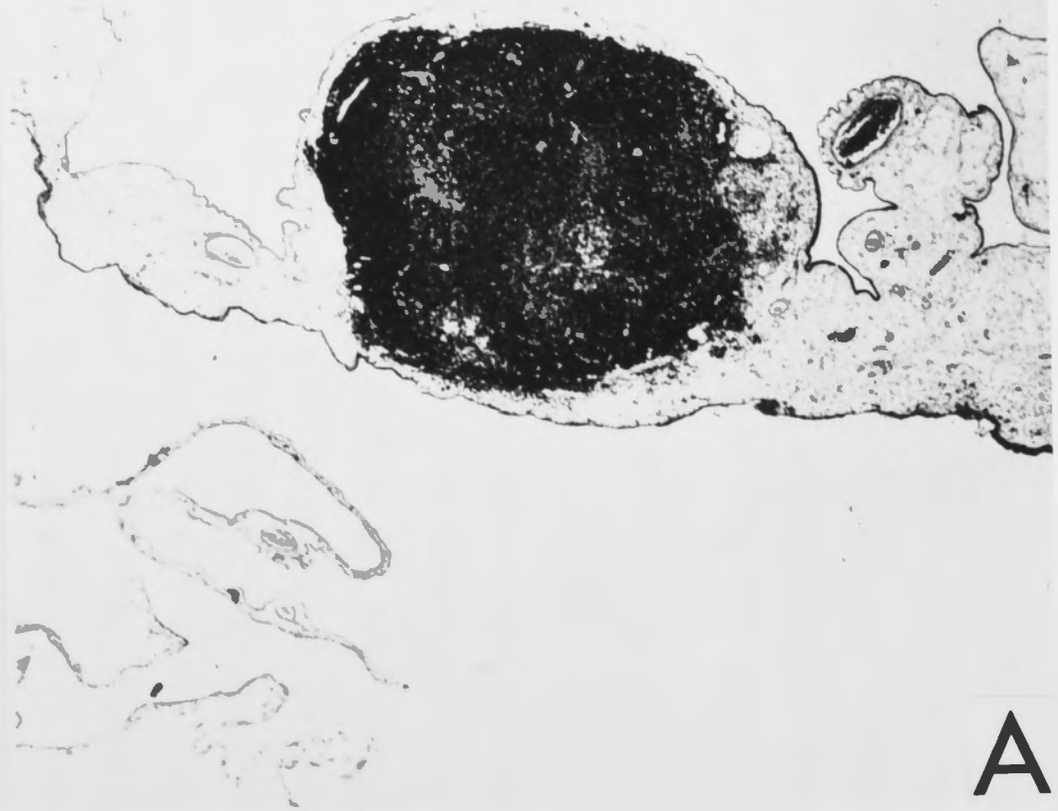
Figure 4.13

A comparison of the histological appearance of γ-irradiated (1000 rads) embryonic spleen with a normal embryonic spleen 6 days after grafting to 11 day old "treated" chicken embryos.

A Irradiated spleen

B Normal spleen

Magnification x50 haematoxylin and eosin



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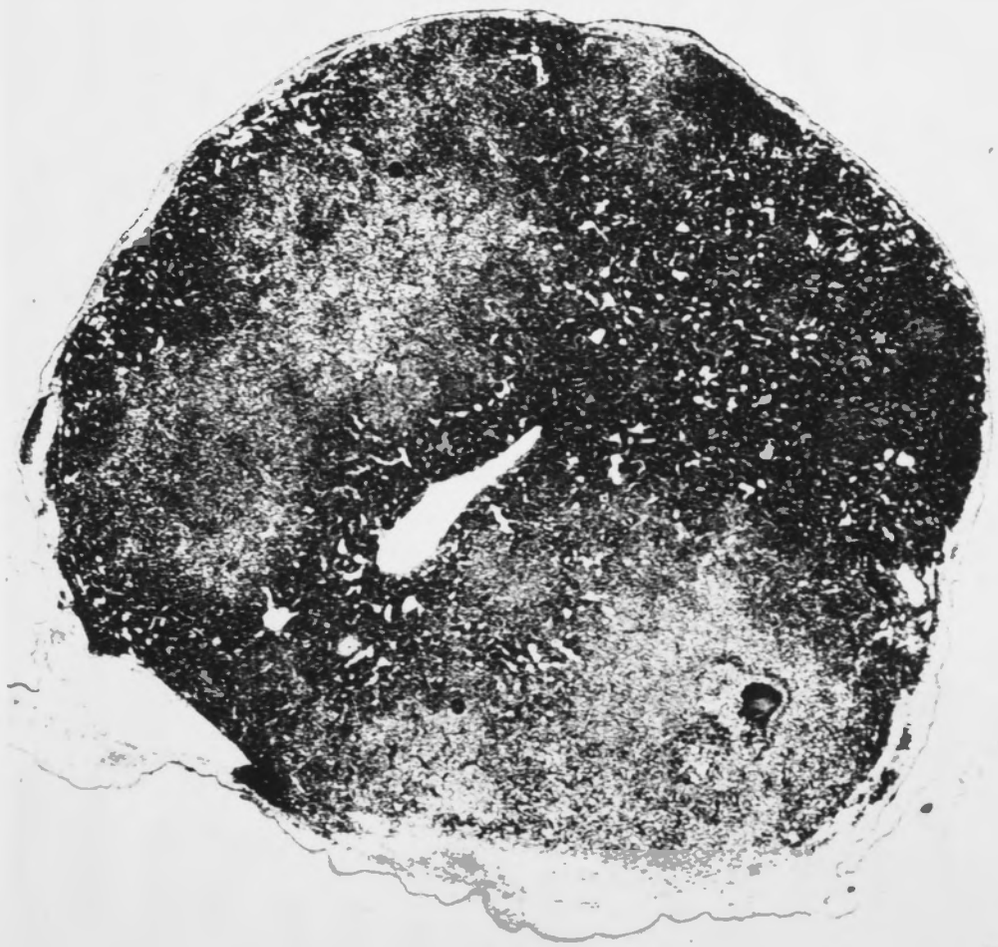


B

Figure 4.14 compares the histological appearance of an unirradiated bone graft with that of an irradiated one that had been transferred to "treated embryos". The marked increase in the cellularity of the unirradiated bone contrasts with the normal histological appearance of the bone which had received 2000 rads of irradiation before grafting. These results indicate that irradiation of bones before grafting affords considerable protection against transplantation damage by the "treated embryo".

Discussion

It could be argued that the lack of GVH activity exhibited by some xenogeneic cells results not from any deficiency in the recognition of foreign antigens by these cells, but rather that the embryonic environment is inadequate for the transformation of the stimulated cells to fully functional effector agents. This proposition is not supported by the experimental findings. Cells, such as those obtained from pigeon spleen, are capable of recognizing and destroying grafts of embryonic pigeon bone carried on the CAM of chicken embryos, but grafts of chicken bone or the embryo itself are in no way damaged by these cells. Conversely, the transfer of adult blood leucocytes to syngeneic chicken embryos renders them competent to reject grafts of allogeneic bone but does not facilitate the rejection of some xenografts by such embryos. One must conclude, therefore, that lymphoid cells fail to initiate a GVH reaction in a xenogeneic environment because they cannot be stimulated by the antigens they contact in this situation. Similarly, the failure of syngeneic lymphoid cells to bring about the destruction of xenografts carried by the chicken embryo can only mean that these cells are not stimulated by xenogeneic antigens even when the cells are in a syngeneic environment.

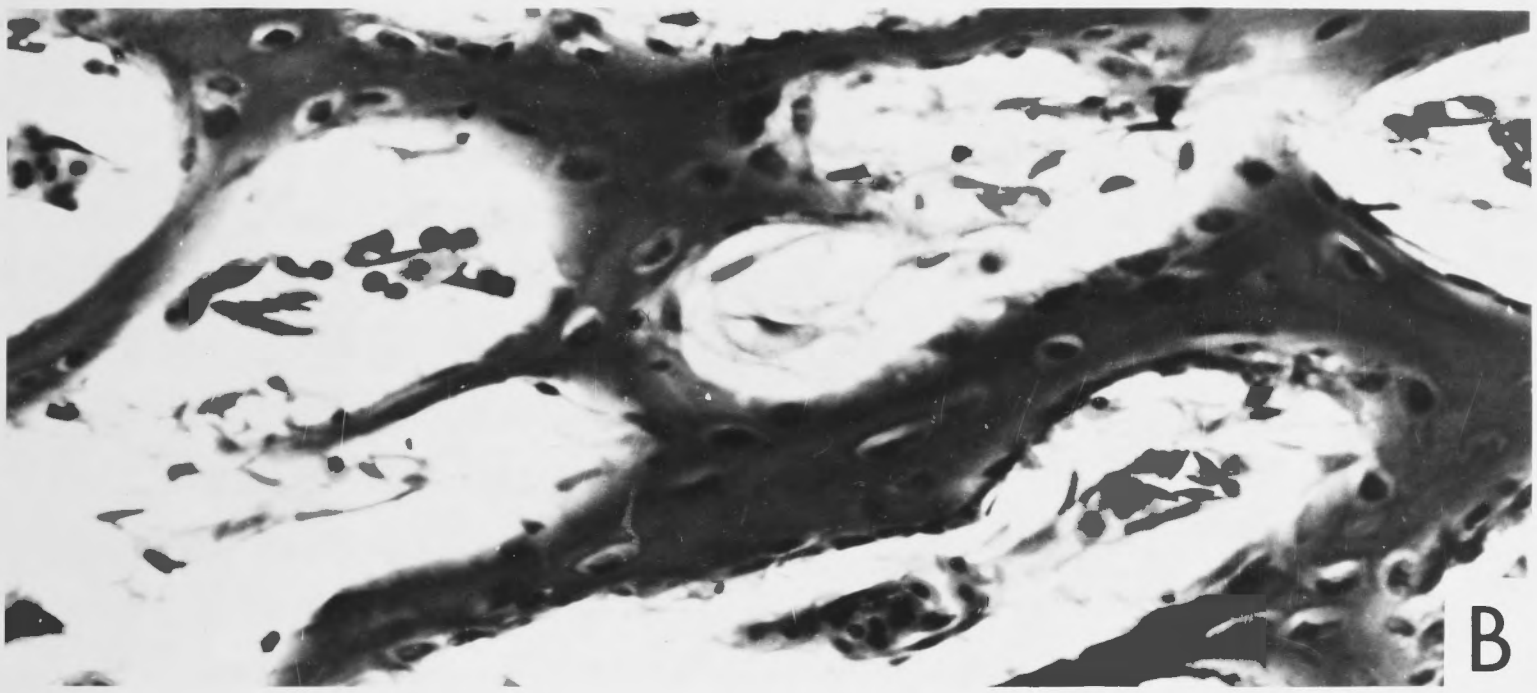
In the light of these observations it would seem possible

Figure 4.14

Comparison of the histological appearances of an unirradiated bone graft 6 days after treatment with allogeneic blood leucocytes (Photograph A) and a bone graft that had received 2000 rads of γ-irradiation before grafting and then treated with allogeneic blood leucocytes. Photographs B and C are the histological appearance of the latter 6 days later.

Magnification x320 haematoxylin and eosin


A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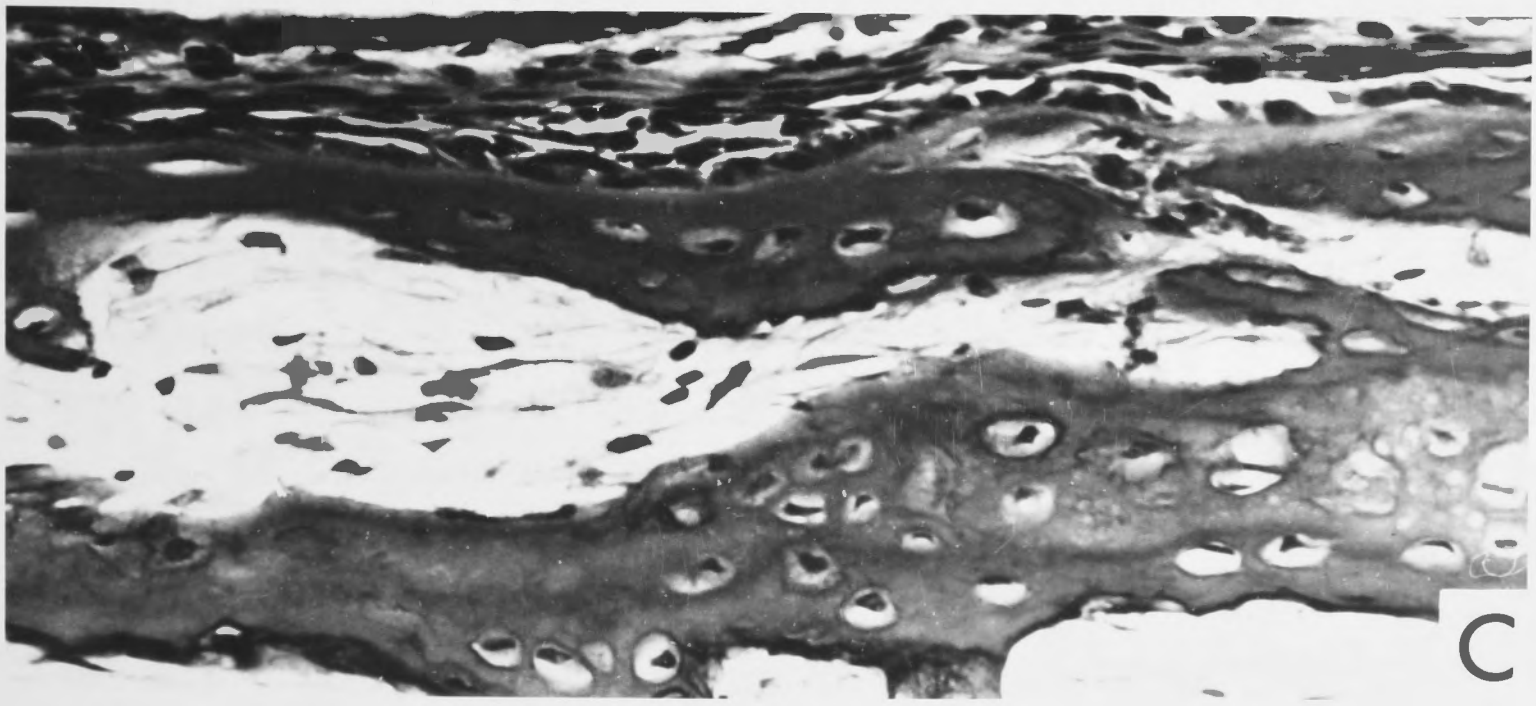

C

that the factor which stimulates transferred normal lymphoid cells to react against allogeneic but not xenogeneic tissues may not be antigen. This immediately raises a question as to the nature of the material present in the grafted tissue that will stimulate allogeneic but not xenogeneic lymphoid cells. A clue as to which cellular component of the graft is the agent responsible for stimulating allogeneic lymphoid cells comes from the work of Ramseier and Billingham (1966) and Elkins (1966). These workers concluded from their studies on the inhibition of local GVH reactions in γ-irradiated hamsters and rats, that the factor responsible for the manifestation of the reaction was not associated with antigen on the surface of epidermal cells but was carried by cells of haematological origin. The effect of γ-irradiation on the GVH reaction in the chicken embryo was investigated by Seto and Albright (1965). These workers found that irradiation of either recipient chicken embryos or adult donor lymphoid cells prior to injection greatly reduced the amount of splenomegaly that occurred. These findings indicate that either some radiosensitive material or cell is responsible for stimulating the grafted lymphoid cells to produce a GVH reaction.

The experimental results recorded above show that prior irradiation of embryonic bone and spleen tissue before grafting protected them from subsequent attack by the adult cells of "treated embryos". The importance of reticular elements of the grafted tissue in the process of graft rejection by "treated embryos", is most strikingly demonstrated in the case of heart muscle grafts. Heart muscle obtained from allogeneic embryos, in contrast to other allogeneic tissues such as bone, spleen or liver, is not damaged when transferred to "treated embryos". If, however, the heart is perfused with embryonic spleen cells before grafting, the muscle cells are destroyed. Thus it would appear that the reticular elements in the graft initiate the

process of graft rejection by stimulating the injected cells which, once stimulated, are able to destroy the surrounding muscle cells.

Summary

The transfer of adult blood leucocytes to syngeneic embryos will confer on the embryo the ability to reject transplants of some xenogeneic tissues. The latter type of transplant (pigeon bone) can be rejected if lymphoid cells, allogeneic with respect to the graft, are introduced into the embryo. Allogeneic transplants are only rejected by "treated embryos" if the transplant contains a relatively large amount of reticular tissue. Treatments, such as γ-irradiation, that reduce the amount of reticular tissue in the graft, protect it from transplantation damage in treated embryos.

It was concluded from these observations that the agent which stimulates allogeneic and not xenogeneic lymphoid cells during GVH reaction is present in reticular tissue.

CHAPTER 5

THE HISTOPATHOLOGY OF GVH REACTIONS IN THE CHICKEN EMBRYO

Chapter 5 - The Histopathology of GVH Reactions in the Chicken Embryo

Introduction

Embryos that have been inoculated with blood leucocytes obtained from a syngeneic adult bird are able to distinguish between syngeneic and allogeneic transplants that have been introduced into these embryos. However the fact that such embryos are unable to reject some xenografts, such as pigeon bone, raises an element of doubt as to whether this distinction between "self" and "not self" components is mediated by the recognition of foreign antigens carried by the transplant. It would seem likely that this type of graft rejection does not represent a primary interaction of the transferred cells with the foreign graft. One possibility is that lymphoid cells obtained from normal adult birds are "naturally" sensitive to allogeneic tissues. Indeed it would seem that this is, in fact, the case. The question that must be investigated further is whether the factor to which these cells are sensitive is antigen.

The fact that cells that are reactive towards chicken tissues arise as the result of the immunization of ducks with chicken spleen cells offered a system for investigating this question further. Such cells are presumably sensitive to chicken antigens and form discrete pocks when inoculated on to the CAM of the chicken embryo. An estimate of the number of antigen sensitive cells circulating in the blood of immunized ducks can be obtained from the number of pocks that are produced by a given number of blood leucocytes, following their inoculation onto the CAM of chicken embryos. The following study is a comparison of the pathogenesis of GVH reactions mediated by immune duck cells and normal allogeneic cells. The cell suspensions injected into the embryos were adjusted to have the same pock forming activity.

Experimental

The Pathology of GVH Reactions Produced in the Chicken Embryo by Normal Allogeneic and Immune Xenogeneic Lymphoid Cells

The numbers of reactive cells contained in each millilitre of blood obtained from a duck immunized against chicken antigens and that from a normal chicken were determined by titrating the pock forming ability of 0.1 mls of samples of each donor's blood on the CAM of chicken embryos. These titrations indicated that 10 million immune duck leucocytes were approximately equal in pock forming ability to 1 million chicken leucocytes. Accordingly, groups of chicken embryos were inoculated intravenously with either 10 million immune duck leucocytes or 1 million normal chicken leucocytes. Simultaneously with these intravenous inoculations, equivalent doses of immune duck or normal chicken cells were inoculated onto the CAM of groups of 11 day old chicken embryos and the numbers of pocks produced by each cell suspension counted after 4 days incubation at 38.5°C. The embryos that had received cells by intravenous inoculation were killed 6 days later and smears prepared from their blood. After the preparation blood smears, the embryos were examined for pathological changes, the weight of their spleens determined and representative samples of liver, thymus, bone, proventriculus, thyroid gland and spleen prepared from each for histological examination.

The results of this experiment are recorded in Table 5.1. It can be seen that the spleens from embryos inoculated intravenously with immune duck cells are much smaller than those from embryos, that had received normal chicken blood leucocytes and although some are slightly enlarged, as compared with uninoculated control embryo spleens (Table 3.1), most show no increase in weight. The variance of the spleen weight from embryos that had received immune duck leucocytes was significantly greater ($P < 0.01$) than that of embryos inoculated with

Table 5.1

Pock formation and spleen enlargement produced by the inoculation of chicken embryos with leucocytes obtained from a normal allogeneic chicken or an immunized duck.

	10^7 Duck Leucocytes	10^6 Chicken Leucocytes
Mean number of pocks per membrane at 4 days	24 (6)*	13 (7)*
Spleen weight (mgm) at 6 days	8.0	97.6
	12.8	97.6
	22.9	57.0
	13.0	126.0
	29.0	83.9
	13.0	176.2
	27.7	74.2

* The bracketed figure is the number of membranes in the group.

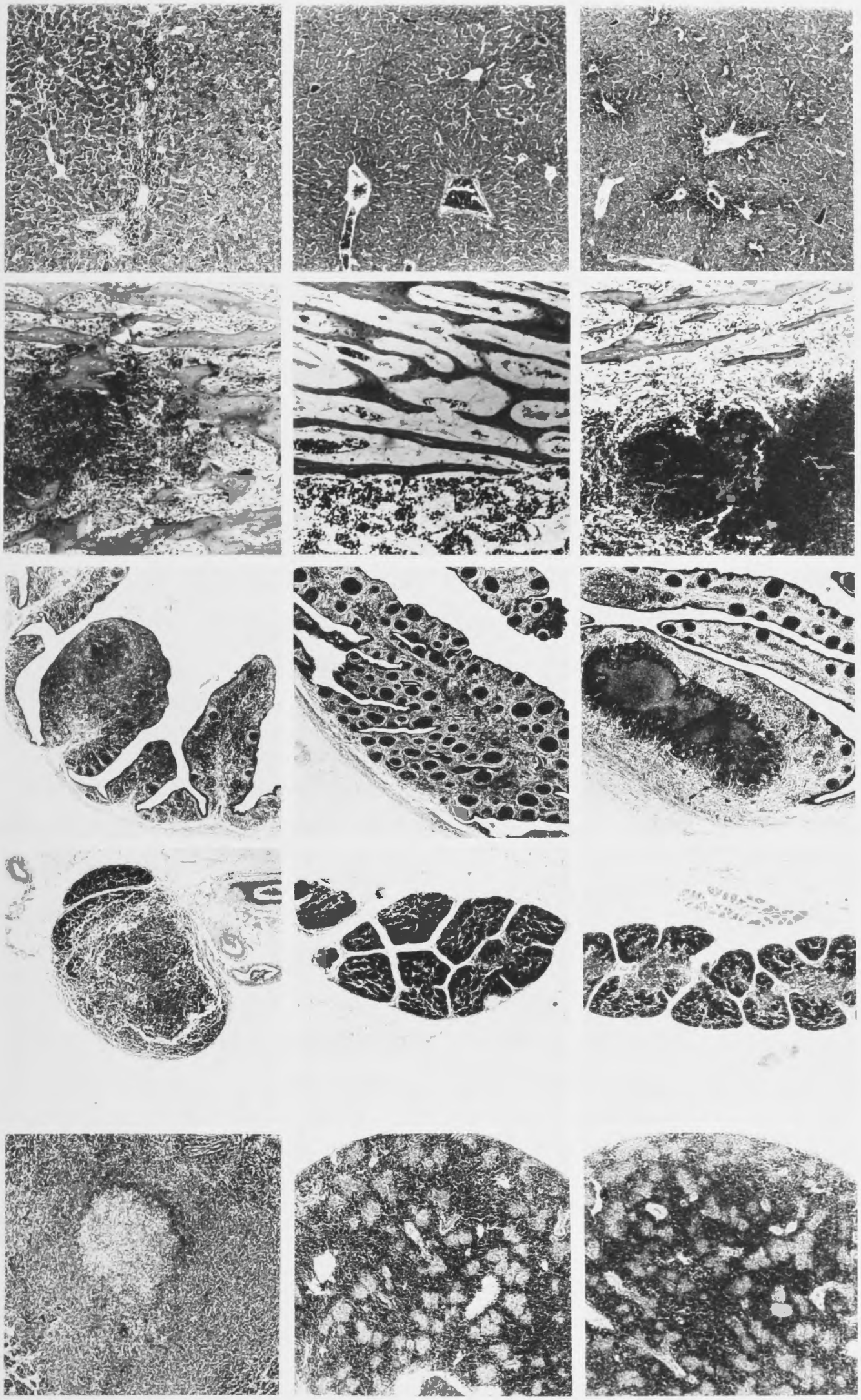
medium alone indicating that the increases in spleen weight in some embryos was significantly different from that likely to occur by chance.

The Histopathological Changes Produced in the Chicken Embryo by Normal Allogeneic and Immune Xenogeneic Lymphoid Cells

Figure 5.1 compares tissues obtained from 17 day old chicken embryos that had been intravenously inoculated 6 days previously with either normal chicken leucocytes or an equivalent number of immune duck leucocytes with those obtained from a normal 17 day old chicken embryo. It can be seen that similar lesions have been produced in the liver, bone marrow, thymus and bursa of fabricius of injected embryos by both types of leucocytes. These lesions do not differ from those already described by other workers and as reviewed by Simonsen (1962). The liver lesions are characterized by the accumulation of granulocytes around the portal tracts. The bone marrow usually has one or more foci of a granulomatous type composed of fibroblasts and mononuclear cells, often with a central necrotic area. The thymi in embryos which had received chicken leucocytes, show the results of more violent reactions than those treated with immune duck leucocytes. The degree of involvement of the thymi varies from lobe to lobe, with necrotic foci occurring occasionally in those from embryos treated with chicken leucocytes. Such necrotic foci are not seen in the thymi of embryos inoculated with immune duck cells. The histological changes seen in bursae of fabricius from both groups of embryos show considerable variation, but the most frequent ones are a reduction in the number of lymphoid follicles in the bursa and an increase in the number of myeloid cells. Focal areasof necrosis in the bursa are seen as a result of inoculation of either immune duck or normal chicken leucocytes. The most marked differences in the histopathological changes are seen to have occurred in the spleen and blood. In the

Figure 5.1

Comparison of the histological changes produced by allogeneic lymphoid cells and immune duck lymphocytes with the normal chicken embryo tissues. The tissues shown in the left hand column come from embryos treated with adult chicken leucocytes, those in the central column come from normal embryos and those in the right hand column were taken from embryos treated with immune duck leucocytes. The tissues in each row from the top down are liver, bone, bursa of fabricius, thymus and spleen. Sections were stained with haematoxylin and eosin.



greatly enlarged spleens of the embryos treated with allogeneic leucocytes, there are usually macroscopic nodules. They consist mainly of reticulum type cells and have the appearance of a tumour with a necrotic centre surrounded by giant syncitial cells. In contrast, spleens from embryos treated with immune duck leucocytes have a histological appearance indistinguishable from that of a normal 17 day old chicken embryo spleen. Even in significantly enlarged spleens, the only change appeared to be a slight hypertrophy of the myeloid elements.

There is also a difference between the blood pictures of embryos inoculated with allogeneic chicken leucocytes and of those which had received immune duck cells. The blood of the former contained increased number of immature cells of both myeloid and erythroid type (Fig. 5.2). These immature cells, which were found to constitute 2 to 3 per cent of the total white cells in the blood, were sometimes seen in mitosis. Chicken embryos treated with immune duck leucocytes did not have an increase in the numbers of immature myeloid and erythroid leucocytes in their blood but they did have a slight increase in the proportion of erythrocyte precursors. It was however no greater than that seen in control embryos that had received either medium or 10 million syngeneic leucocytes.

In addition to the above tissues in which lesions were detected, sections of kidney, thyroid gland, salivary gland, proventriculus and intestinewere examined from embryos undergoing GVH reactions. No histological changes were detected in any of these tissues as compared with tissues from untreated embryos.

Discussion

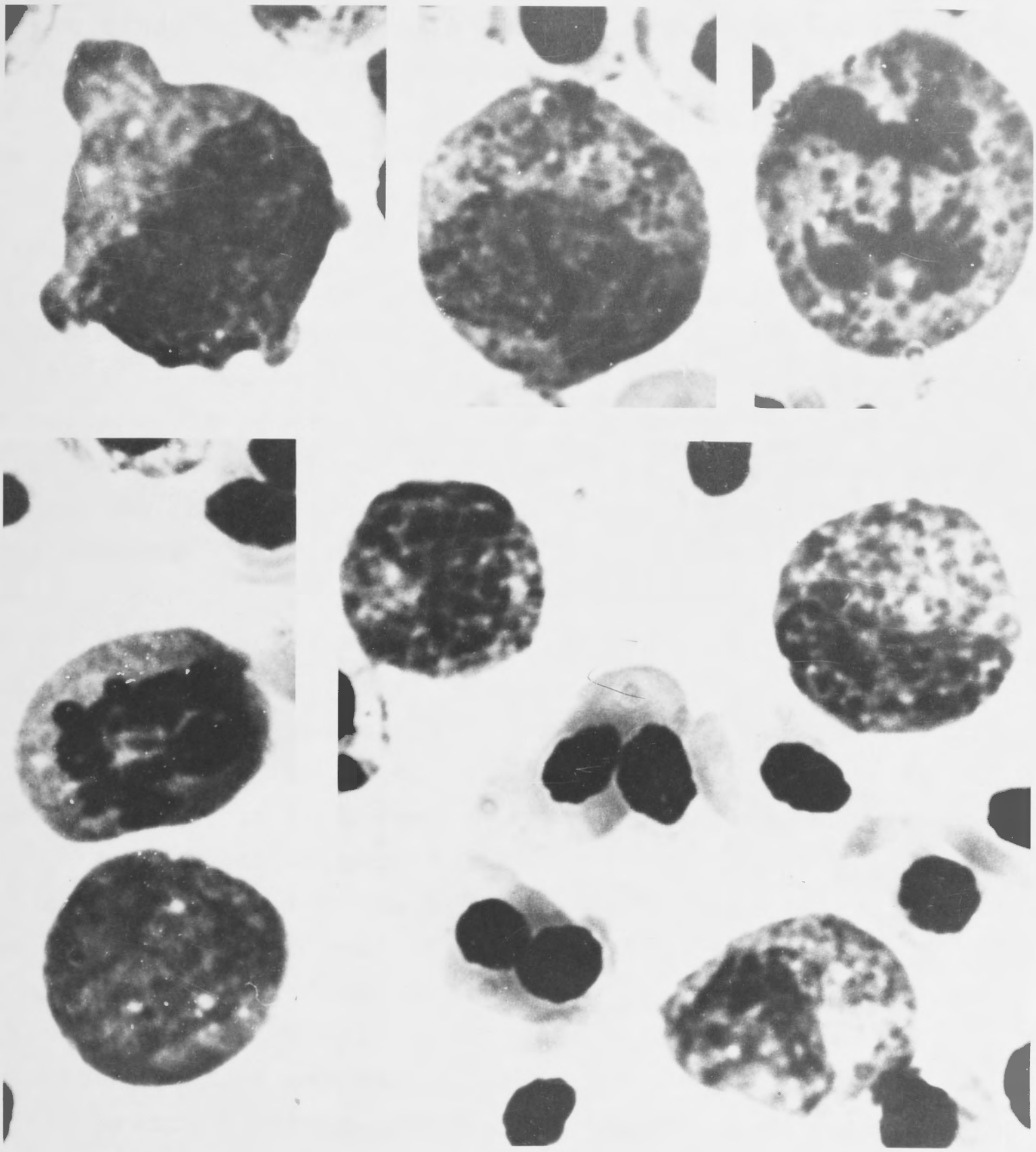
Several groups of workers have shown that antigen sensitive cells appear in the blood of animals subsequent to their immunization with antigen (Pearmain *et al.*, 1963;

Figure 5.2

Immature myeloid and erythoid cells seen in the blood of 17 day old chicken embryos undergoing GVH reaction produced by the inoculation of 1 million allogeneic chicken blood leucocytes.

Magnification x2000

Leishman



Hirschhorn et al., 1963). Since appreciable numbers of cells that are reactive against the chicken embryo only appear in the blood of ducks after the immunization of these birds with chicken tissues, it is reasonable to assume that such cells are reactive towards chicken antigens.

The comparison of the pathological effects that result from the inoculation of chicken embryos with equivalent doses of immune duck cells or normal allogeneic cells emphasizes the difference between "allogeneic reactions" and reactions that are mediated by the interaction of immune cells and what one assumes must be embryonic antigens. Allogeneic reactions are characterized by the formation of tumour-like lesions in the recipient's spleen. Embryos inoculated with immune duck cells, on the other hand, show little or no splenomegaly, and tumour-like lesions are never seen in histological sections of these spleens. The fact that immune duck cells do not stimulate this proliferative response in the spleen of the recipient embryo, makes it unlikely that the splenic reaction produced by allogeneic cells is the result of the interaction of elements in the recipient's spleen with antigen sensitive cells. If it were, one would expect the immune duck cells to be at least as potent in this respect as normal allogeneic cells.

Additional support for the previous observation that allogeneic lymphocytes react against reticular tissue elements present in the recipient's body comes from the failure to find any manifestations of GVH reaction in organs containing few reticulo-endothelial elements. A similar observation was reported by Nisbet and Heslop (1962) in "runt disease" in rats. The appearance of large numbers of immature myeloid and erythroid cells in the blood of treated embryos is more readily understood when considered in the light of recent finding of Nisbet and Simonsen (1967). These workers found that in 14 day old chicken embryos undergoing GVH reaction, all but

7 per cent of the cells proliferating in the spleen were of host origin. This being the case, it is possible sufficient of these proliferating cells could escape from the spleen into the blood to account for the 2 to 3 per cent that were observed in blood smear from embryos undergoing GVH reaction.

Summary

When equal numbers of reactive blood leucocytes from a normal allogeneic chicken and an immunized duck are inoculated separately into chicken embryos, different pathological changes are produced. The splenic weights of the embryos that had received the allogeneic cells were very much greater than those of embryos that had received immune duck cells, which were only slightly enlarged as compared with normal chicken embryo spleens. Leucocytes from both types of donor produced similar histopathological changes in the liver, bone marrow, thymus and bursa of fabricius. The most marked histological changes were in the appearance of immature myeloid and erythroid cells in the blood and macroscopic nodules in the spleens of embryos inoculated with allogeneic leucocytes, both of which changes were absent from embryos that had received immune duck leucocytes. It was concluded that the proliferative response produced in the spleen of recipient embryos was unlikely to be due to the interaction of elements of the recipient's spleen with antigen sensitive cells.

CHAPTER 6

THE NORMAL LYMPHOCYTE TRANSFER REACTION IN SHEEP

Chapter 6 - The Normal Lymphocyte Transfer Reaction in Sheep

Introduction

The comparative study described in the previous chapter showed that the histopathological changes produced in the chicken embryo by adult allogeneic chicken cells were different from those produced by immune xenogeneic (duck) lymphoid cells. The most striking difference was that failure of the duck cells to produce the proliferative changes seen in the spleens of embryos that had been inoculated with normal allogeneic leucocytes. Since immune duck cells are probably reacting against antigen their failure to produce the proliferative changes in the spleen of treated embryos suggests that the stimulus for this proliferative response may not be antigen. Before such a suggestion can be entertained, the possibility that the interaction of transferred lymphoid cells with allogeneic reticular tissue may be a phenomenon which is specific for an embryonic host must be investigated. To discover whether the manifestations of the proliferative response which follows the interaction of lymphoid cells with reticular tissue can occur in other environments besides that of the chicken embryo, a study of local GVH reactions produced in the skin of normal sheep by allogeneic lymphocytes was undertaken.

Experimental

The normal lymphocytes transfer (NLT) reaction was conducted in sheep in a similar manner to that described for guinea pigs by Brent and Medawar (1963). The methods used to cannulate efferent lymphatics, collect the lymph and obtain the lymphocytes together with the technique for producing and measuring the NLT reaction in sheep have been described in Chapter 2.

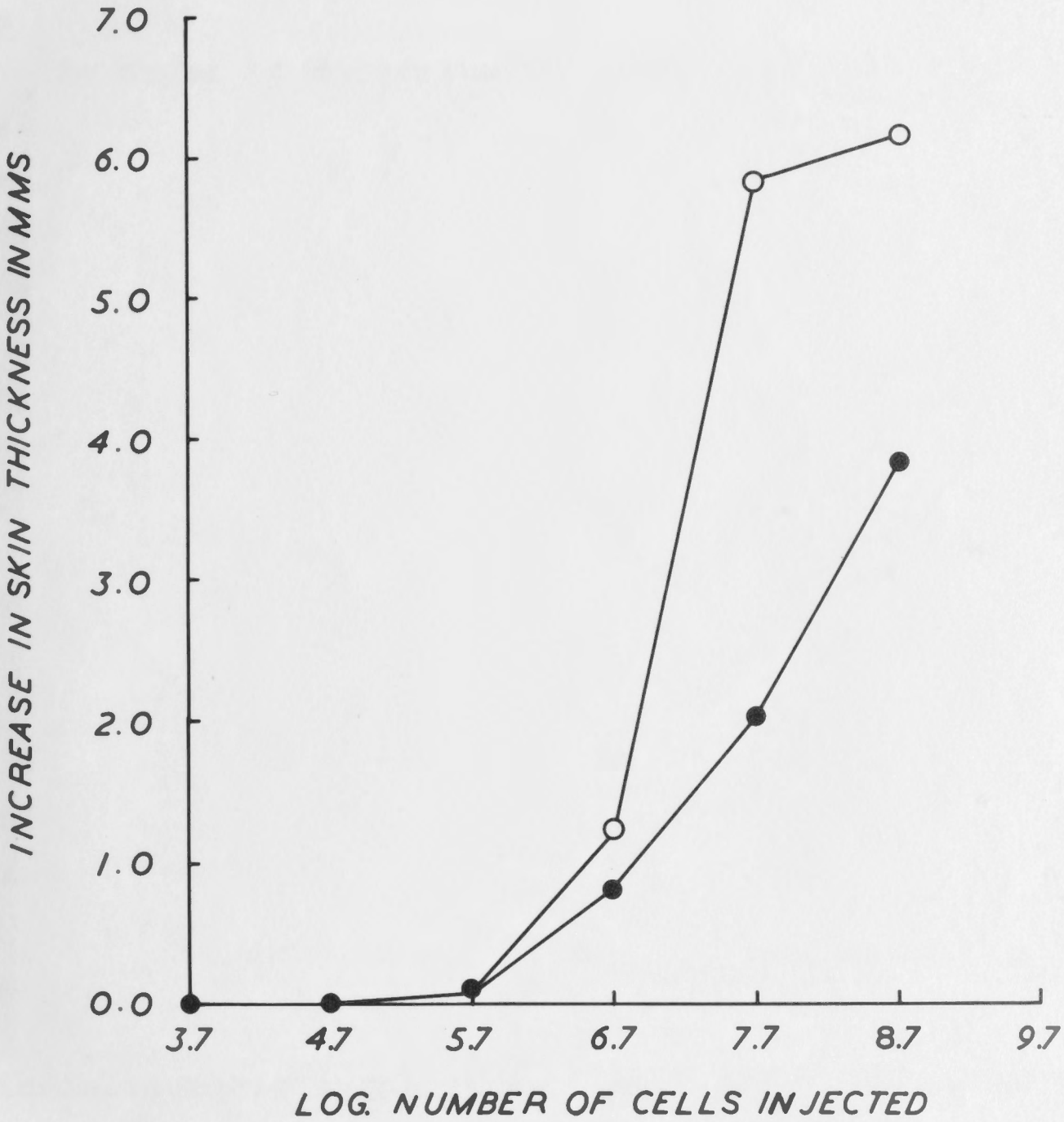


Figure 6.1

The effect of the number of allogeneic lymphocytes injected on the increase in skin thickness in a sheep 6 days later. The open circles represent the changes in sheep1 and the filled circles those in sheep 2.

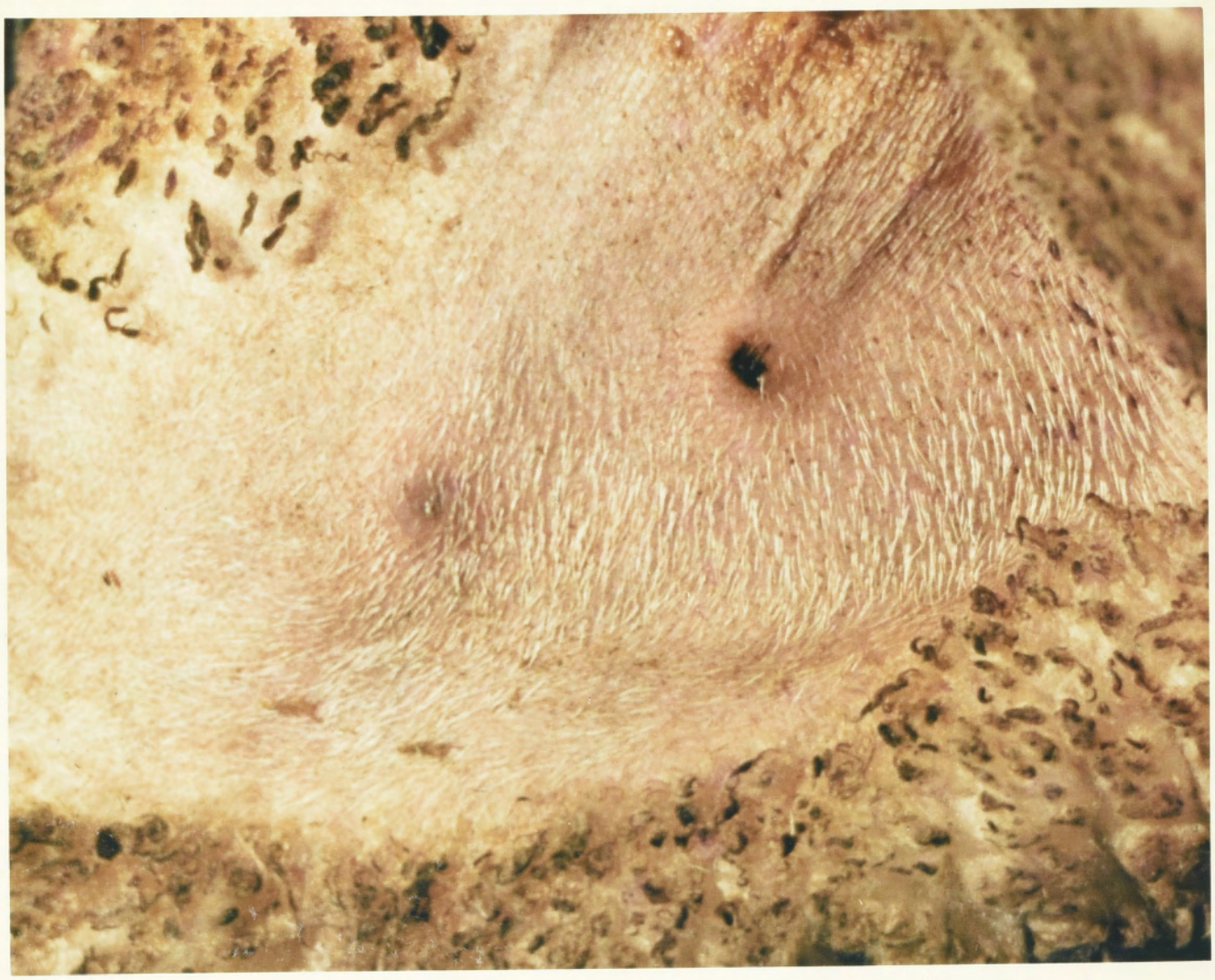


Figure 6.2

NLT reactions in sheep 5 days after the intradermal inoculation of allogeneic lymphocytes. The reaction of the left was produced by the injection of 5 million lymphocytes while the other with a darkened centre resulted from the inoculation of 50 million lymphocytes.

The Effect of the Numbers of Allogeneic Lymphocytes Injected on the Size of the NLT Reaction Produced in the Recipient Sheep

In order to determine the number of allogeneic lymphocytes that will consistently produce detectable NLT reactions in sheep, a panel of recipients each received 6 separate intradermal injections that contained doses of cells ranging from 5 thousand to 500 million.

Figure 6.1 illustrates the degree of dependence of NLT reactions on the number of lymphocytes injected by comparing the increases in skin thickness 6 days after the injection of lymphocytes into two sheep, one a strong reactor, the other a weak one. It can be seen that although doses of fewer than 500 thousand lymphocytes failed to produce detectable changes, 5 million lymphocytes resulted in significant responses in both recipients. It was not possible to inject more than 500 million lymphocytes in 0.1 ml of EBM because spontaneous agglutination of cell suspensions occurred at higher concentrations.

The Macroscopic Changes Produced in the Skin of Sheep During an NLT Reaction

Immediately after the intradermal inoculation of 5 million allogeneic lymphocytes, there appeared a small area of erythema and oedema at the injection site, which gradually disappeared during the next 10 hours. Erythema reappeared 6 to 14 hours later, and showed a steady increase until 5 days after the inoculation. About this time, the centre of the lesion sometimes darkened and slowly became necrotic (see Fig. 6.2); such necrotic changes were commonly seen where more than 5 million allogeneic lymphocytes had been injected. At the same time, the thickness of the sheep's skin at the injection site steadily increased to reach a maximum sometime between 6 and 8 days after the injection before slowly declining to a constant level over the next 3 to 4 days. Often

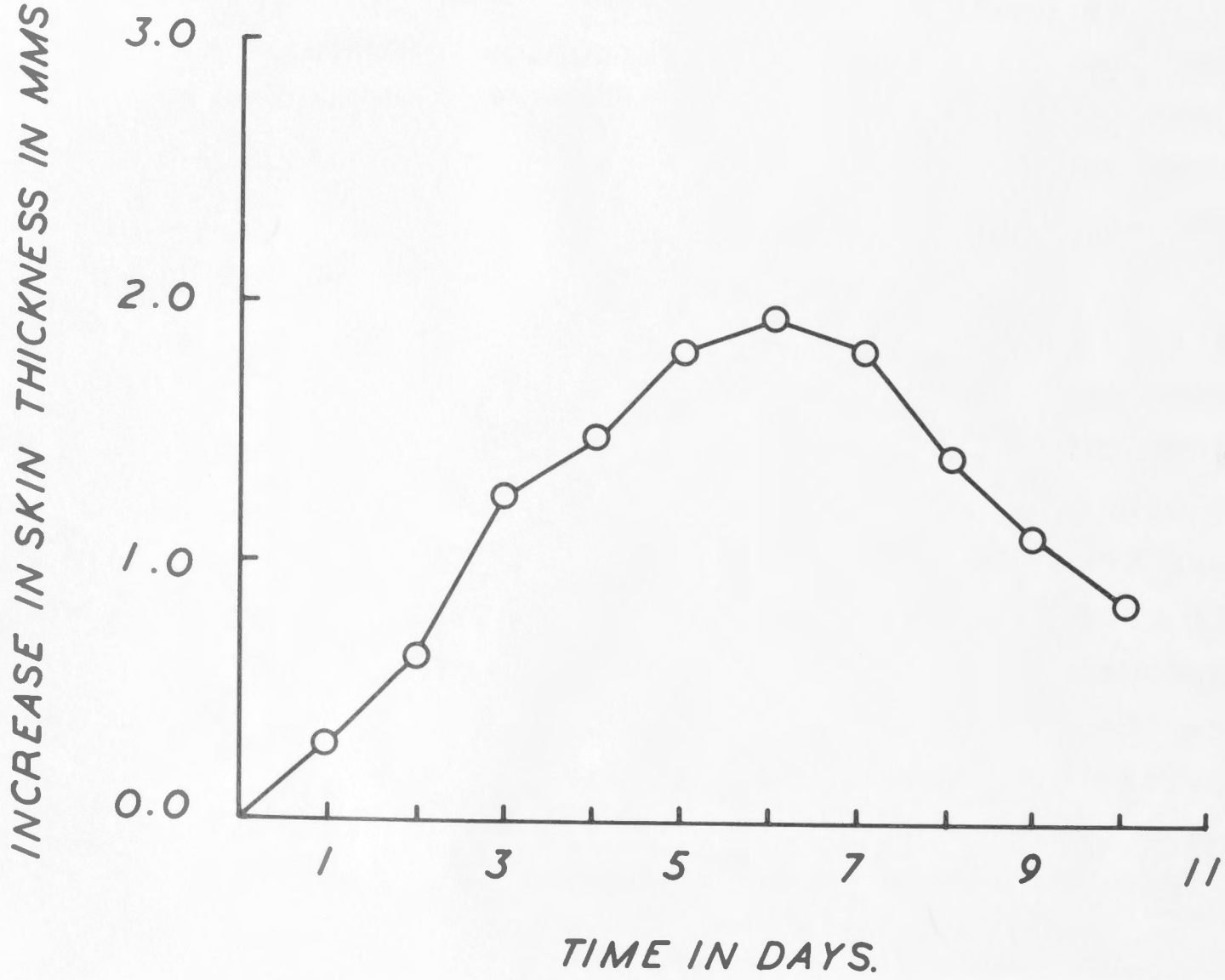


Figure 6.3

The daily increases in skin thickness at the site of an intradermal injection of 5 million allogeneic lymphocytes in a sheep.

when the reaction had subsided, there remained some slight permanent increase in skin thickness, following the formation of scar tissue at the injection site. Figure 6.3 shows typical increases in skin thickness, as measured by skin calipers, that occurred after 5 million allogeneic lymphocytes had been injected intradermally into the medial aspect of a sheep's thigh.

To determine what constituted a real increase in skin thickness, attributable to the intradermal injection of allogeneic lymphocytes, the errors associated with the measurement of the NLT reaction were determined (see Appendix 1). It was found that the standard deviations of repeated measurements of skin thickness at the sites of NLT reactions had a range of 0.1 to 0.2 mms. The skin of the sheep around the area used for NLT reaction tests is usually 1 to 2 mms thick before injection. Any reactions that lead to an increase in skin thickness of 1 mm or more were considered to be highly significant; changes of this magnitude represented a 100 per cent increase in the thickness of the skin at the injection site and were outside the 95 per cent confidence interval for the determination of the mean skin thickness.

The Proportion of Sheep that Respond to the Intradermal Injection of Lymphocytes Obtained from a Single Donor

In early experiments it was apparent that not all sheep responded equally to intradermal injections of allogeneic lymphocytes as measured by the NLT reaction. The proportion of sheep responding with a significant increase in skin thickness was determined by inoculating 145 randomly bred merino sheep intradermally with lymphocytes from a single donor. Each sheep received 5 million and 50 million lymphocytes injected into two different sites. When the resultant increases in skin thickness were compared 6 days later, 15 per cent of the sheep did not have significant reactions at the sites where

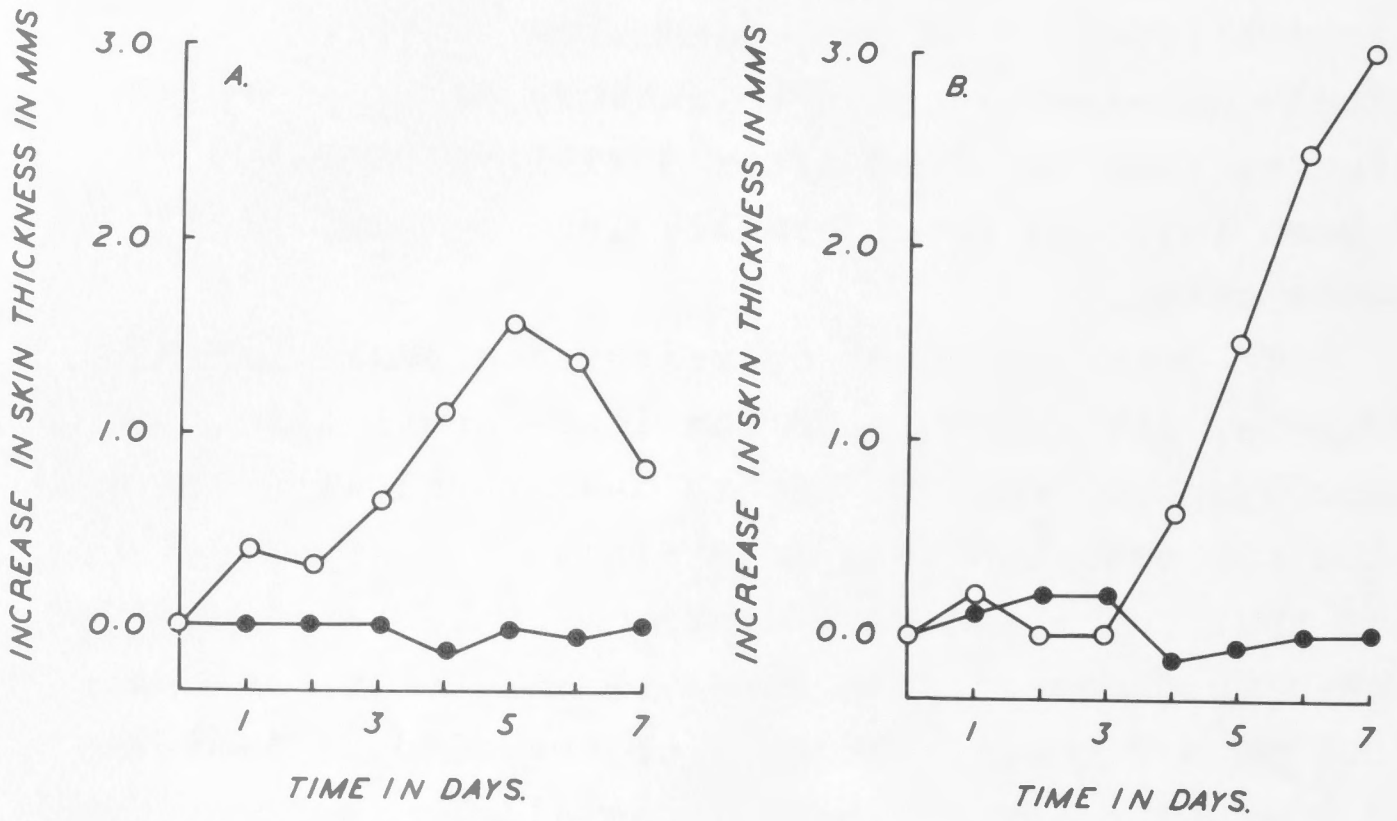


Figure 6.4

A comparison of the daily increases in skin thickness produced by irradiated lymphocytes (filled circles) with those produced by 5 million normal allogeneic lymphocytes from the same donor. Graph A records the results when the irradiated cells were injected 1 day after the normal lymphocytes and graph B records the changes when the irradiated cells were injected 5 days after the normal cells.

the 5 million lymphocytes had been injected, and only 1.5 per cent of the sheep failed to show a significant increase in skin thickness at the site inoculated with 50 million cells. It is clear from these findings that only a small number of sheep fail to show a skin reaction when injected with adult sheep lymphocytes, and the proportion of unreactive animals decreases as the dose of donor cells is increased.

The Importance of a Reaction by Grafted Lymphocytes in the Production of the NLT Reaction

The NLT reaction, unlike the GVH reaction in the chicken embryo, is produced in a host capable of rejecting allogeneic and xenogeneic tissue grafts. For this reason, it is possible that the manifestations of the NLT reaction may be the result of a reaction by the recipient against the antigens on the surface of injected allogeneic cells. Evidence that active participation of the injected lymphocytes was essential for the production of NLT reactions was obtained by the intradermal injection into sheep of allogeneic lymphocytes that had been killed by 6000 rads of gamma-irradiation. Sheep lymphocytes that had received 6000 rads were antigenic when tested in guinea pigs hypersensitive to sheep antigens (see Chapter 7). If the manifestations of the NLT reaction consist in part of a delayed-type hypersensitivity response by the recipient, then it would be predicted that the inoculation of dead lymphocytes during an NLT reaction induced by lymphocytes from the same donor would result in dermal reactions occurring at the site of injection. Figure 6.4 compares the daily increases in skin thickness in 2 sheep that were injected intradermally with 5 million viable lymphocytes and then 1 or 5 days later the same sheep were inoculated intradermally with an equal number of heavily irradiated lymphocytes obtained from the initial donor. It can be seen that no increase in skin

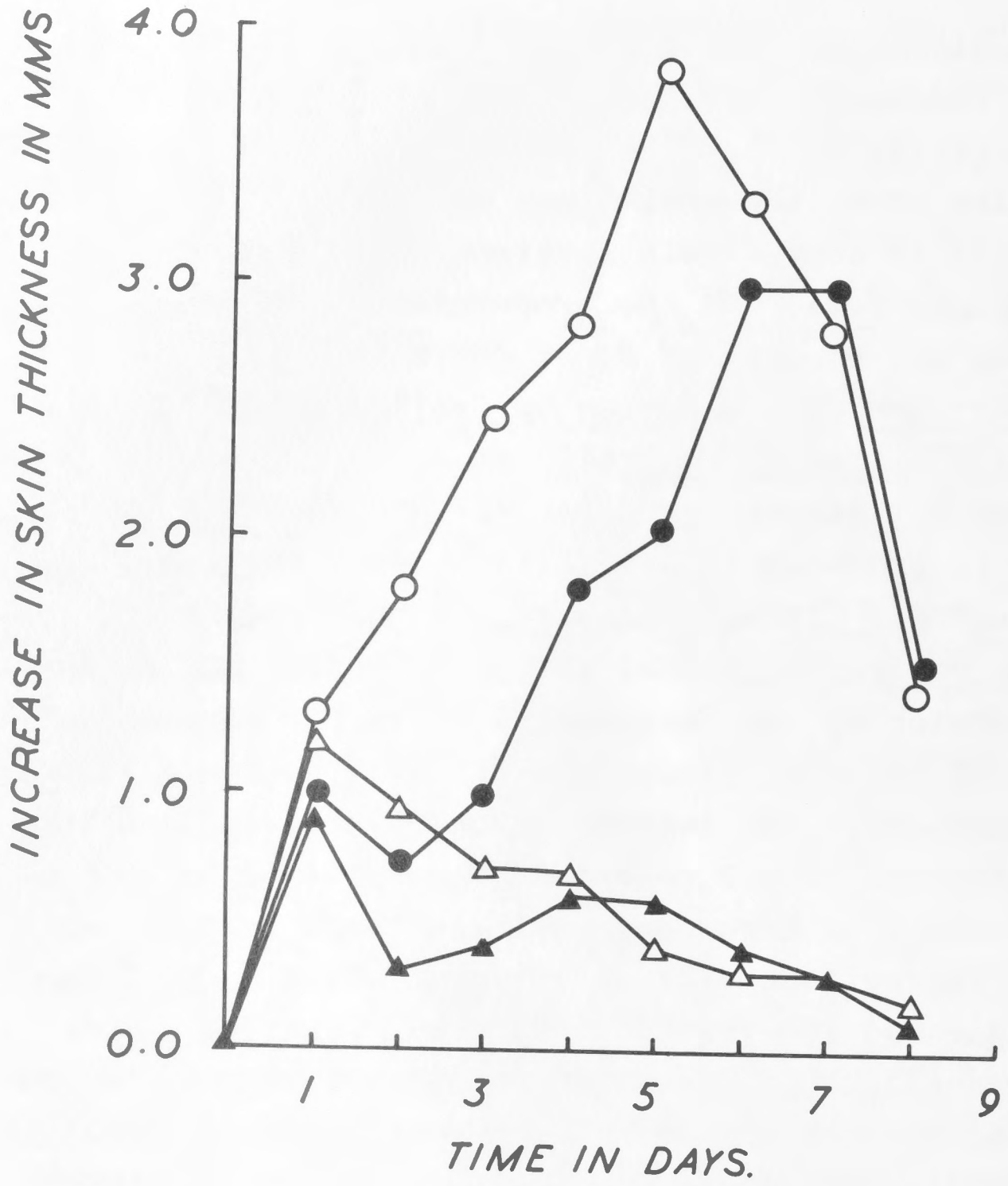


Figure 6.5

A comparison of the daily increases of skin thickness resulting from the intradermal injection of allogeneic lymphoid and tissue cells into sheep. The open circles represent the changes produced by spleen cells and the filled circles those caused by lymph node cells. The changes produced by liver and kidney cells are represented by open and filled triangles, respectively.

thickness is produced by the irradiated cells in either animal. These observations indicate that the presence of donor antigen in the form of dead lymphocytes is insufficient to induce increases in skin thickness comparable with those associated with the NLT reaction.

It is possible that the NLT reaction represents a response by the host against antigens only present on grafted viable cells, in the same manner as the host would reject solid tissue grafts. This proposition was examined by comparing the dermal reactions produced by injecting dissociated cells from allogeneic tissues other than lymphoid organs, with NLT reactions. In this experiment 3 sheep were inoculated intradermally with 0.1 ml of EBM containing 5 per cent calf serum and 5 million dissociated allogeneic cells. These cells, which were obtained from the liver, kidney, lymph node and spleen of a sheep as described in Chapter 2, were inoculated individually into different sites on the thighs of each sheep. Increases in skin thickness were measured daily and the measurements recorded from one sheep, as it was typical of the three used in the experiment, are shown in Figure 6.5. It can be seen that no prolonged increases in skin thickness occurred when liver or kidney cells were injected, in contrast, spleen and lymph node cells produced reactions indistinguishable from NLT reactions. It may be concluded from the failure of dead allogeneic lymphocytes and allogeneic tissue cells to produce dermal reactions comparable to NLT reactions, that in the absence of a reaction by injected allogeneic lymphocytes, no dermal response occurs. Yet when 5 million lymphocytes are exchanged between two sheep, by means of intradermal injections, then NLT reactions are produced in both as is shown in Table 6.1. This finding indicates that the donated allogeneic lymphocytes are sensitive to some component of the host, which is absent from the injection of irradiated lympho-

Table 6.1

Comparison of the increases in skin thickness produced by intradermal injections of lymphocytes exchanged between two sheep.

Lymphocyte donor	Recipient	Increases in skin thickness in mms						
		Time after inoculation in days						
		1	2	3	4	5	6	7
Sheep 1	Sheep 2	0.4	0.6	0.8	1.5	2.0	2.2	1.8
Sheep 2	Sheep 1	0.8	1.0	1.8	2.8	2.8	3.2	2.6

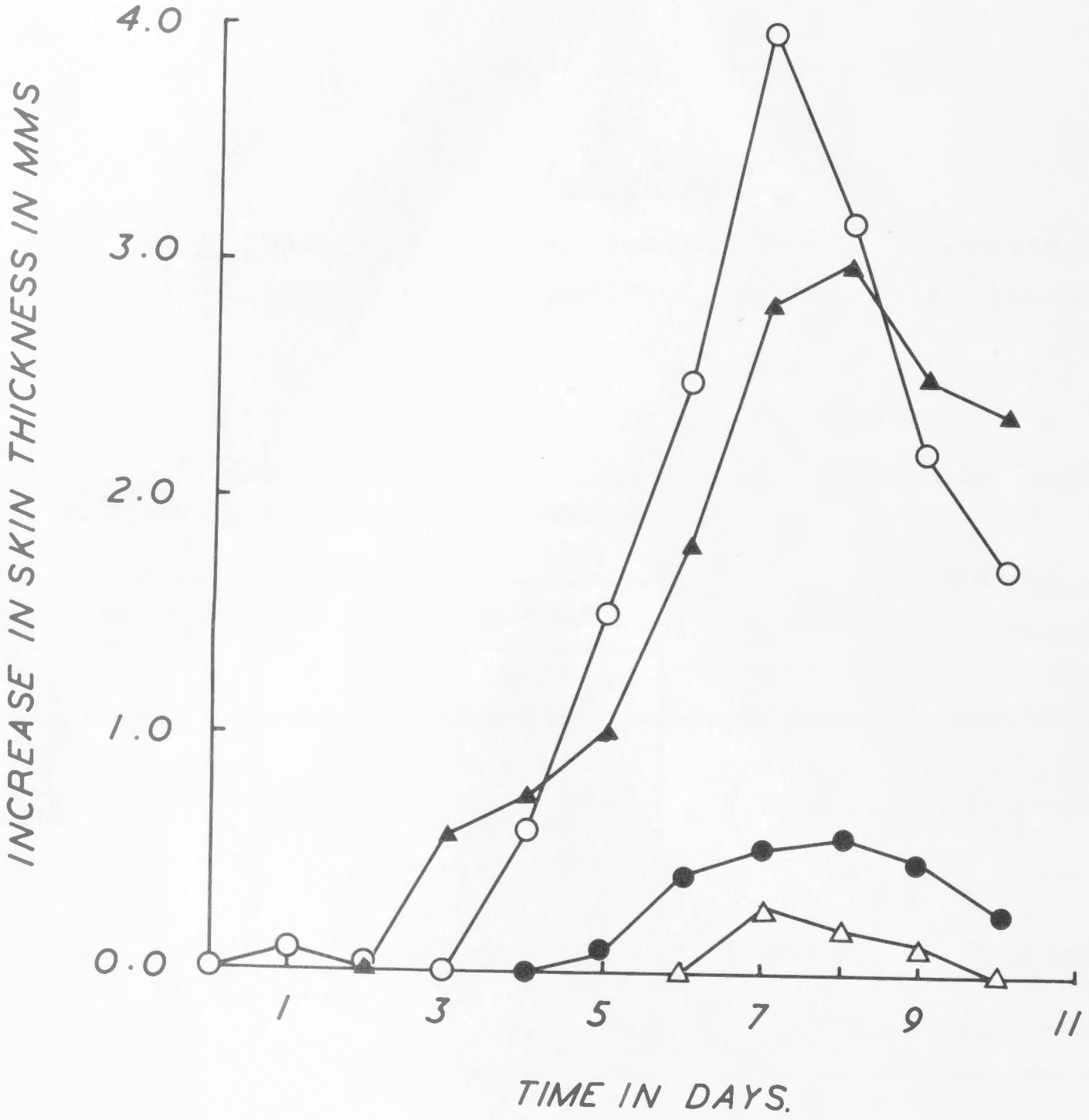


Figure 6.6

Increases in skin thickness produced by intradermal inoculation of lymphocytes from a single donor at intervals of 2 days into a sheep. The open circles, filled triangles, filled circles and open triangles represent the changes produced by injections given at the beginning of the experiment and 2, 4 and 6 days later, respectively.

cytes and allogeneic tissue cells. Moreover, since it is known that lymphocytes that have received similar amounts of irradiation are antigenic (see Chapter 7), these observations suggest the interaction of viable lymphocytes from donor and recipient. It is this dependence upon active participation by viable allogeneic lymphocytes, that the NLT reaction in sheep is similar to a GVH reaction.

The above evidence suggests that the recipient sheep plays more than a passive role in the NLT reaction. To determine the contribution made to the NLT reaction by the recipient, 3 sheep were given multiple daily injections of 5 million allogeneic lymphocytes from the same donor. Since there had been no change in the donor's contribution up to the time of injection, all observed differences in the multiple reactions must be directly attributable to the host. The increases in skin thickness at the sites of inoculation were recorded daily and observations in one sheep typical of all three are shown in Figure 6.6. It can be seen that the 3 day time lag following the first injection, before a substantial increase in skin thickness occurred, has disappeared from the dermal reaction produced at the site of an injection given 2 days later. This latter reaction produced a rapid, almost linear increase in skin thickness, paralleling that which was occurring at the site of the first inoculation. Injections given on or after the fourth day, following the first injection, fail to reach the same size and begin to regress at the same time as the earlier reactions. These results clearly indicate a substantial host reponse, both in early increases in skin thickness and subsequent decline in the NLT reactions.

Dermal Reactions Produced in Sheep by Xenogeneic Lymphocytes

Earlier studies of NLT reactions in inbred guinea pigs and rats have justified the inclusion of the NLT reaction in the class of local GVH reactions (Brent and Medawar, 1964;

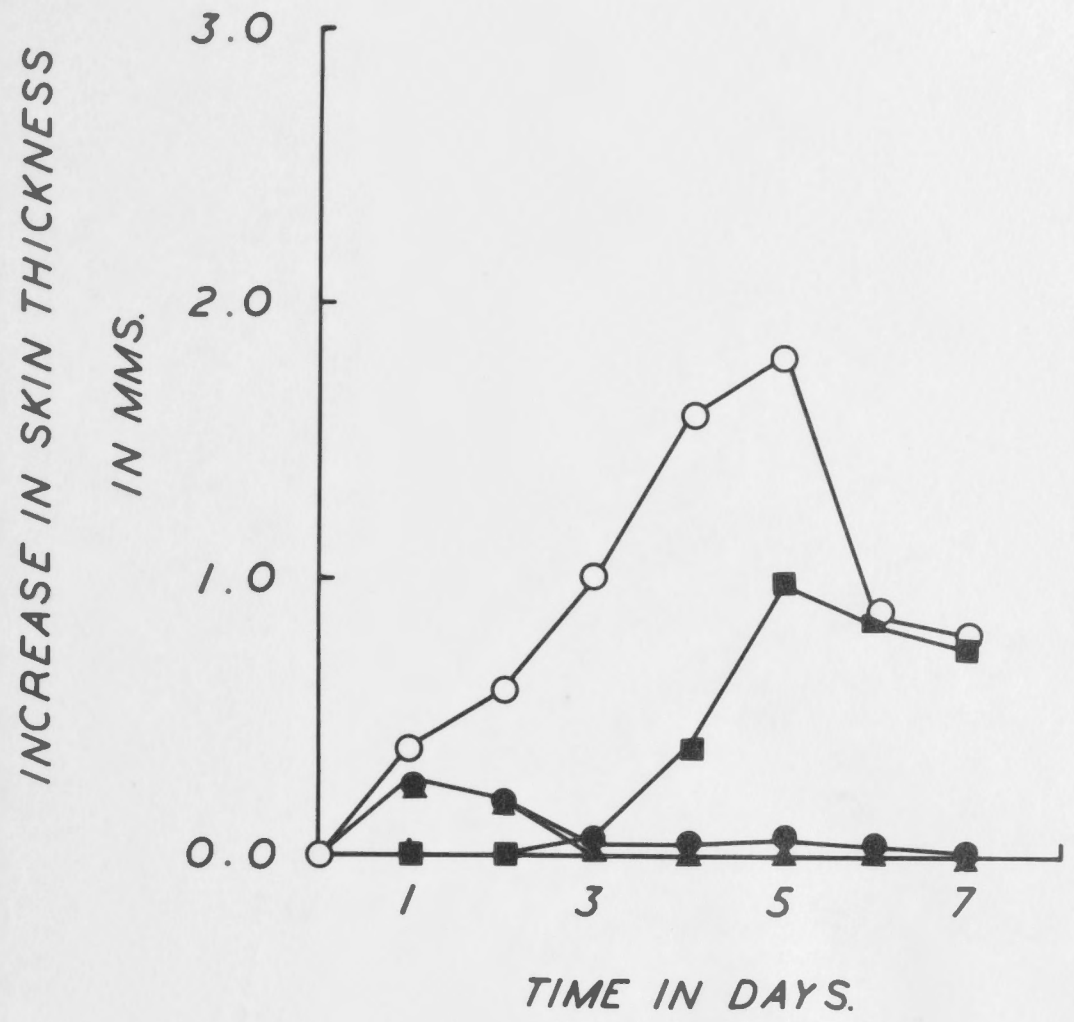


Figure 6.7

A comparison of the daily increases in skin thickness produced by the intradermal injection of xenogeneic lymphoid cells. The open circles represent the changes induced by goat lymphocytes, and the filled squares those produced by bovine lymphocytes. The filled triangles and circles represent the changes produced at the sites of inoculation of guinea pig and chicken lymphoid cells, respectively.

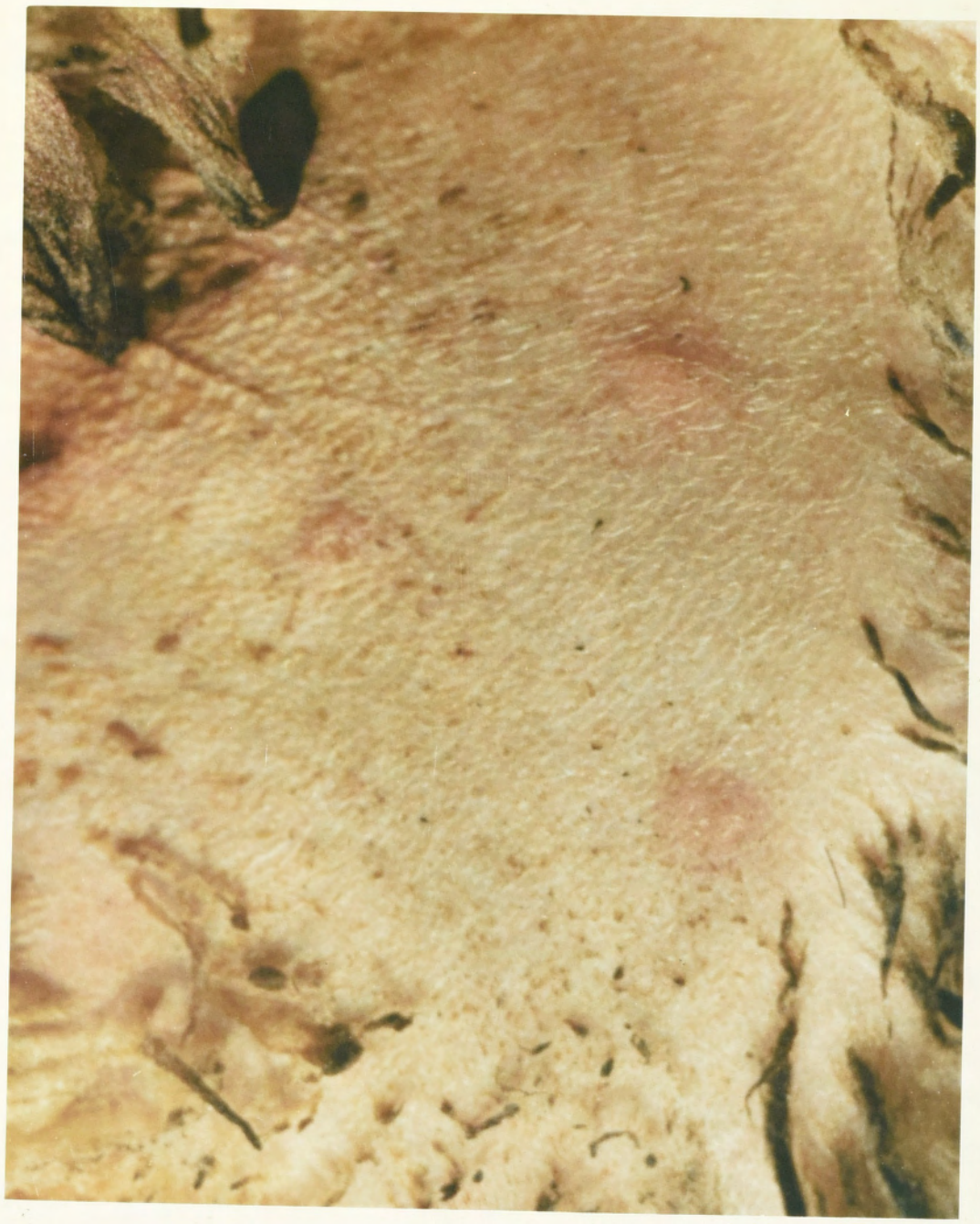


Figure 6.8

The dermal responses produced 4 days after the injection of 5 million normal bovine lymphoid cells into sheep. The smaller dermal reaction on the left of the two bovine reactions has occurred at the site of the injection of 5 million spleen cells obtained from an immune chicken 2 days previously.

Elkins, 1966). The NLT reaction as a local GVH reaction could well provide an opportunity to study whether the emasculated ability of transferred lymphocytes to react against xenogeneic tissues can be shown to occur in recipients other than the chicken embryo. In the chicken embryo, the capacity of xenogeneic cells to produce reduced GVH reactions was shown to decrease to an undetectable level as the phylogenetic differences between the lymphoid cell donor and the recipient embryo increased. Bearing this in mind, members of four species were selected to act as donors of lymphoid cells for injection into sheep. The species selected were two closely related to sheep, the goat and the ox, and two unrelated ones, the guinea pig and the domestic chicken. Dispersed suspensions of lymphoid cells were obtained from lymph nodes or spleens from these species as described in Chapter 2. When 5 million lymphoid cells obtained from each of these species were inoculated intradermally into recipient sheep, significant increases in skin thickness were detected only at the sites where goat and bovine cells had been placed (Fig. 6.7). The macroscopic changes at the site where goat cells had been injected were indistinguishable from those produced by allogeneic sheep cells. On the other hand, the reactions produced by bovine lymphoid cells were often not detectable for the first 4 days after injection, when there was a sudden and distinct increase in skin thickness with a marked area of erythema around the site of inoculation (Fig. 6.7 and 6.8). Chicken and guinea pig lymphoid cells did not produce any detectable changes at the doses used. The results of this experiment indicate that as the genetic and presumably antigenic disparity between the donor and recipient increases, so the size of the NLT reaction decreases, until, as in the case of guinea pig and chicken donors, there is no detectable reaction at all.

Dermal Reactions Produced in Sheep by Immune Xenogeneic Lymphocytes

It is quite possible that guinea pig and chicken lymphoid cells do not survive long enough in the sheep to produce a dermal reaction. To test whether chicken lymphoid cells do survive in sheep, prospective lymphoid cell donors were immunized against the sheep as described in Chapter 2. Throughout the long course of immunization, chickens were killed at intervals of 2 weeks and single cell suspensions prepared from their spleens. It was found that after a period of 2 or more months, suspensions of chicken spleen cells produced distinct areas of erythema and slight but regular increases in skin thickness where the cells had been injected into recipient sheep. No detectable dermal reaction was produced by injections of washings from the spleen cells or by the injection of 0.1 ml of immune donor serum into the same recipient sheep. These observations establish the capacity of xenogeneic cells to survive long enough in the skin of sheep to produce a lymphocyte transfer reaction.

Discussion

The dermal inflammatory response produced in sheep by the intradermal injection of 5 million allogeneic lymphocytes appears to be very similar to that described by Brent and Medawar (1963) in the guinea pig. Where a large number of sheep were injected intradermally with both 5 and 50 million lymphocytes from the same donor, 15 per cent of the sites where the lower dose was injected failed to show an NLT reaction. In contrast only 1.5 per cent of the sites where 50 million lymphocytes were injected had no detectable increase in skin thickness 6 days later.

When xenogeneic lymphoid cells are injected into sheep, dermal inflammatory reactions are produced by cells from donors

which are phylogenetically closely related to the sheep. Cells obtained from more distantly related or unrelated species (chicken and guinea pig) failed to induce a detectable dermal reaction unless they were from a donor that had been immunized against sheep antigens. It should be mentioned that no dermal response is produced by lymphoid cells from chickens, following intradermal injections into sheep, until more than 2 months after the initial immunization of the animals. A similar period elapsed after the initial immunization of guinea pigs before the immune cells were capable of producing pocks on the CAM of chicken embryos. The mechanism leading to the appearance of reactive lymphoid cells 2 months after immunization is not understood. The fact that lymphoid cells from immunized chicken donors can induce dermal reactions in the sheep indicates that the injected cells are able to survive in the environment of the sheep. It could be argued that the NLT reaction and the dermal responses by injected xenogeneic lymphoid cells resulted from a reaction by the host against the antigen on the grafted cells. The stongest host reaction being against allogeneic antigens, decreasing through antigens of closely related species to disappear entirely in unrelated species. The experimental findings do not support the proposition that the injected lymphoid cells merely act as a source of antigen, since antigenic dead allogeneic lymphocytes and dispersed allogeneic kidney cells do not produce significant skin reactions. Liver cells produce small significant increases in skin thickness which persist for a shorter period than those associated with NLT reactions. These reactions could possibly be accounted for by the presence of contaminating lymphoid cells in the liver cell suspension that was injected. These observations suggest that an active part is played by the injected allogeneic lymphocytes in the production of an NLT reaction.

The recipient plays more than a passive role in the NLT reaction. It is clear from the inhibition of a second NLT reaction, after the subsidence of an initial reaction in the same recipient, that the host develops some forms of immunity. In addition the host contributes to the size of the NLT reaction in some way. This is shown by a more rapid rate of increase in skin thickness at the sites of subsequent injections of lymphocytes from the same donor, up to 5 days after the initial injection into the recipient. The suggestion, by Ramseier and Streilein (1965), that the host cells play an important part in the size of the NLT reaction has been substantiated by the recent work of Ramseier and Billingham (1966) and Streilein and Billingham (1967) who found allogeneic lymphoid cells unable to produce NLT reactions in leucopaenic X-irradiated hamsters and rats. These workers concluded that the failure of allogeneic lymphoid cells to produce an NLT reaction in irradiated hosts was due to a deficiency in the skin of cells of haematogeneous origin, possibly leucocytes.

It is inferred from these conclusions that the antigens present in the skin are insufficient to stimulate allogeneic lymphocytes to produce a NLT reaction, but that some interaction between the injected lymphocytes and the cells of haematogenous origin is necessary to bring about the manifestation of the NLT reaction. The production of NLT reactions in sheep by the intradermal injection of lymphocytes exchanged between two donors (A and B) also suggested that the NLT reaction is the result of a lymphocyte-lymphocyte interaction. This is seen by the failure of lymphocyte antigens from donor A to produce a skin reaction when injected intradermally into donor B, although donor B's lymphocytes will react in donor A's skin to produce an NLT reaction and vice versa, indicating that both donors are sensitive to some material in the recipient which is not present in this injected irradiated lymphocyte

preparation.

It may be concluded, from the results of these experiments, that the NLT reaction in the sheep is similar to those described in the guinea pig and is likely to be a local GVH reaction. Using the NLT reaction in sheep as a measure of reactivness of injected cells against the tissues of the host it was found that transferred lymphoid cells showed a similar emasculated ability to recognise xenogeneic tissues as was shown by xenogeneic cells in the chicken embryo. These observations indicate that at least some of the findings concerning the behaviour of lymphoid cells in xenogeneic hosts are not specific to an embryonic environment.

Summary

The NLT reaction produced in sheep by the intradermal inoculation of allogeneic lymphocytes was studied as an example of a local GVH reaction. Justification for the inclusion of the NLT reaction as a local GVH reaction was provided by the demonstration that no characteristic dermal response occurred when dead allogeneic lymphocytes or allogeneic tissue cells were injected instead of viable lymphocytes from the same donor. The capacity of xenogeneic lymphoid cells from goats, oxen, guinea pigs and chickens to produce NLT reactions in sheep was examined. It was found that goat and bovine cells produced NLT reactions, the latter slower and smaller than those produced by allogeneic lymphocytes in sheep. Guinea pig and chicken lymphoid cells failed to produce a reaction unless obtained from a donor immunized extensively against sheep antigens over the proceeding 2 months. It was concluded that cells from unrelated lymphoid cell donors were unable to be primarily stimulated to react against the tissues of the xenogeneic host.

CHAPTER 7

THE MIXED LYMPHOCYTE REACTION

Chapter 7 - The Mixed Lymphocyte Reaction

Introduction

The earlier discussions of the interaction of normal lymphoid cells with allogeneic tissue have emphasized the fact that the presence of reticular elements in the target tissue appears to determine whether the lymphoid cells will attack such alien tissue. Moreover, there is considerable doubt that such reactions are initiated by the interaction of the normal lymphoid cells with antigen. *In vivo* reactions are always complicated by the response of the host to tissue damage, and because of this, it would be desirable to study the question of the involvement of antigen in these reactions in an *in vitro* system.

In 1964 Bain *et al.* described the mixed lymphocyte reaction. This reaction is a proliferative response that occurs when allogeneic lymphocytes are cultured together *in vitro*, and is thought to be an *in vitro* immunological reaction initiated by the contact of immunologically competent lymphocytes with foreign antigens (see Dutton, 1966b; Wilson, 1967; Wilson *et al.*, 1967). In view of these findings it would seem likely that the mixed lymphocyte reaction is an *in vitro* analogue of the initial events that take place in reactions of the GVH type.

The following experiments were designed to investigate two aspects of the mixed lymphocyte reaction. Firstly an attempt was made to determine whether or not such reactions represent primary immunological reactions and secondly the role played by cellular antigen in the initiation of these reactions was investigated.

Experimental

The Mixed Lymphocyte Reaction (MLR) with Sheep Lymphocytes

The lymphocytes to be cultured together were obtained from lymph collected over a 24 hour period from the cannulated

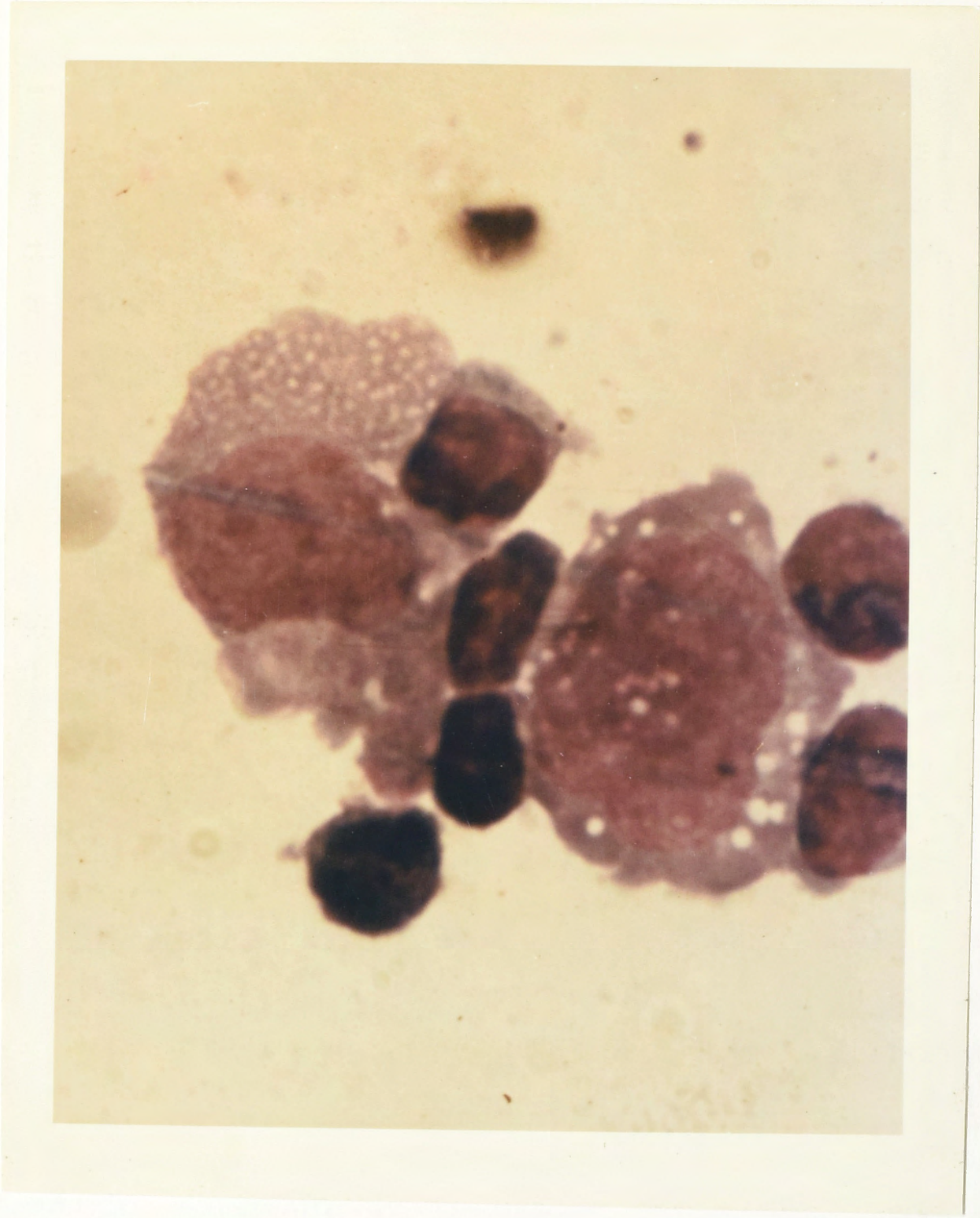


Figure 7.1

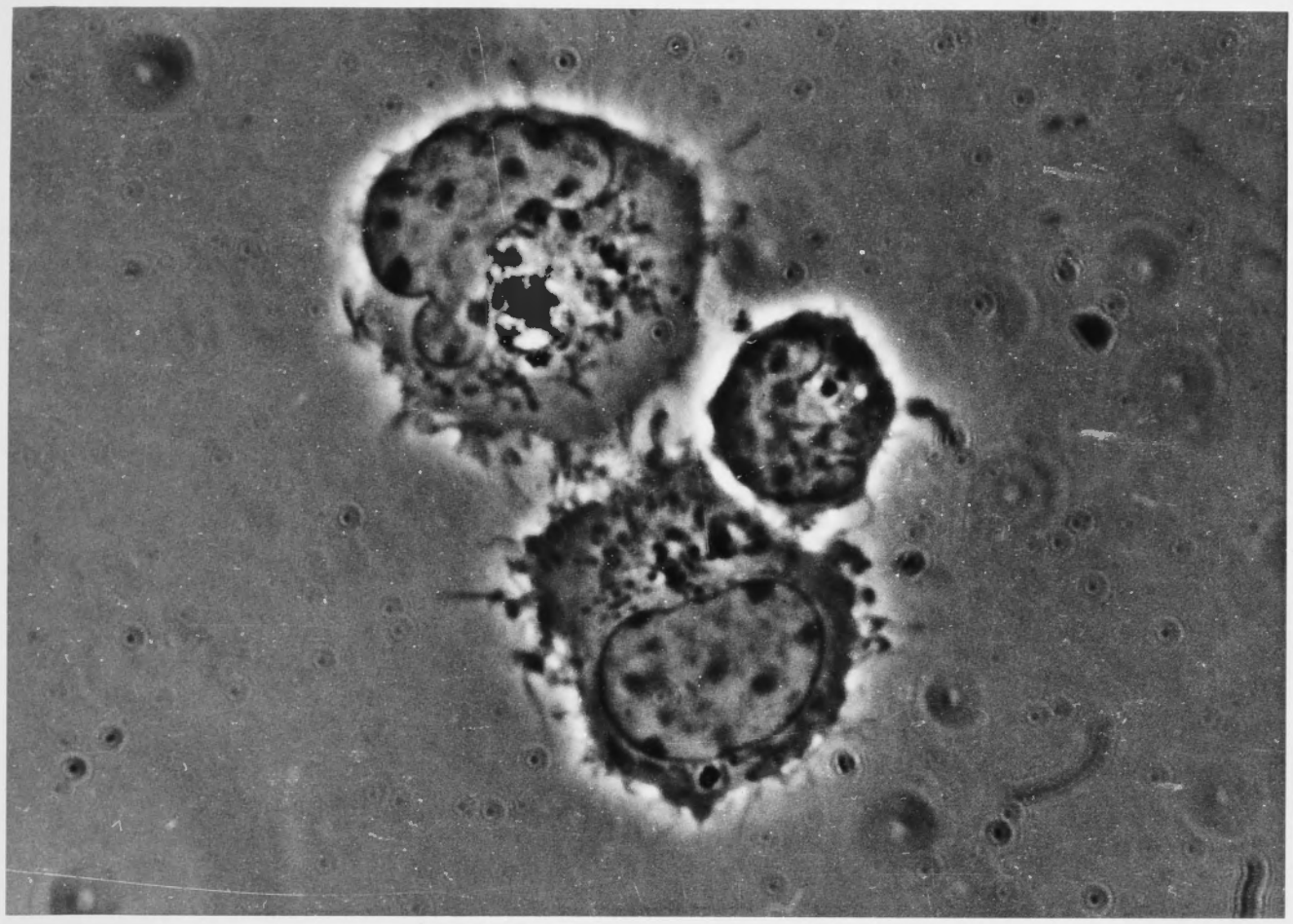
Large blast cells in mixtures of allogeneic sheep lymphocytes after 4 days incubation. Magnification x1500. Leishman stain.

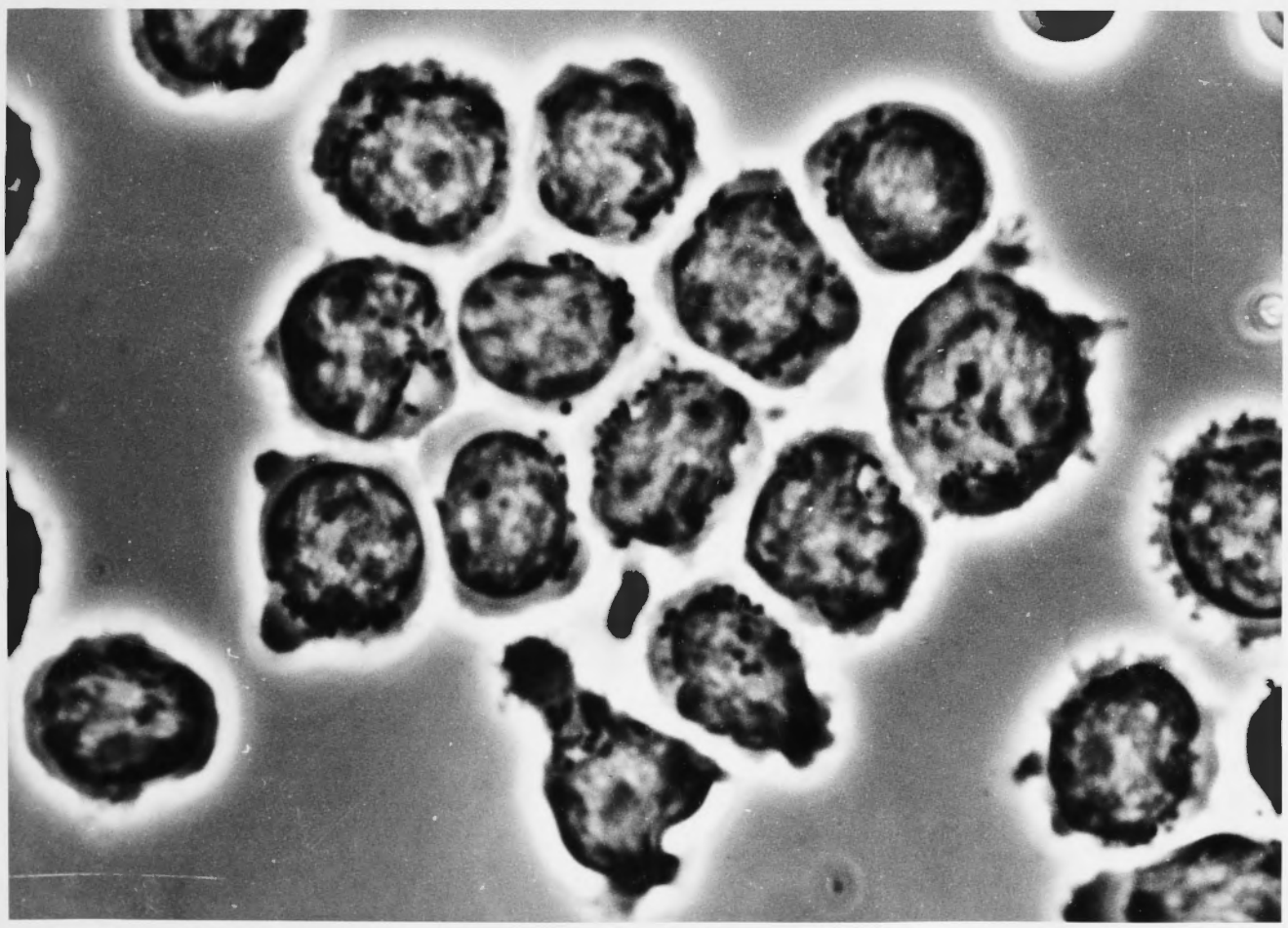
efferent ducts of popliteal lymph nodes of allogeneic sheep. The collections of lymph were made in sterile glass bottles containing 5 mls of anticoagulant solution. After collection, the lymphocytes were washed in Hank's balanced salt solution and resuspended at a concentration of 3 million per ml in EBM supplemented with 5 per cent inactivated sheep serum. Equal volumes (1.5 mls) of lymphocyte suspensions from two different donors were placed in screw capped vials 1.5 cms in diameter. Cultures consisting of 3 mls of unmixed lymphocyte suspensions in similar vials were treated in the same manner to act as controls. The mixtures and controls were incubated for 4 days at 37°C in air to which 5 per cent carbon dioxide had been added. The occurrence of a proliferative response in lymphocyte mixtures was determined by the appearance in leishman stained smears of blastoid cells with a large palely staining nucleus and abundant cytoplasm which was often vacuolated (Fig. 7.1). When cells from lymphocyte mixtures were examined by phase microscopy, the large blastoid cells were found to be highly motile and were often surrounded by medium lymphocytes (Fig. 7.2). An alternative method used to determine the occurrence of lymphoid cell proliferation in a mixture of allogeneic sheep cells was to measure the uptake of ^{3}H-thymidine by the lymphocytes as described in Chapter 2.

When the uptake of ^{3}H-thymidine by mixtures of allogeneic and xenogeneic lymphocytes was compared (Fig. 7.3) it was found that while there was a marked increase in the uptake of ^{3}H-thymidine by cells in mixtures of lymphocytes from allogeneic sheep donors, no such increase occurred in mixtures of sheep and goat or sheep and goose lymphocytes. These preliminary experiments showed that mixtures from xenogeneic donors did not stimulate the uptake of ^{3}H-thymidine in one another when cultured <u>in vitro</u> in spite of an antigenic difference. Thus lymphocytes show the same inability to react to xenogeneic

Figure 7.2

Phase photomicrographs showing the presence of large highly motile cells, closely associated with small lymphocytes in cultures of allogeneic sheep lymphocyte mixtures, 96 hours after mixing. The lower photograph is from unmixed cultures. Magnification x1200.





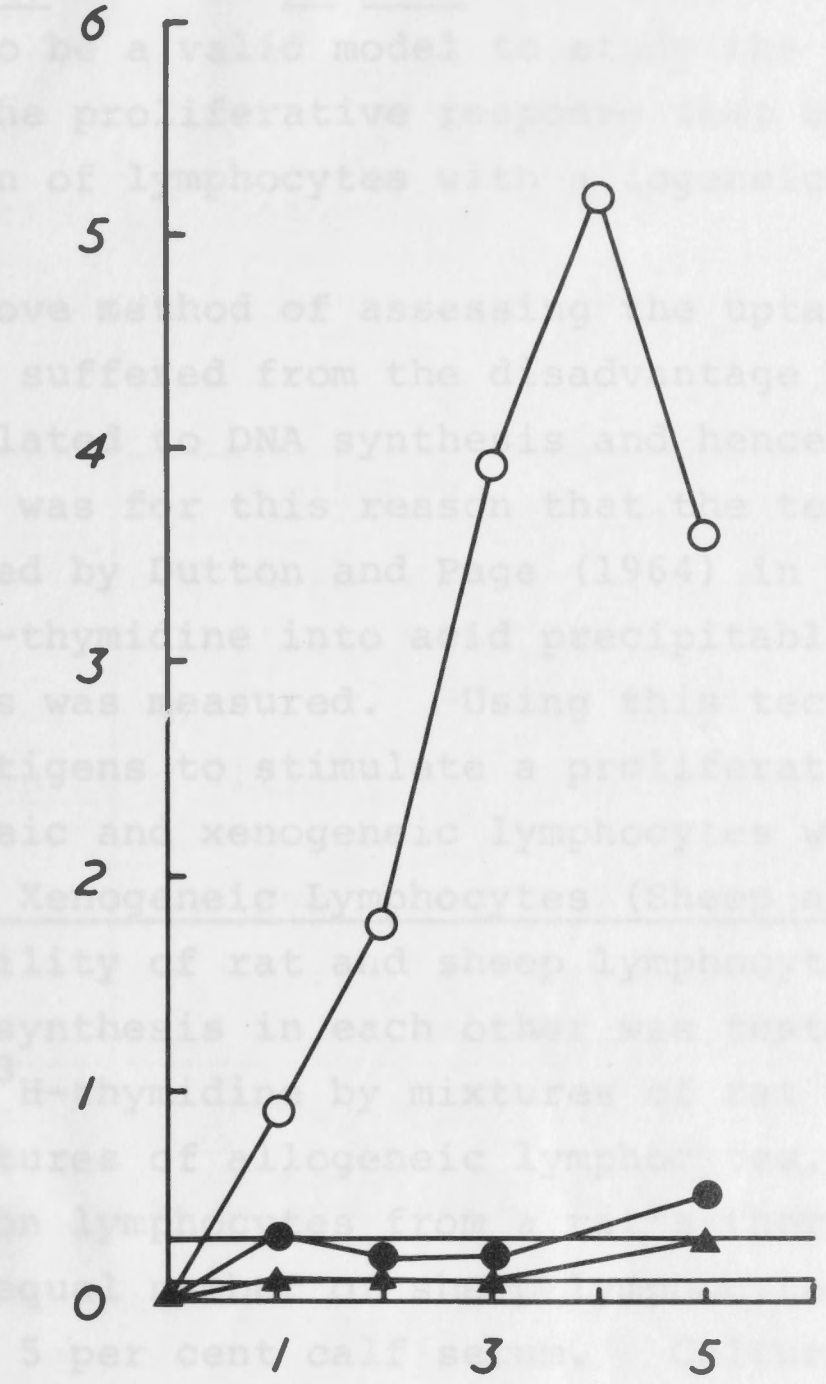


Figure 7.3

A comparison of the daily uptake of ^{3}H-thymidine (counts per minute) by mixtures of allogeneic and xenogeneic lymphocytes. The open circles represent mixtures of allogeneic sheep lymphocytes and filled circles and triangles represent sheep-goat and sheep-goose mixtures respectively.

tissues in vitro as seen in vivo and in this respect the MLR would appear to be a valid model to study the nature of the stimulus for the proliferative response that occurs following the interaction of lymphocytes with allogeneic reticular tissue.

The above method of assessing the uptake of ^{3}H-thymidine by lymphocytes suffered from the disadvantage that it could not be directly related to DNA synthesis and hence to a proliferative response. It was for this reason that the technique was changed to one described by Dutton and Page (1964) in which the incorporation of ^{3}H-thymidine into acid precipitable components of the lymphocytes was measured. Using this technique the ability of cellular antigens to stimulate a proliferative response in normal allogeneic and xenogeneic lymphocytes was studied.

Interaction of Xenogeneic Lymphocytes (Sheep and Rat)

The ability of rat and sheep lymphocytes to stimulate increased DNA synthesis in each other was tested by comparing the uptake of ^{3}H-thymidine by mixtures of rat and sheep lymphocytes with mixtures of allogeneic lymphocytes. In this experiment 4.5 million lymphocytes from a rat's thoracic duct were mixed with an equal number of sheep lymphocytes in 3 mls of EBM containing 5 per cent calf serum. Cultures containing 9 million unmixed lymphocytes and mixtures of allogeneic lymphocytes were treated in the same manner and used as controls. ^{3}H-thymidine was added to both test and control cultures after 3 days incubation and treated as described by Dutton and Page (1964). The results of the experiments are recorded in Table 7.1 where it can be seen that lymphocytes from allogeneic sheep when mixed in vitro incorporated significantly more thymidine than lymphocytes in mixtures of rat and sheep origin or control mixtures. These results confirm those obtained in the preliminary experiments and indicate that sheep lymphocytes are not stimulated in vitro by xenogeneic cells to syn-

TABLE 7.1

The incorporation of ^{3}H-thymidine (CPM) into the DNA of allogeneic and xenogeneic lymphocytes when mixed in vitro

Components of Mixture	MIXTURE Mean CPM SE(1)	CONTROLS Mean CPM SE(1)
Sheep 1 + Sheep 2	1275 ± 153 (4)*	468 ± 56 (4)
Rat + Sheep	474 ± 57 (4)	440 ± 52 (4)

* The figure in brackets refers to number of replicate cultures in each group.

(1) Method of calculation of SE given in appendix.

thesize DNA. Such observations do not support the proposition that the proliferative changes seen in the mixed lymphocyte reaction are primary immunological responses mediated by antigen. It is possible that lymphocytes are naturally hypersensitive to tissue antigens from allogeneic hosts. However, if these reactions are a true *in vitro* analogue of the events that occur when allogeneic lymphocytes are injected into chicken embryos, it is unlikely that this is the case (see Chapter 5).

The Nature of the Stimulus of the Mixed Lymphocyte Reaction

The role of antigen as the stimulus for the proliferative response seen in lymphoid cells during mixed lymphocyte reactions has never been conclusively demonstrated. But rather the importance of antigen in initiating such reactions has been inferred from the finding that mitotic activity is not stimulated when mixtures of syngeneic cells are cultured *in vitro*. If cellular antigens are the initiators of the MLR, then mild irradiation of lymphocytes from one sheep should not affect their ability to induce DNA synthesis in allogeneic lymphocytes with which they are mixed. To test this proposition, lymphocytes from two sheep were collected for 24 hours and prepared as described in Chapter 2. Lymphocyte suspensions from each donor received either 1000 or 6000 rads of γ-irradiation from a ^{60}Co source at the rate of 196 to 198 rads per minute. The irradiated cell suspensions were then divided into two equal parts, one of which was mixed with an equal number of unirradiated lymphocytes from the other donor sheep to provide 9 million cells per 3 mls of medium contained in each culture vial. The remaining portion of irradiated cells were cultured for a period of 24 hours before being mixed with an equal number of fresh lymphocytes from the other sheep. Lymphocytes from the same donors, both mixed and unmixed, were cultured in an identical manner, as controls. The incorporation of ^{3}H-thymidine was determined as described previously and the results are shown in Table 7.2. It can be seen that

TABLE 7.2

Effect of irradiation on the incorporation of ^{3}H-thymidine into mixed lymphocyte cultures

Cultured Lymphocytes	^{3}H-thymidine incorporation CPM/Culture ± S.E.(1)	
	Mixed Cultures	Control Cultures
Cells cultured immediately after the irradiation of one partner		
Sheep 1 + Sheep 2	2940 ± 220	186 ± 14
Sheep 1 + Sheep 2 (1,000r)	706 ± 71	82 ± 8
Sheep 1 + Sheep 2 (6,000r)	150 ± 15	84 ± 8
Sheep 1 (1,000r) + Sheep 2	197 ± 19	142 ± 14
Sheep 1 (6,000r) + Sheep 2	84 ± 8	142 ± 14
Cells cultured 24 hours after the irradiation of one partner		
Sheep 1 + Sheep 2 (1,000r)	85 ± 9	95 ± 10
Sheep 1 + Sheep 2 (6,000r)	75 ± 8	96 ± 10
Sheep 1 (1,000r) + Sheep 2	91 ± 9	213 ± 21
Sheep 1 (6,000r) + Sheep 2	130 ± 13	212 ± 21

(1) Method of calculation of SE given in appendix.

the irradiation of one component of the mixed culture caused a considerable depression of the amount of thymidine incorporated by the viable lymphocytes in a MLR. The effect of γ-irradiation was dose dependent and when cultures were prepared immediately after irradiation, it differed according to which of the two components of the system had been irradiated. This latter finding would suggest that the interaction between the two populations of lymphocytes was not completely reciprocal.

The reduced incorporation of thymidine that occurred when one member of the lymphocyte mixture was irradiated before mixing disappeared entirely if the irradiated cells were cultured for 24 hours before being mixed with unirradiated lymphocytes from an allogeneic donor. This reduced incorporation of thymidine in the mixtures of sheep lymphocytes with irradiated allogeneic lymphocytes may have been due to the release of toxic material or unlabelled nucleic acid by the irradiated cells. The degree by which the irradiated lymphocytes appeared to depress the thymidine incorporation into DNA of lymphocytes with which they are cultured was estimated using a mixture containing irradiated and unirradiated autologous lymphocytes. A sample of DNA synthesizing lymphoid cells leaving an antigen stimulated lymph node was subjected to either 1000 or 6000 rads of γ-irradiation and then mixed with equal numbers of unirradiated autologous cells to a final concentration of 3 million per ml of medium. Triplicate cultures each of 3 mls were prepared together with similar cultures of unmixed cells as controls and the uptake of ^{3}H-thymidine during a 24 hour period was determined in each. The results of the experiment are recorded in Table 7.3 where it can be seen that the addition of irradiated cells reduced ^{3}H-thymidine incorporation by about two fold. This reduction was not sufficient to account for the very much larger (20 to 30 fold) decrease in ^{3}H-thymidine uptake seen when one component of a lymphocyte mixture was irradiated 24 hours

TABLE 7.3

^{3}H-thymidine incorporation by a stimulated lymphocyte population in the presence and absence of irradiated autologous cells

Cultured Cells	^{3}H-thymidine incorporation CPM/Culture ± S.E.
Stimulated cells alone	2,370 ± 290
Stimulated cells + equal number of autologous cells (1,000r)	952 ± 270
Stimulated cells + equal number of autologous cells (6,000r)	1,160 ± 160

TABLE 7.4

Dermal response of sensitized guinea pigs to the inoculation of 5×10^6 normal or irradiated sheep lymphocytes

Animal	Time tested hrs. after inoculation	Increase in skin thickness at the site of inoculation, mm. The bracketed figure is the diameter of the red flare around the inoculation site in cm.		
		Normal lymphocytes	Irradiated lymphocytes (1,000r)	Irradiated lymphocytes (6,000r)
		Cells inoculated immediately after irradiation		
1	24	1.1 (3.0)	1.1 (2.5)	1.0 (1.5)
	48	2.4 (1.5)	2.4 (1.5)	1.0 (1.0)
2	24	1.7 (2.5)	2.0 (1.5)	2.1 (1.5)
	48	0.3 (2.0)	0.1 (1.5)	0.9 (1.5)
3	24	2.1 (2.0)	2.0 (2.0)	1.7 (1.5)
	48	1.0 (1.0)	1.2 (1.0)	1.6 (1.0)
		Cellsinoculated 24 hrs. after irradiation		
4	24	2.0 (2.0)	1.8 (2.5)	2.0 (3.0)
	48	0.5 (1.0)	1.0 (1.0)	1.5 (1.0)
5	24	2.5 (3.0)	2.0 (3.0)	3.1 (3.0)
	48	1.5 (1.5)	2.0 (1.5)	2.0 (1.5)
6	24	0.9 (1.5)	-	2.6 (2.0)
	48	1.8 (2.0)	-	1.1 (2.0)

before mixing.

It is possible that the radiation effect is the result of damage to cellular antigen. This is unlikely as Haskovà and Hilgert (1965) found that irradiation doses of up to 15000 rads did not destroy the antigenicity of spleen cells. In an experiment to examine the effect of irradiation on antigenicity, sheep lymphocytes that had received either 1000 or 6000 rads of γ-irradiation were inoculated intradermally into hypersensitive guinea pigs. The resultant dermal responses that were produced by the irradiated lymphocytes were compared with those resulting from intradermal injections of unirradiated lymphocytes from the same cell suspension. The results of this experiments are recorded in Table 7.4 where it can be seen that the increases in skin thickness and areas of erythema produced by 50 million irradiated lymphocytes both before and after 24 hours culture are the same as those produced by unirradiated lymphocytes. These observations would suggest that the antigens on sheep lymphocytes, to which the guinea pigs had been sensitized, were not affected by the doses of irradiation used in this experiment.

Discussion

The experimental results presented in this chapter demonstrate that the cells in mixtures of lymphocytes obtained from xenogeneic donors are not stimulated to incorporate ^{3}H-thymidine during culture *in vitro*. This failure of xenogeneic lymphocytes to stimulate each other cannot be ascribed to a lack of antigenic stimulation, since under similar conditions lymphocytes from the same preparation will induce increased thymidine incorporation in allogeneic lymphocytes. It might be argued that the mutual stimulation of allogeneic lymphocytes is not a primary response but rather it is a response of sensitized cells to antigen, similar to that described by Pearmain

et al. (1963). This possibility can be discounted since the results of experiments have shown that lymphocytes which have been subjected to 1000 rads of γ-irradiation followed by 24 hours incubation at 37°C were unable to stimulate DNA synthesis in allogeneic lymphocytes. The irradiated lymphocytes were shown to be antigenic, by their ability to produce delayed-type hypersensitivity reactions in hypersensitive guinea pigs. The finding that the material present in lymphocytes responsible for stimulating DNA synthesis is sensitive to ionizing radiation whilst cellular antigens are not is different from observations of other workers (Kasakura and Lowenstein, 1967; Main et al., 1967). These workers were able to produce unidirectional stimulation of allogeneic leucocytes with γ-irradiated leucocytes from both man and dog. Kasakura and Lowenstein (1967) found that human blood leucocytes even after 6000 rads were capable of stimulating DNA synthesis in the other human leucocytes in a mixture. It is difficult to compare these observations with the findings reported for sheep lymphocytes since the leucocyte preparations used by Kasakura and Lowenstein were able to withstand much higher doses of irradiation than sheep lymphocyte preparations. This was clearly shown by the ability of leucocyte preparations to incorporate ^{3}H-thymidine in response to phytohaemagglutinin stimulation after they had received doses of x-irradiation of more than 5000 rads. This contrasts markedly with the tolerance of sheep lymphocytes to γ-irradiation in which 1000 rads was sufficient to reduce the incorporation of ^{3}H-thymidine into lymphocytes from a stimulated node to background level.

In view of the above findings, it is difficult to maintain the hypothesis that antigen is the factor responsible for the stimulation of mitotic activity that occurs when allogeneic lymphocytes are cultured in vitro. If this reaction is a valid model of reactions of the GVH type, and the more recent experiment

reported by Wilson (1967) and Wilson et al. (1967) suggest very strongly that it is, then the experiments described above add further support to the concept that reactions of the GVH type do not result from the interaction of immunologically competent lymphocytes with foreign antigen.

Summary

When a mixture of equal numbers of lymphocytes obtained from two allogeneic sheep are cultured together a response is obtained that may be measured either in terms of the incorporation of radioactive thymidine into an acid precipitable cell fraction or by the numbers of large, motile blast cells formed. Lymphocytes from sheep are not stimulated to incorporate radioactive thymidine by lymphocytes obtained from different species of animals such as the rat, goose or goat. The treatment of one population of sheep lymphocytes with γ-rays abolished their ability to stimulate the uptake of radioactive thymidine in allogeneic lymphocytes in vitro 24 hours later. γ-irradiated sheep lymphocytes were shown to be antigenic in hypersensitive guinea pigs. It was concluded that stimulation of the uptake of ^{3}H-thymidine by cultures of allogeneic lymphocytes was unlikely to be by antigen.

CHAPTER 8

THE HISTOPATHOLOGY OF THE NLT REACTION IN SHEEP

Chapter 8 - The Histopathology of the NLT Reaction in Sheep

Introduction

The NLT reaction has been described by Brent and Medawar (1966) as a local GVH reaction. The experiments dealt with in the foregoing chapters made it apparent that the prominent lymphoid cell proliferation in spleens of chicken embryos undergoing GVH reactions is a result of the interaction of the donor's lymphocytes with elements of the reticular tissue of the recipient. The MLR, and it is likely, the NLT reaction, are also manifestations of the same interaction of allogeneic lymphoid cells. When mixtures of sheep lymphocytes obtained from allogeneic donors are cultured *in vitro*, blast cells appear in the cultures after 3 days. These large blast cells, which result from the interaction of allogeneic lymphocytes, resemble the large pyroninophilic cells which Gowans *et al.*(1963) described as occurring in recipients' spleens during GVH reactions in rats. This GVH reaction was produced by lymphoid cells syngeneic to the recipient, reacting against allogeneic lymphoid cells that had colonized the recipient's spleen following their previous injection to induce tolerance in the young animal. As a result of this produced tolerance, the recipient bore a skin allograft. This skin graft was being rejected by effector cells that were identical to the large pyroninophilic cells seen in the spleen, where a GVH reaction was occurring. It has been shown in previous experiments (Chapter 4) that tissue destruction of allografts in "treated" chicken embryos occurred in close association with large reticular cells which were morphologically similar to cells that appeared in the spleens of embryos undergoing a GVH reaction. These large reticular cells, like the blast cells which appeared in lymphocyte cultures, and during GVH reactions, probably resulted from the interaction of allogeneic lymphocytes. The production of large blast cells does

not occur following the confrontation of lymphocytes from donors of distantly or unrelated xenogeneic species.

On the basis of the morphological similarities between the blast cells which were seen to occur following lymphocyte-lymphocyte interaction both *in vitro* and *in vivo*, the histological changes which occurred in the dermal responses evoked by intradermal injections of allogeneic and xenogeneic lymphoid cells in sheep were examined for the presence of these cells. At the same time an attempt was made to establish whether there was any relationship between the presence of large blast cells and tissue destruction which may occur within NLT reactions.

Experimental

The Histopathological Changes Occurring in the Normal Lymphocyte Transfer (NLT) in Sheep

The histopathology of the NLT reaction in sheep was studied by the examination of serial biopsy specimens. The biopsy specimens were removed at daily intervals from a sheep that had received multiple intradermal injections of 5 million allogeneic lymphocytes. After being fixed in formol saline the biopsy samples were prepared for histological examination as described in Chapter 2.

Figure 8.1 compares the histological appearance of daily biopsy specimens that were obtained immediately before and up to 7 days after the inoculation of the allogeneic lymphocytes. It can be seen that there is an increase in the cellularity of the dermis which was most pronounced during the period 4 to 7 days after the injection of lymphocytes. The cellular infiltration during this period tended to occur as discrete collections of cells separated by bands of connective tissue. When these sections were examined at a greater magnification, the cells infiltrating the dermis during the first 24 hours were found to be mainly small round cells together with a few polymorphonuclear

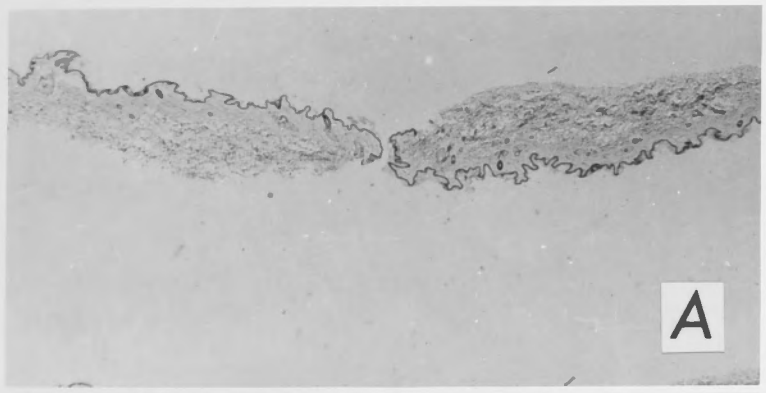
Figure 8.1

A comparison of the cellular infiltration of the dermis of a sheep during NLT reactions produced by the inoculation of 5 million allogeneic lymphocytes. Biopsy specimens were removed at daily intervals.

A Normal sheep's skin
B 1 day NLT
C 2 days NLT
D 3 days NLT
E 4 days NLT
F 5 days NLT
G 6 days NLT
H 7 days NLT

Magnification 6.5x

Stain haematoxylin and eosin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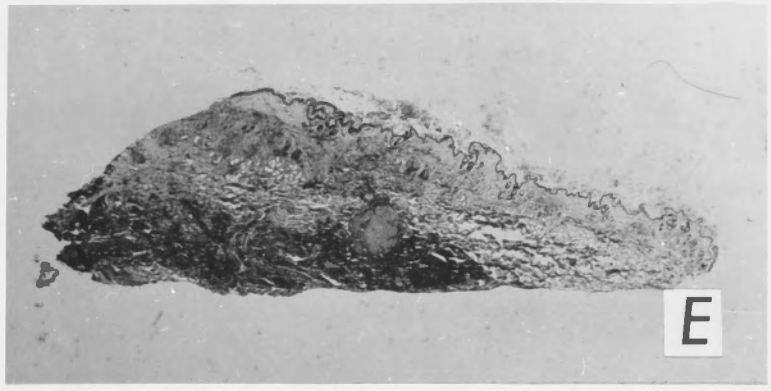
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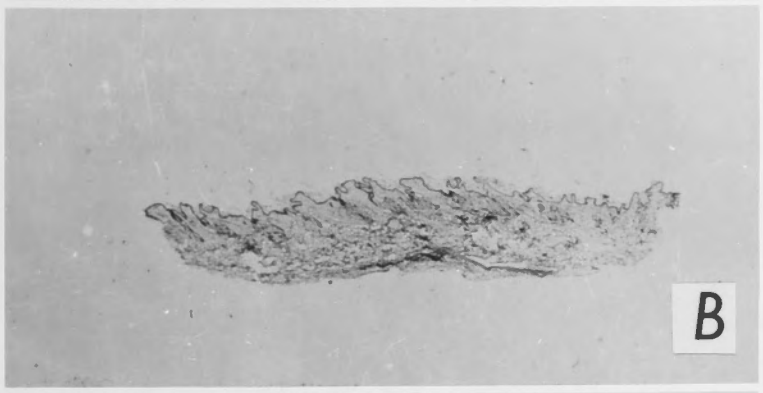
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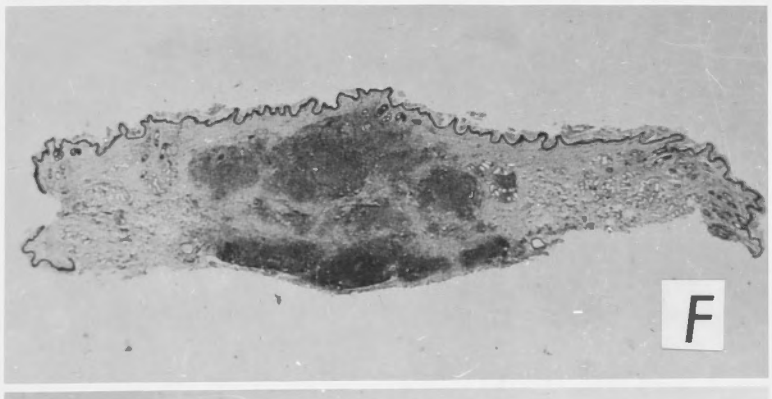
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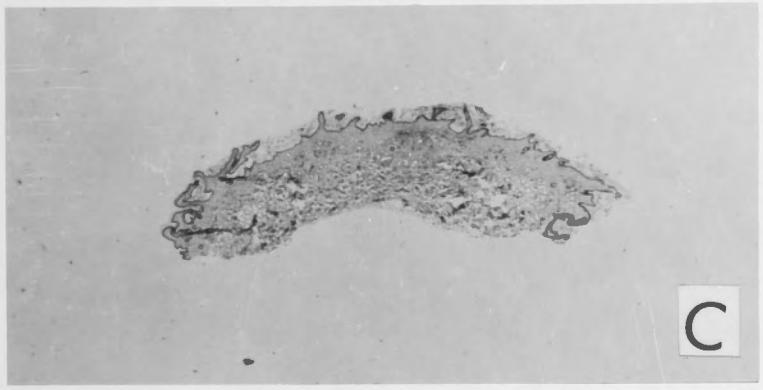
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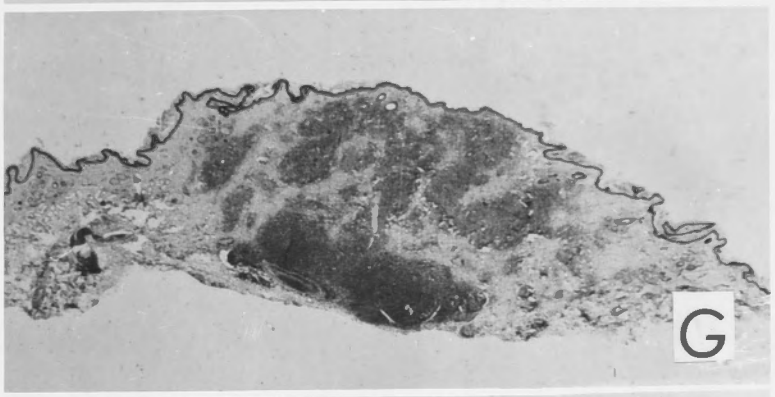
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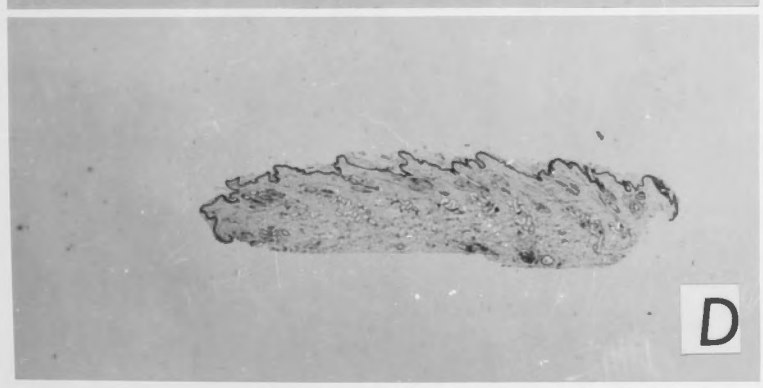
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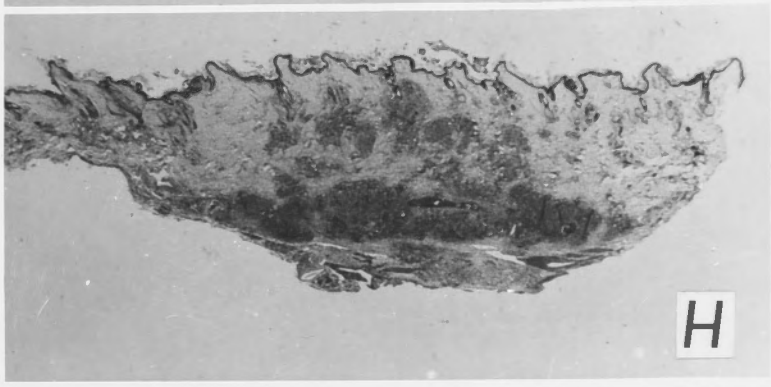
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neutrophils (Fig. 8.2A, B and C). Two days after the lymphocytes had been injected, there was an increased diffuse cellular infiltration of the dermis (Fig. 8.2D) with collections of cells occurring around blood vessels in the deeper layers, and around sebaceous glands and wool follicles in the more superficial layers. At this stage the infiltrating cells were still mostly small round cells, but a few pleiomorphic cells with large palely staining nuclei, 1 to 3 prominent nucleoli and pyronino-philic cytoplasm were present (Fig. 8.3A). When 1 µc of ^{3}H-thymidine in 0.1 ml EBM was injected into the NLT reaction, many of these large cells were found to incorporate the labelled thymidine during the next 8 hours which preceded the biopsy of the lesion (Fig. 8.4). These large cells will be referred to as blast cells. Three days after the intradermal injection of allogeneic lymphocytes, the collections of mononuclear cells at the site of injection had become much more conspicuous. Figure 8.3 (B and D) shows one such perivascular collection of cells consisting mostly of small round cells but including a greater number of blast cells as compared with similar cell collections in NLT reactions of one and two days duration.

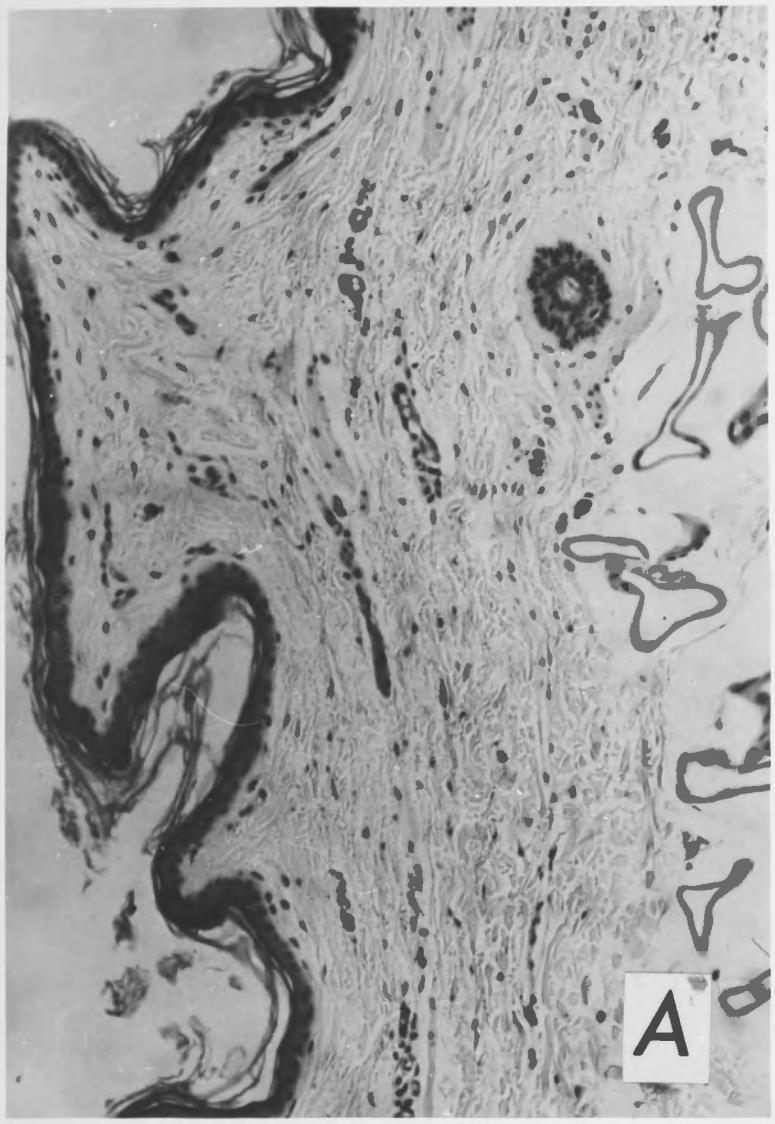
A careful examination of cellular structures situated within an NLT reaction and surrounded by cells that have infiltrated the area do not show any signs of destruction or invasion. However, 4 to 5 days after the injection of allogeneic lymphocytes, it will be seen in Figure 8.3C that there has been a rapid increase in the amount of cellular infiltration of the dermis and a marked increase in the number of blast cells in the collections of mononuclear cells within the NLT reactions (Figs. 8.3D and 8.5C and D). The epidermis overlying the NLT reaction becomes markedly thickened 5 to 6 days after the inoculation of the lymphocytes (Fig. 8.5A and B) and when the cellular structures of the dermis situated within such reactions are examined, many of these show signs of invasion by mononuclear

Figure 8.2

A comparison of the histological appearance of NLT reactions produced in sheep by 5 million allogeneic lymphocytes with normal skin.

A and B	Normal skin
C	NLT reaction after 1 day
D	NLT reaction after 2 days

Magnification 130x Stain haematoxylin and eosin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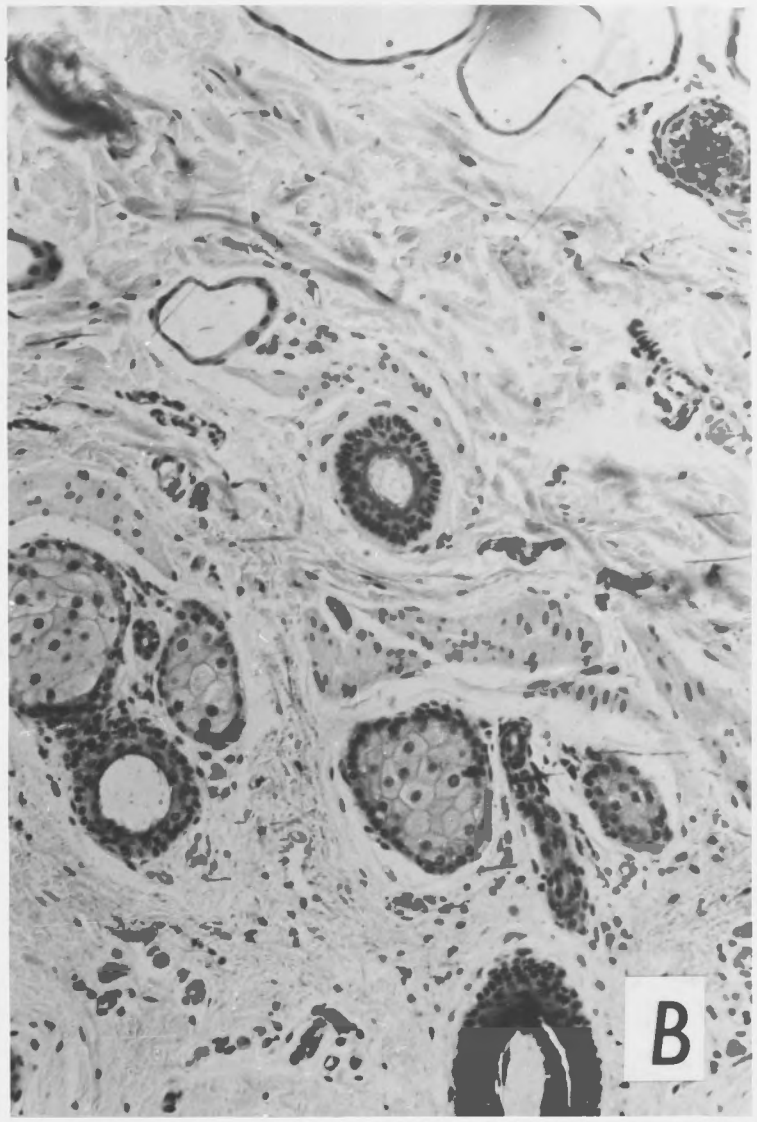
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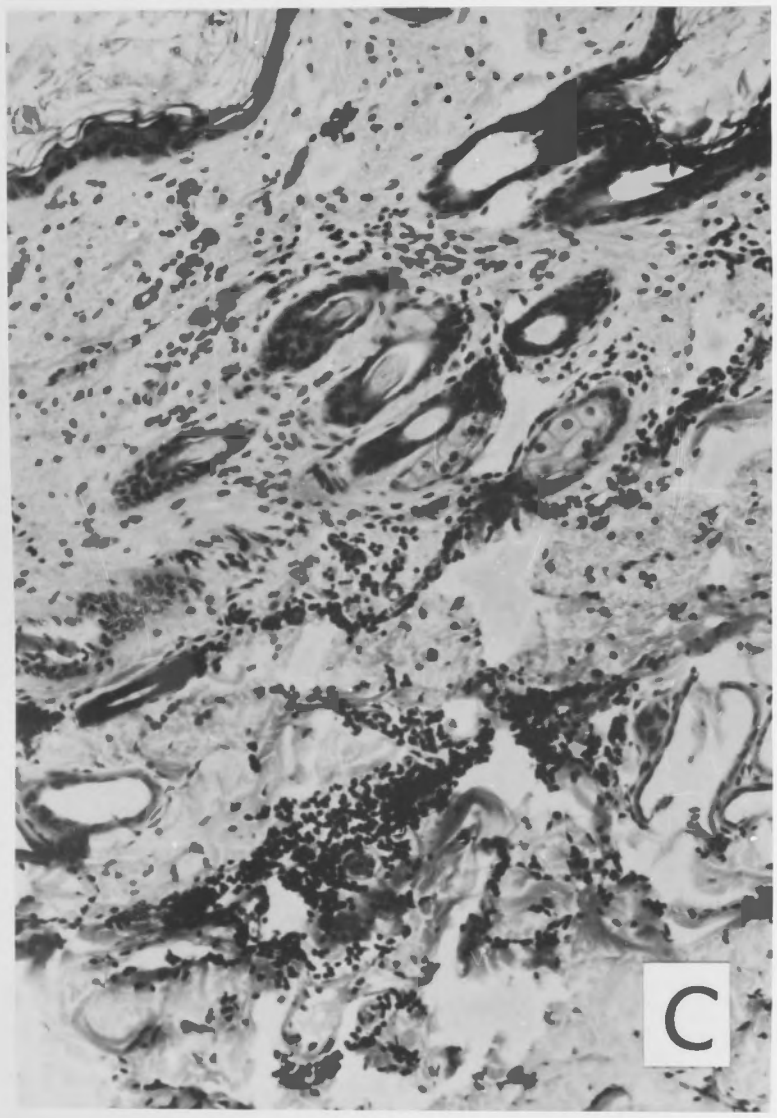
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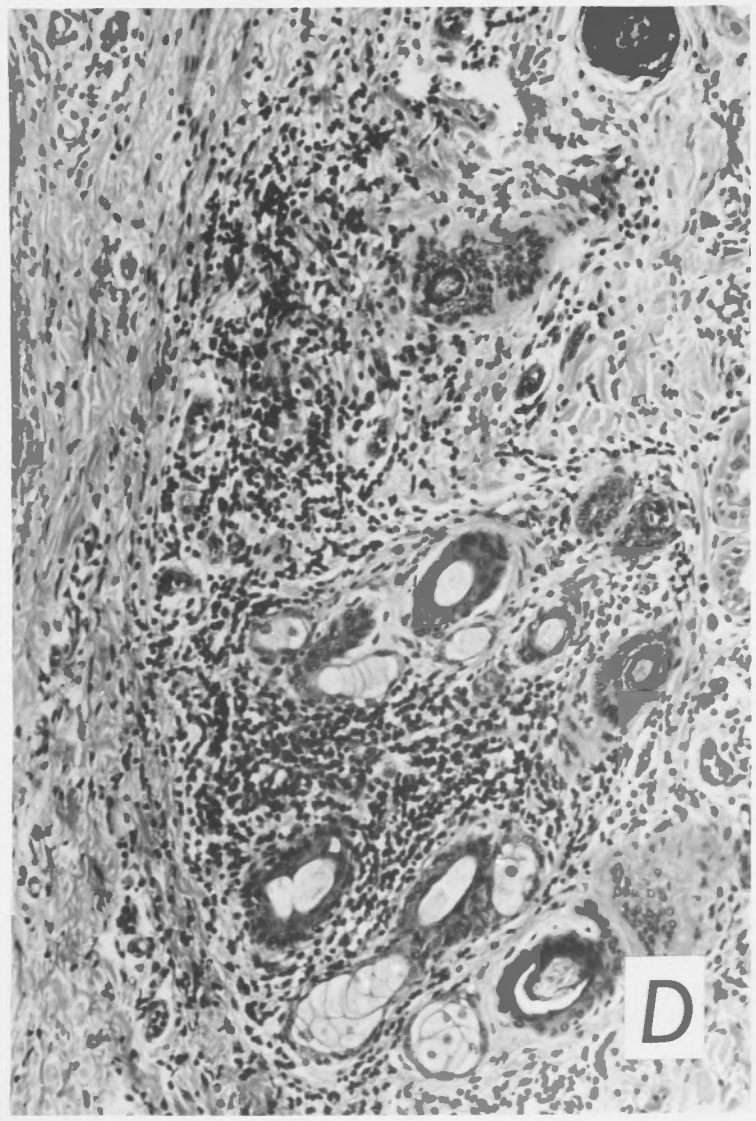
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Figure 8.3

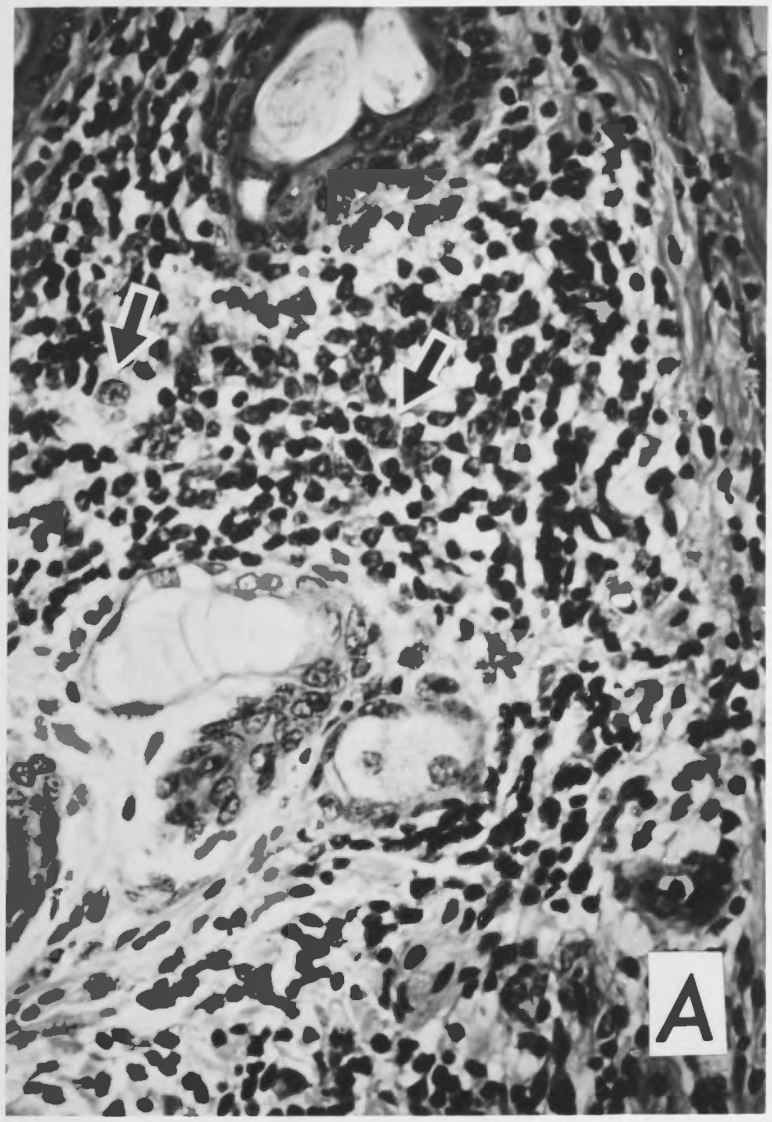
The histological appearance of NLT reaction produced in the sheep by 5 million allogeneic lymphocytes at different time intervals.

A Appearance of "blast" cells (arrowed) in cellular collections after 2 days. Magnification 330x, haematoxylin and eosin.

B Perivascular collection of cells after 3 days. Magnification 330x, haematoxylin and eosin.

C Collection of cells after 5 days showing necrosis (arrowed) around a capillary. Magnification 130x, haematoxylin and eosin.

D A comparison of the pyroninophilic cytoplasm of blast cells (arrowed). The pyronin appears black. Magnification 330x, methyl green and pyronin.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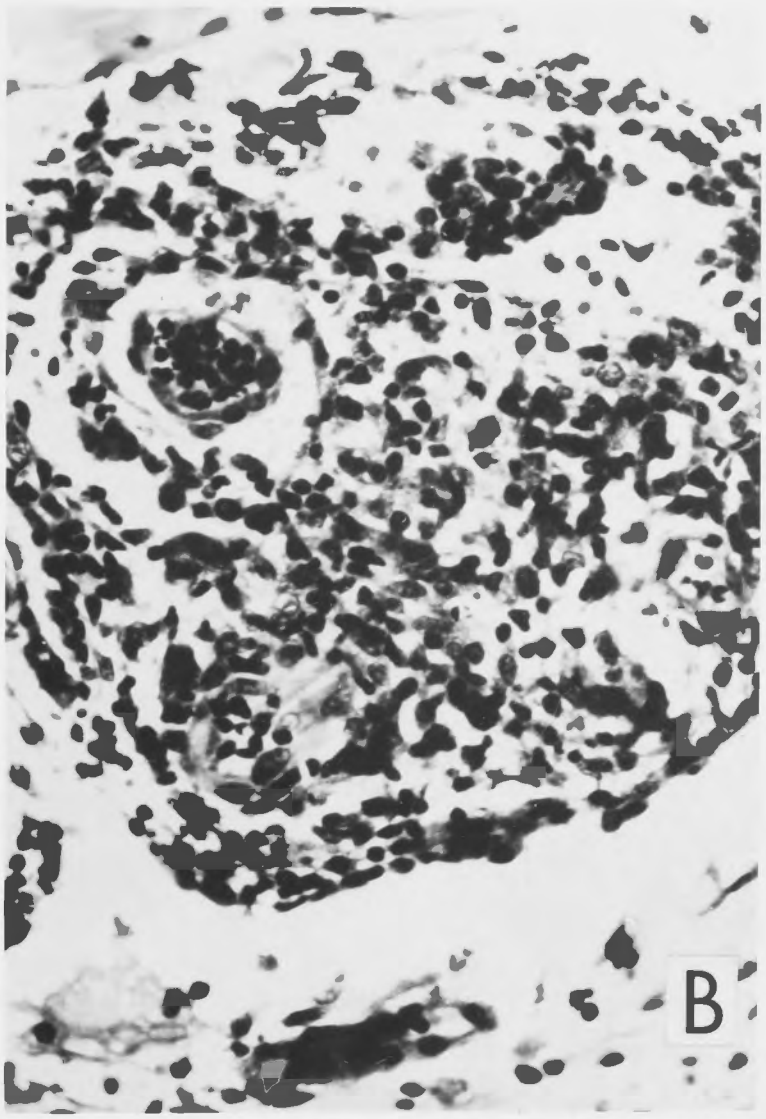
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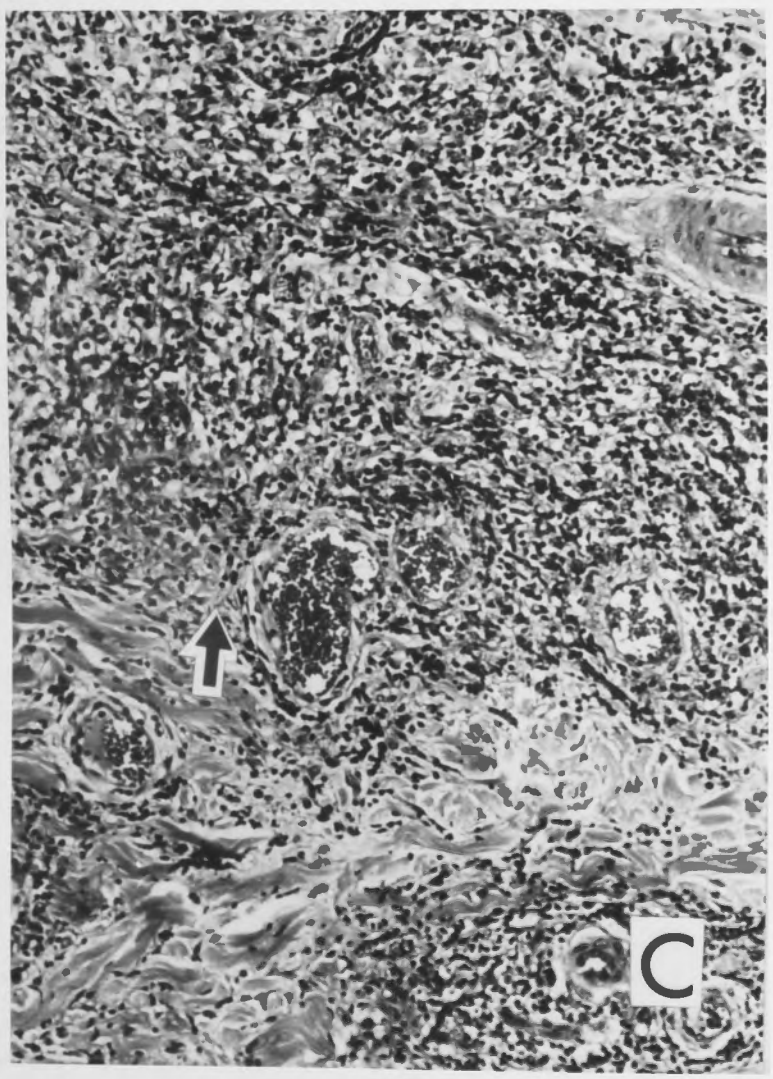
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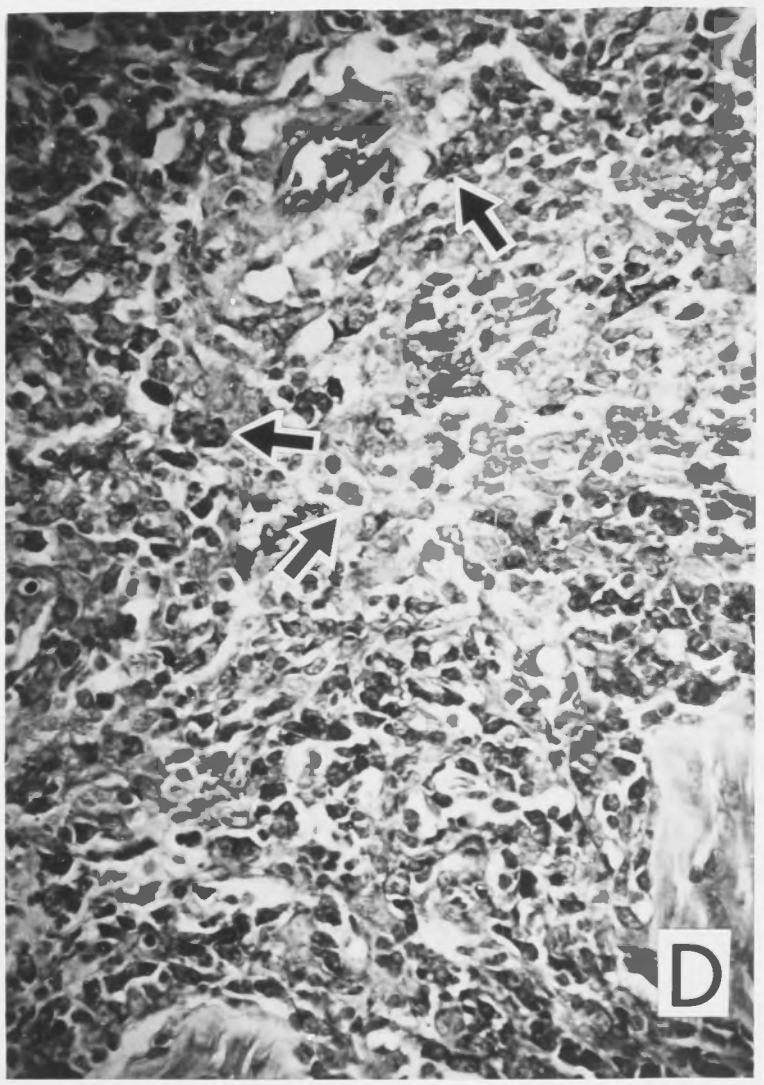
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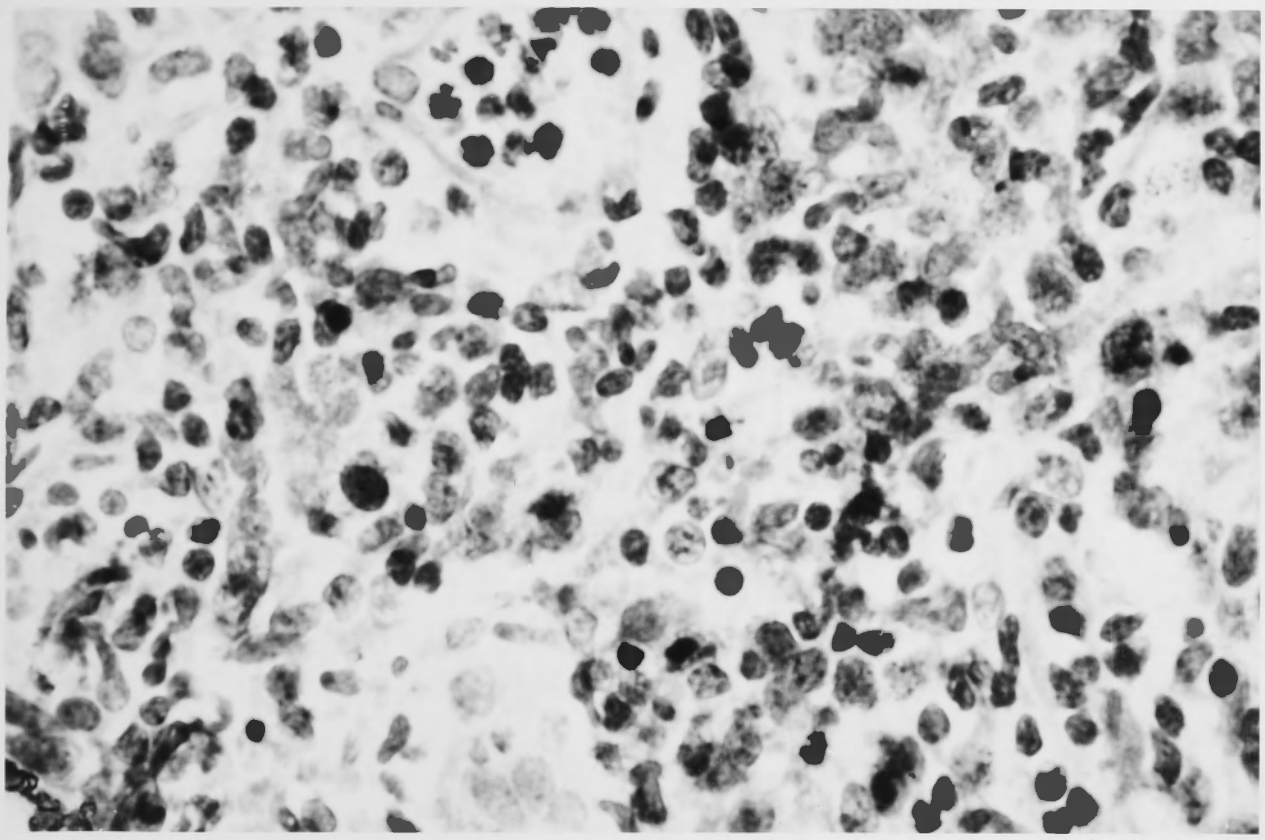
Figure 8.4

Demonstration of the uptake of ^{3}H-thymidine into blast cells in a 5 day old NLT reaction in sheep. 1 µc of ^{3}H-thymidine was injected into the NLT reaction 8 hours before biopsy. The upper photograph demonstrates the cellular morphology of the cells which have incorporated ^{3}H-thymidine as is demonstrated by the presence of grains in the lower photograph.



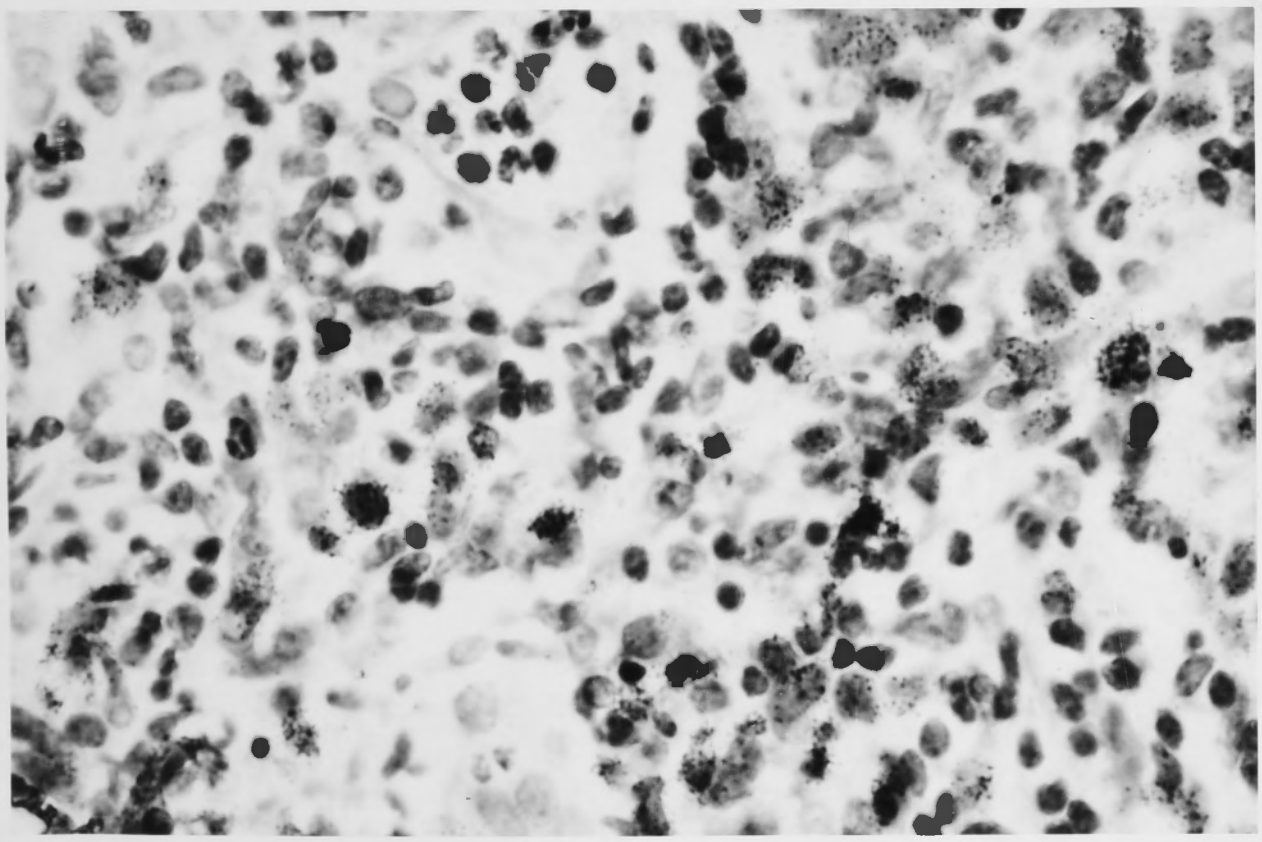


Figure 8.5

A comparison of the changes seen in the epidermis overlying a 6 day old NLT reaction with the normal sheep's epidermis. Together with a demonstration of the collection of blast cells and their morphological appearance in a 5 day old NLT reaction.

A Epidermis from a normal sheep.
Magnification 330x, haematoxylin and eosin.

B Epidermis over a 6 day old NLT reaction showing hyperplasia. Magnification 330x, haematoxylin and eosin.

C Collection of blast cells, showing invasion of adjacent cellular structures. Magnification 130x, haematoxylin and eosin.

D Higher magnification of a collection of blast cells from C, showing their large palely staining nucleus, prominent nucleolus and abundant cytoplasm. Magnification 540x, haematoxylin and eosin.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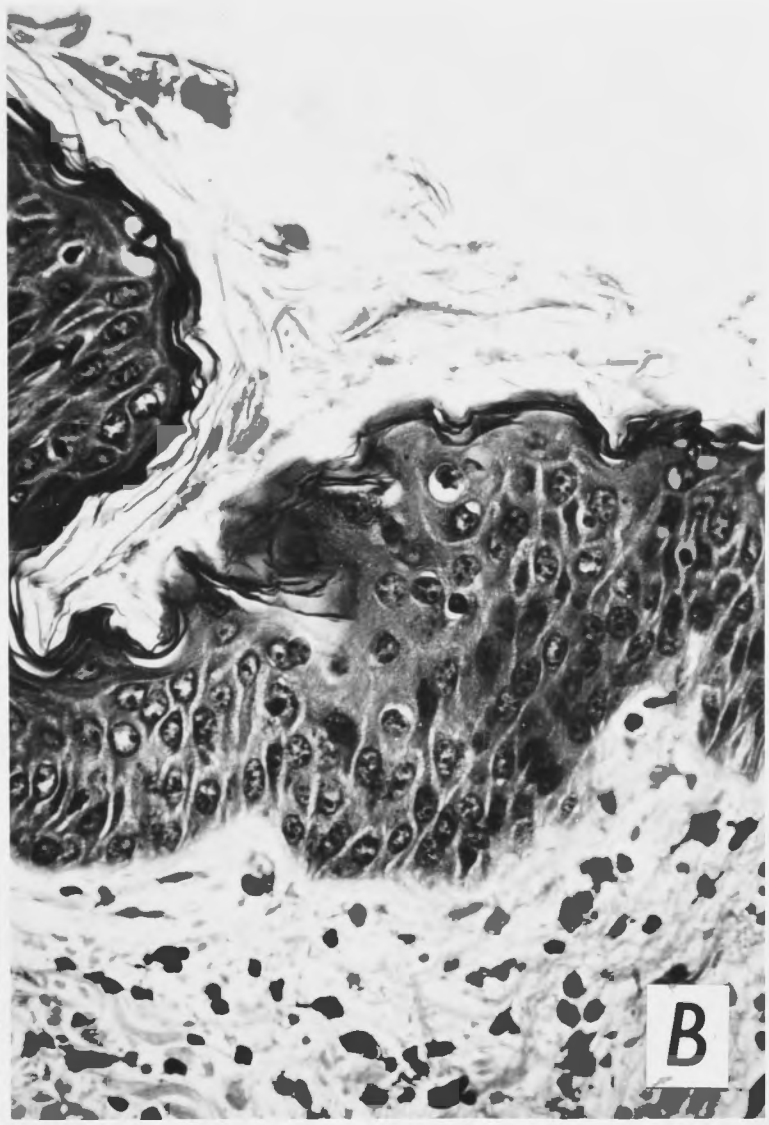
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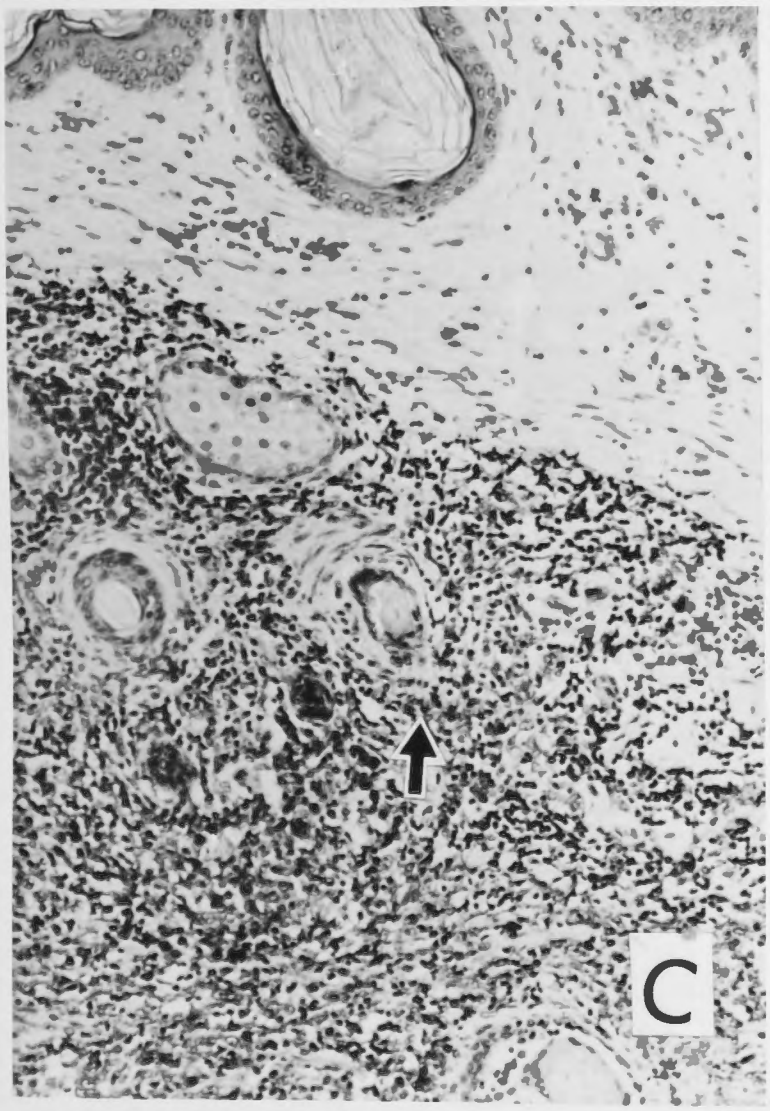
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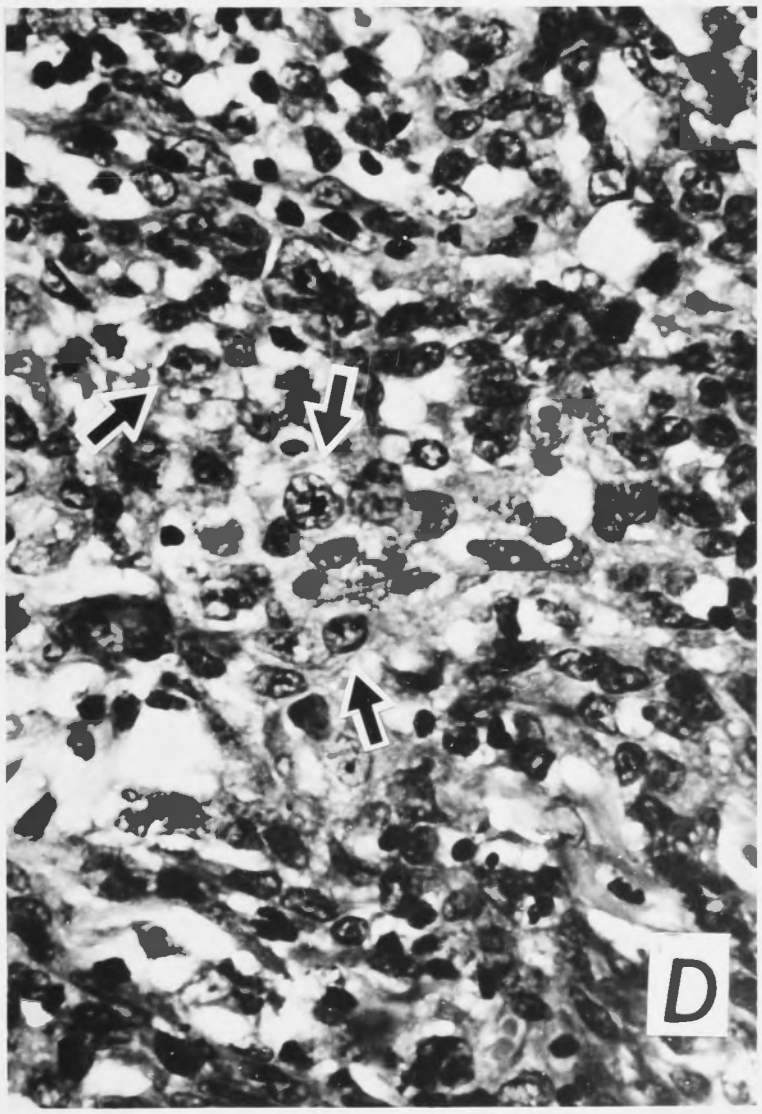
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cells migrating from the surrounding collections of infiltrating cells. The structures which show signs of invasion are the walls of small arteries (Fig. 8.6), sebaceous glands (Fig. 8.7C) and wool follicles (Figs. 8.7D and 8.8A and B). Not all the cellular structures present in the skin, where an NLT reaction occurs, become invaded by mononuclear cells (Figs. 8.6D and 8.7A and B), but the uninvaded structures are usually surrounded by collections of cells that are devoid of blast cells.

5 to 6 days after the injection of lymphocytes, areas of necrosis appear throughout the parts of the NLT reaction where cellular infiltration has occurred. These areas of necrosis were often adjacent to the many prominent blood vessels in the lesion (Fig. 8.9A) and were surrounded by large blast cells (Fig. 8.9B). The necrotic areas sometimes coalesced until the whole central portion of the reaction was filled by necrotic material (Fig. 8.9D).

In addition to the cellular changes seen in the NLT reaction, there was some oedematous swelling of the surrounding dermis with distention of the tissue spaces and lymphatics (Fig. 8.9C). Another feature of the NLT reaction was the build up of reticulin fibres as a supporting framework which began 1 day after the inoculation of the allogeneic lymphocytes (Fig. 8.10).

Histopathology of NLT Reactions Produced by Normal Xenogeneic Lymphocytes

When dermal reactions were produced by 10 million normal xenogeneic lymphocytes, they were usually smaller than those seen at the site of injection of allogeneic lymphocytes. Lymphoid cells from normal guinea pigs and chicken failed to produce any detectable histopathological changes when injected into the skin of sheep.

The histopathological changes that occurred in the skin of the sheep following the intradermal injections of goat or

Figure 8.6

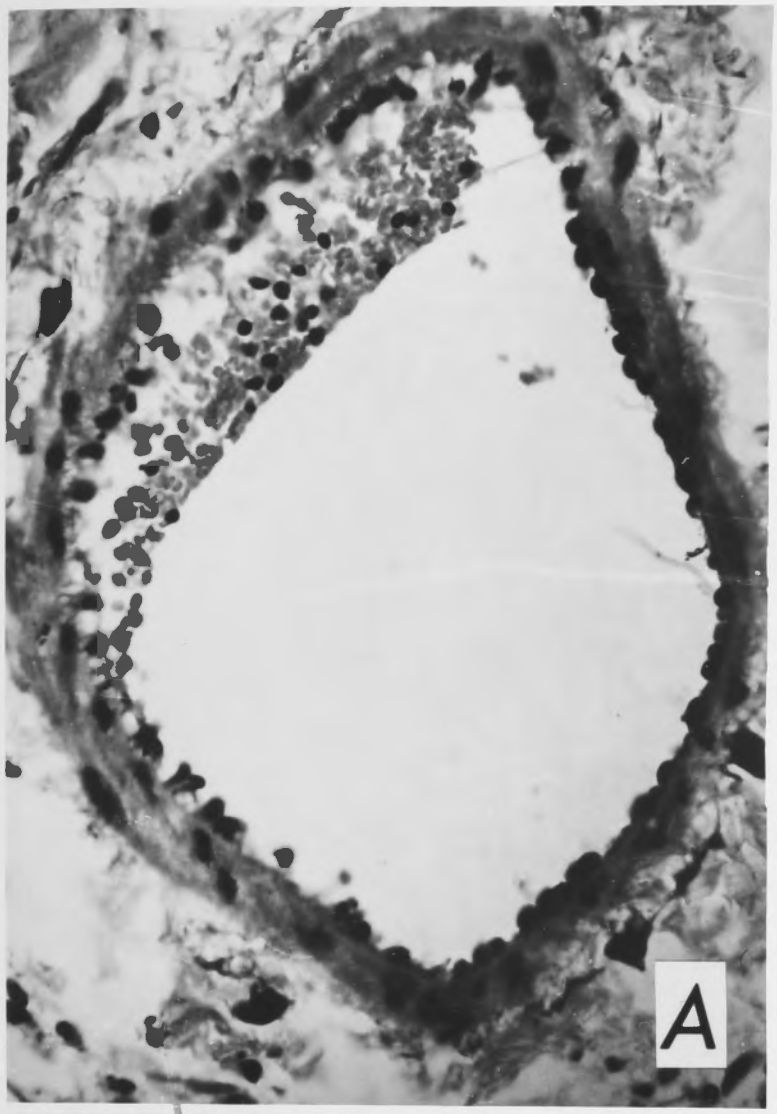
The invasion of the walls of small blood vessels by leucocytes and blast cells in 5 and 6 day old NLT reactions.

A Margination and invasion of the wall of small venule (5 days). Magnification 330x, haematoxylin and eosin.

B Invasion of the wall of a small artery. Magnification 130x, haematoxylin and eosin.

C Higher magnification of the area of B indicated by arrow. Large blast cells can be seen within the vessel wall. Magnification 330x, haematoxylin and eosin.

D Higher magnification of the area of blood vessel wall (from B) not invaded by cells showing the absence of blast cells. Magnification 330x, haematoxylin and eosin.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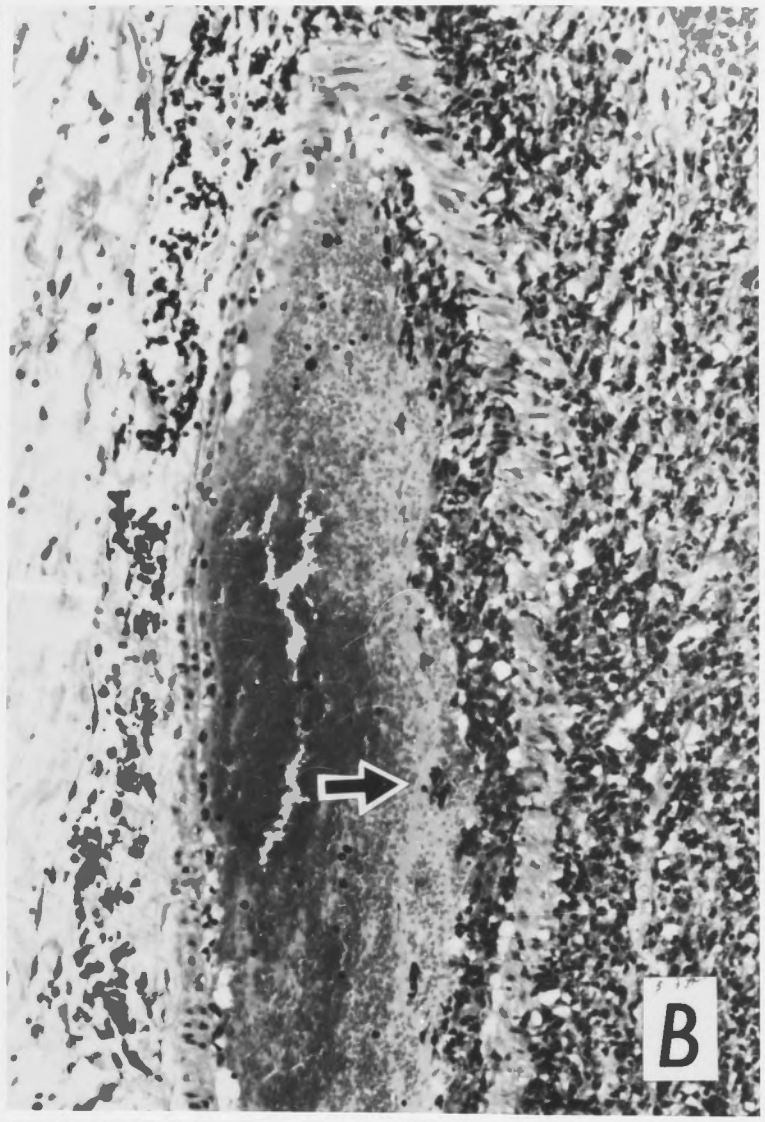
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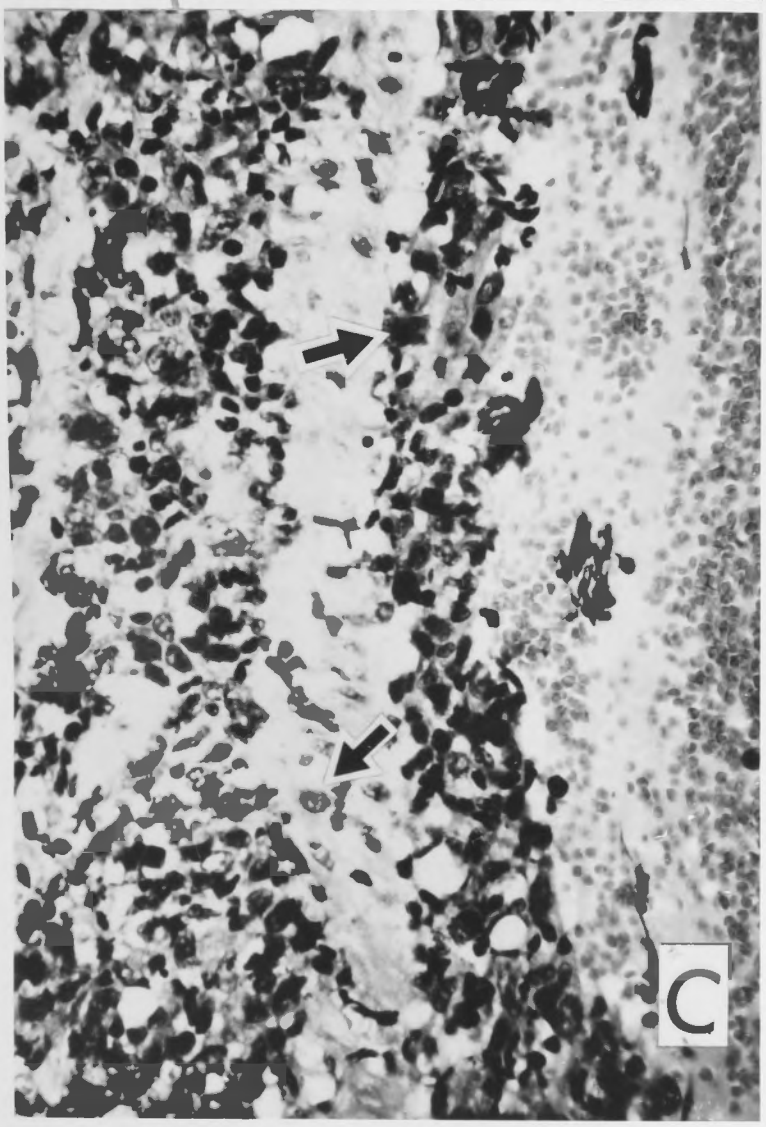
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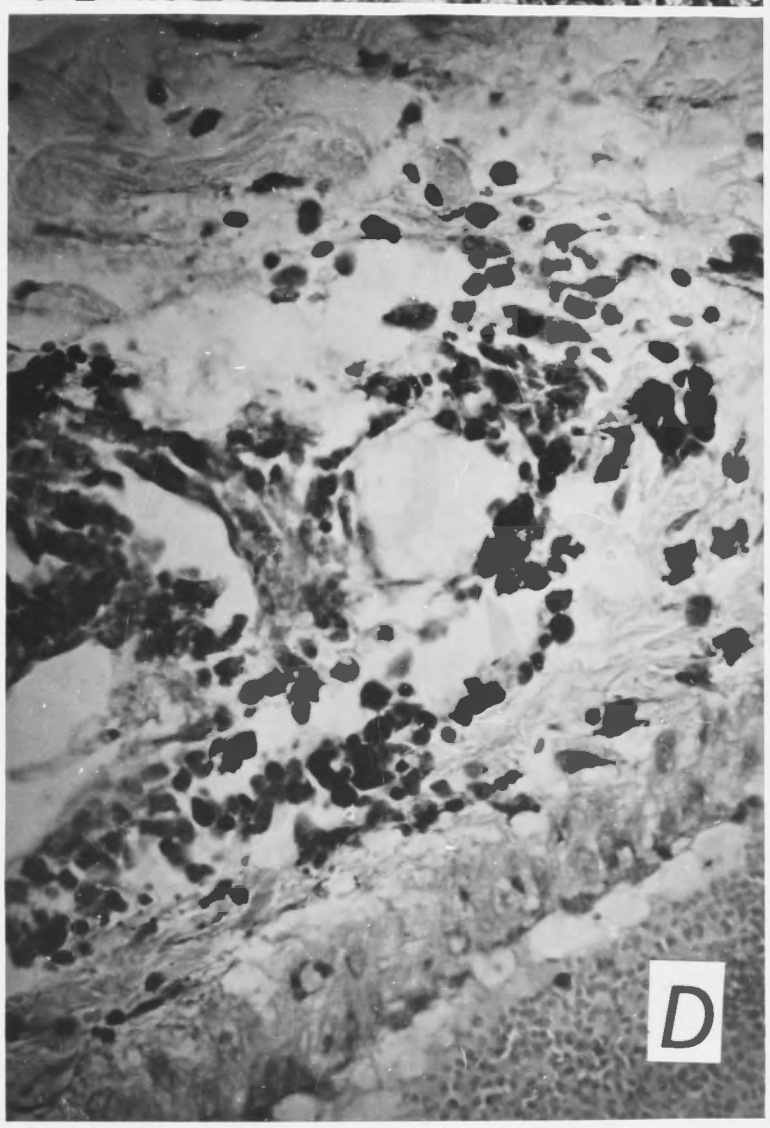
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Figure 8.7

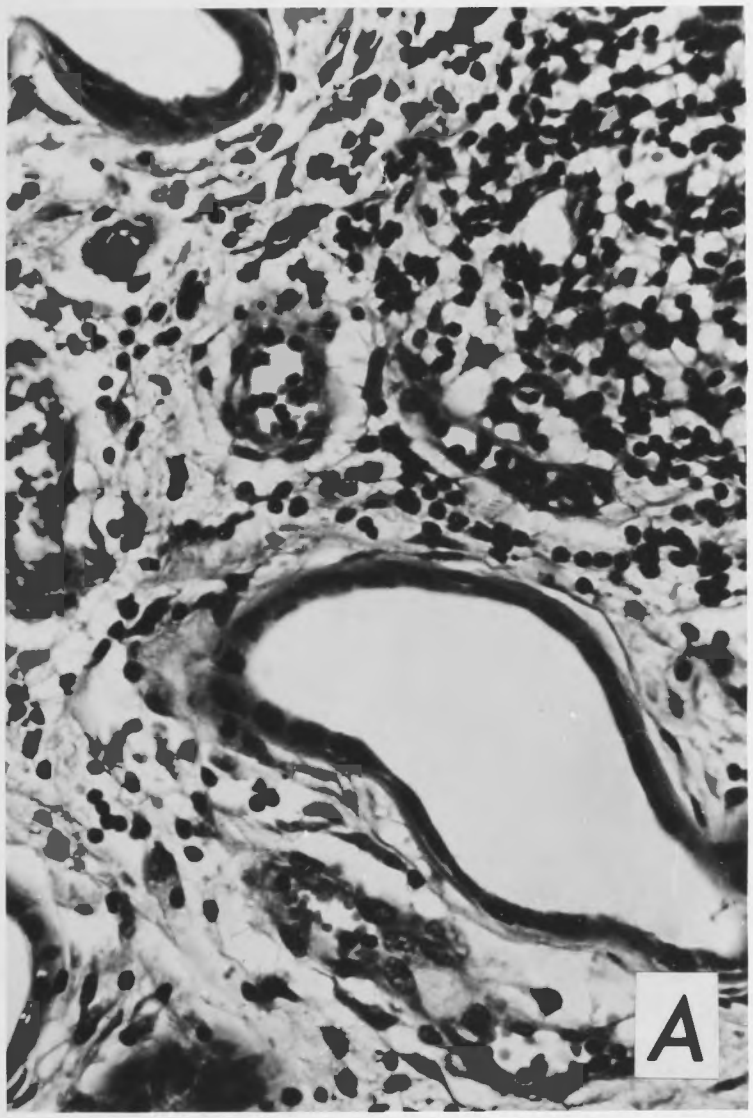
A comparison of the invasion of cellular structures situated within collections of blast cells by surrounding mononuclear cells in 5 and 6 day old NLT reactions.

A and B Coiled sweat glands in 5 day old NLT reaction showing no invasion of their walls.

C Invasion of sebaceous gland by mononuclear cells in a 5 day old NLT reaction.

D Invasion of wool follicle by mononuclear cells in a 5 day old NLT reaction.

Magnification 330x haematoxylin and eosin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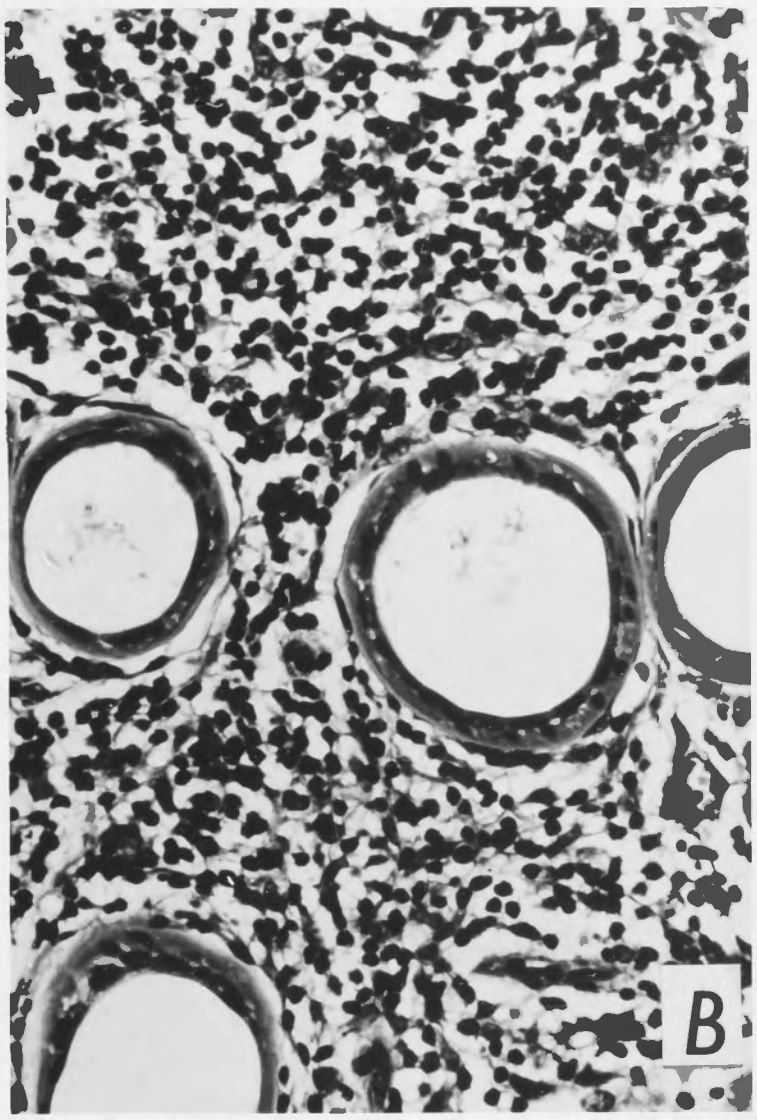
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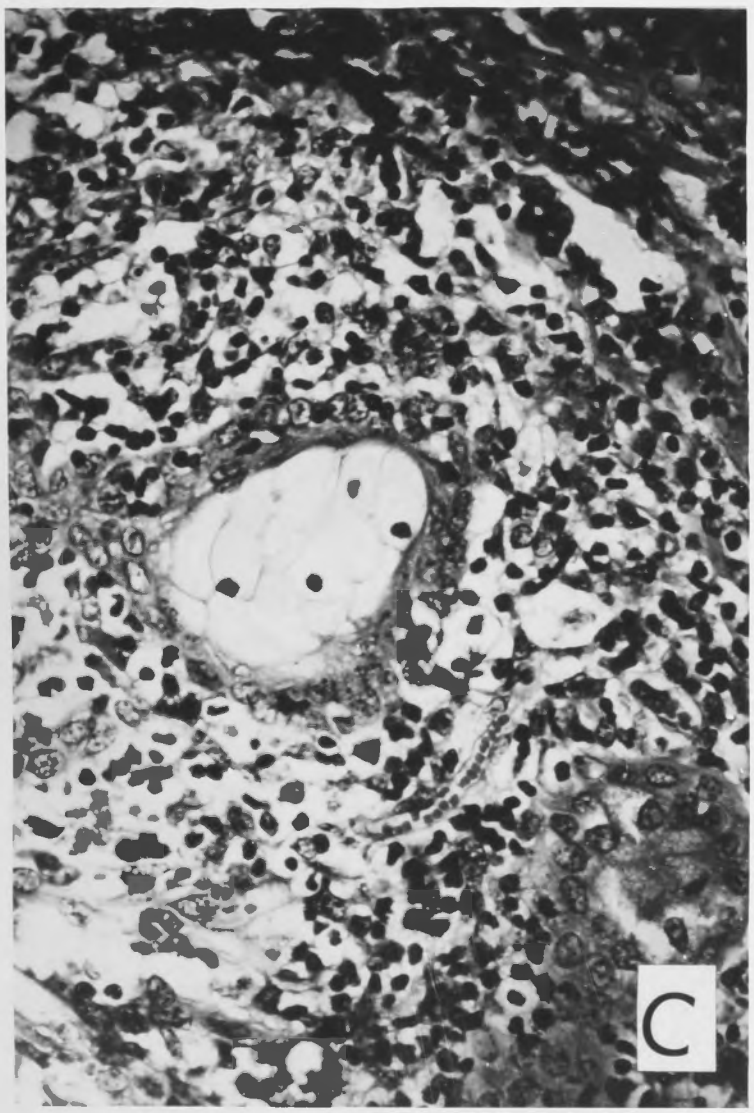
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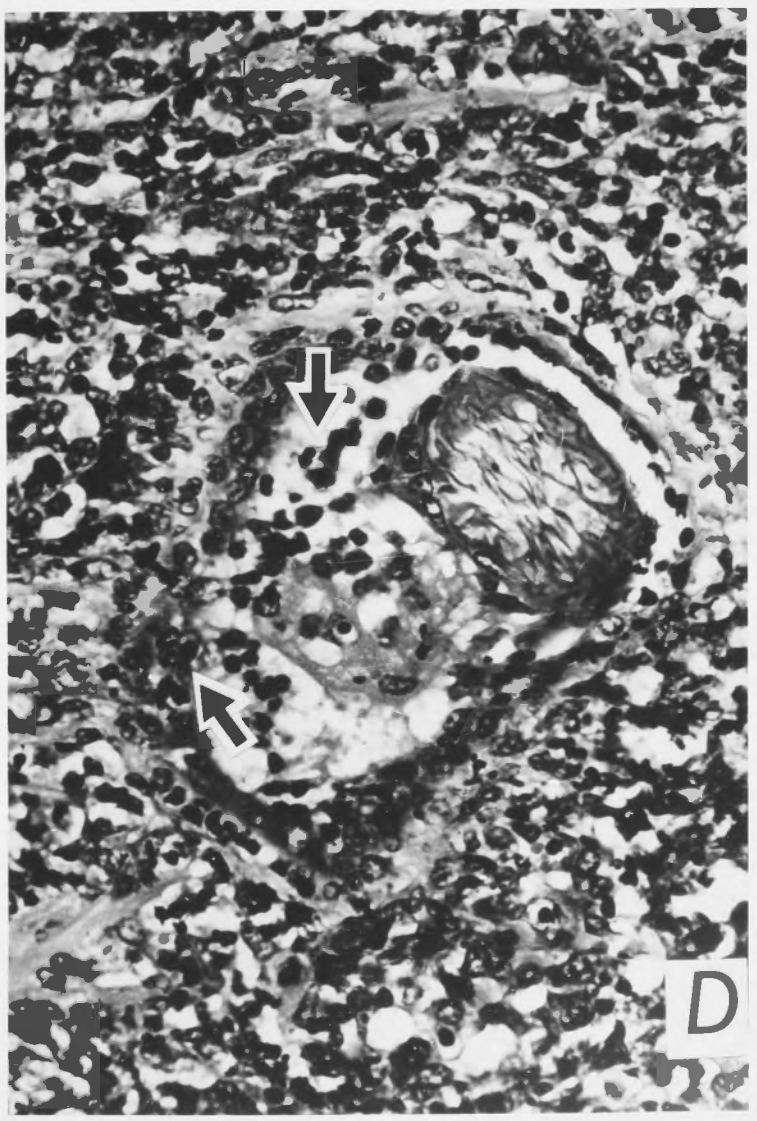
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Figure 8.8

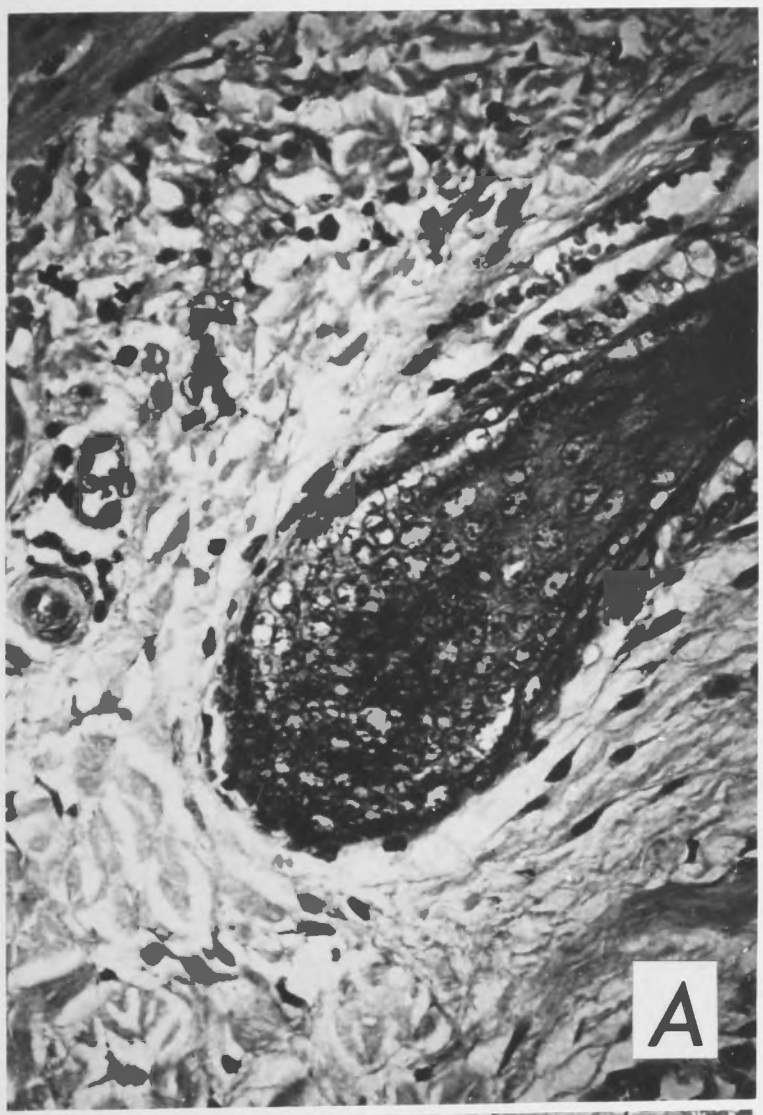
A comparison of a normal wool follicle with one situated in the midst of a collection of blast cells in a 6 day old NLT reaction. Also illustrated is an area surrounding one of the blood vessels that have appeared with the increased blood supply to 7 day old NLT reactions.

A Bulb of a normal wool follicle. Magnification 330x, haematoxylin and eosin.

B Bulb of a wool follicle situated in blast cells in a 6 day old NLT reaction showing invasion by mononuclear cells. Magnification 330x, haematoxylin and eosin.

C Marked increase in blood supply to dermal area of NLT reaction. Magnification 130x, haematoxylin and eosin.

D Higher magnification of a perivascular area from C showing nuclear fragments from necrotic cells. Magnification 330x, haematoxylin and eosin.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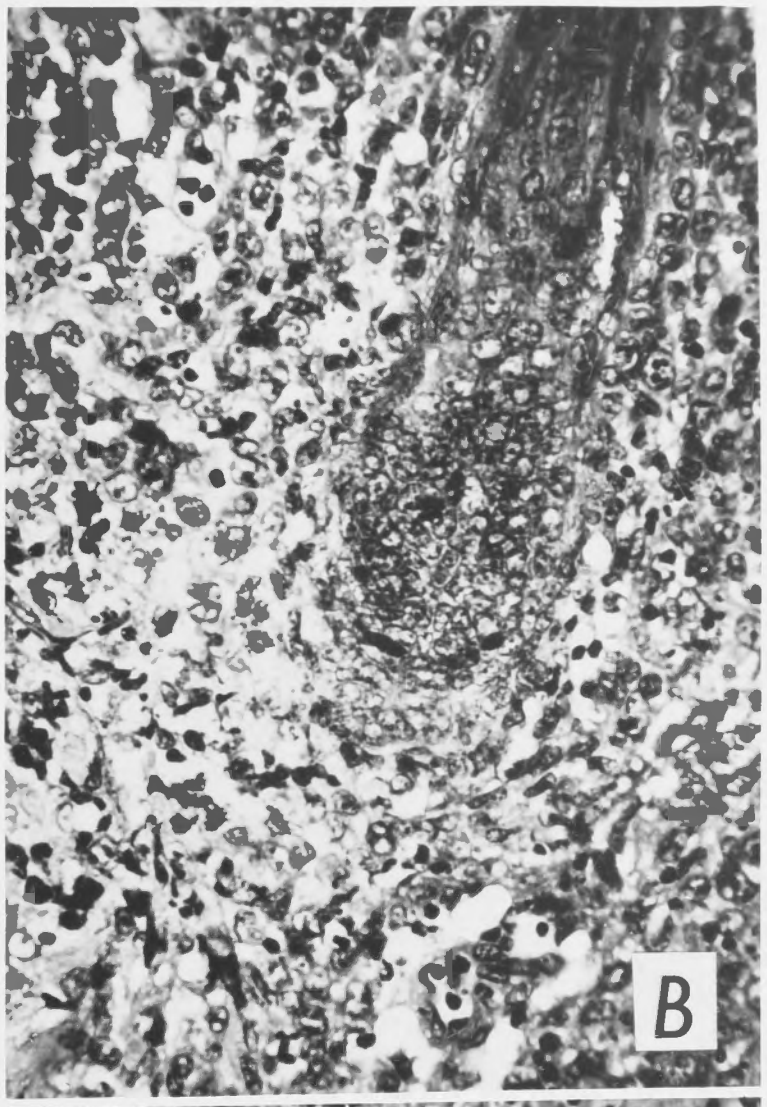
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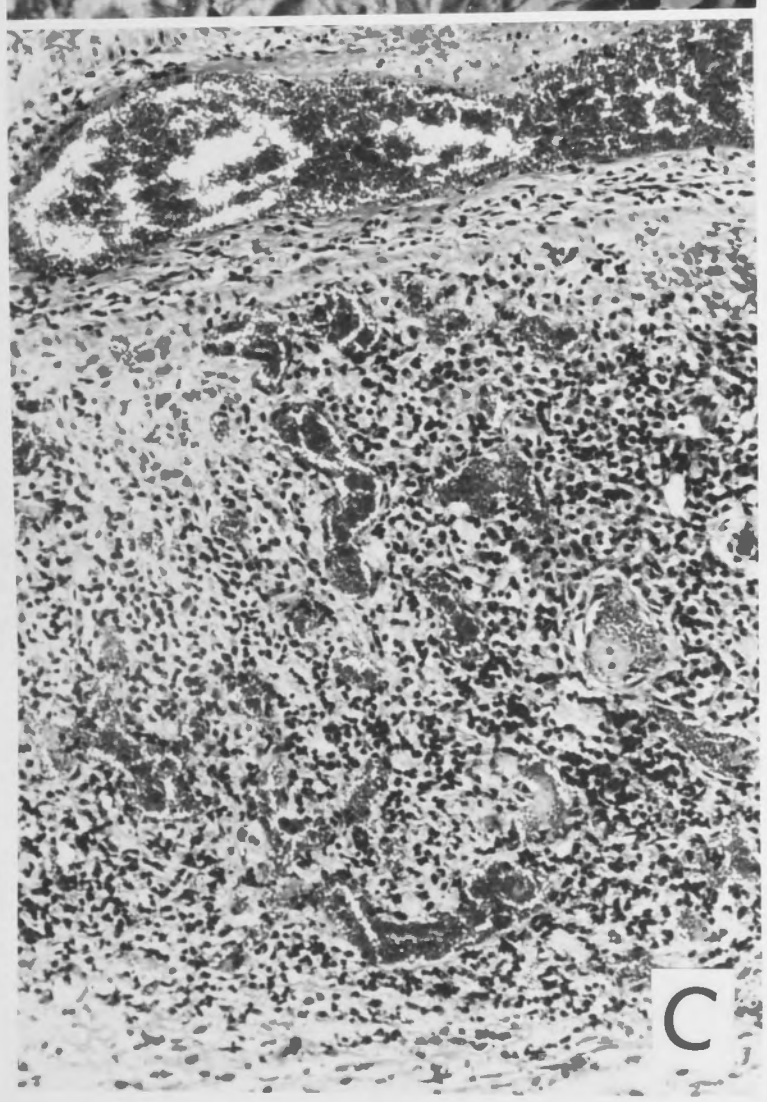
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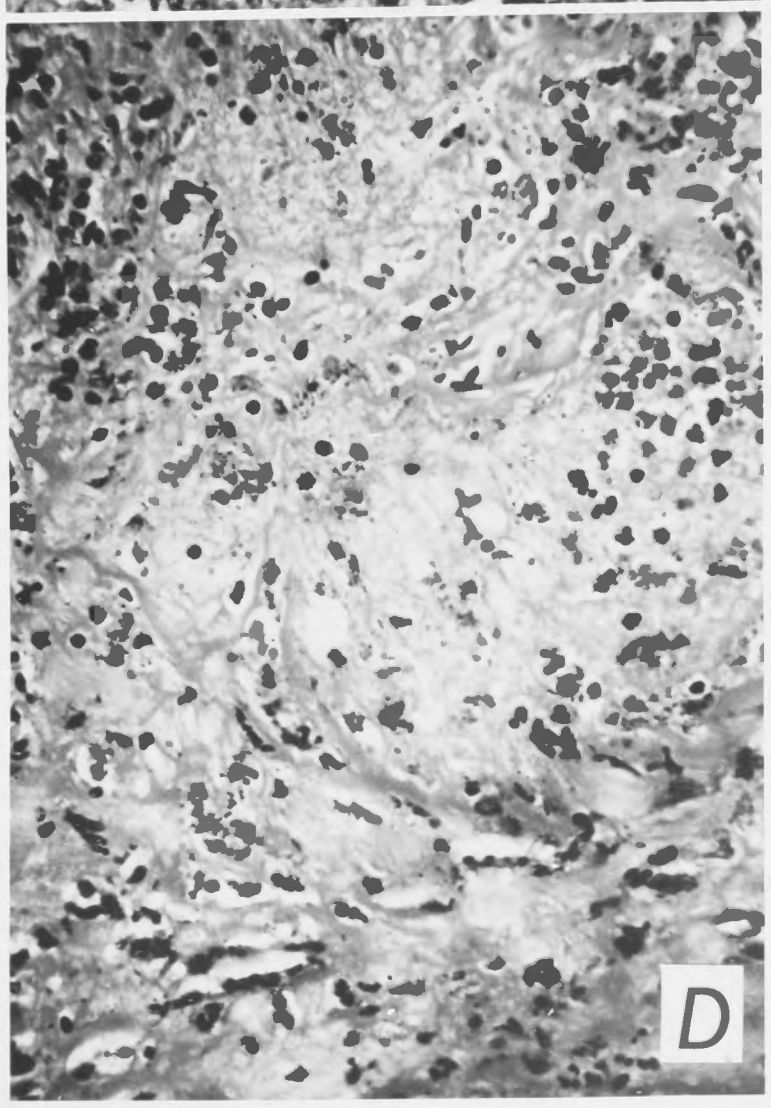
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Figure 8.9

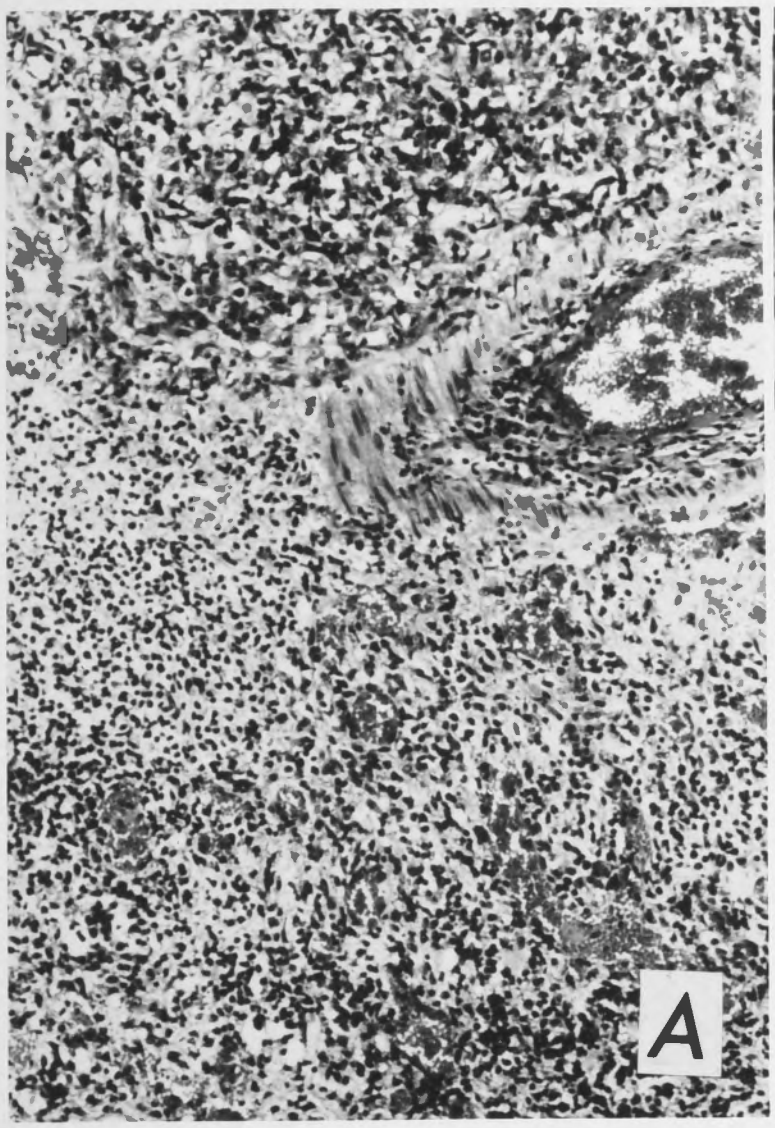
Further histopathological changes in 6, 8 and 9 day old NLT reactions produced by 5 million allogeneic sheep lymphocytes.

A An area of necrosis in a 6 day old NLT adjacent to a small artery whose wall shows signs of being invaded by mononuclear cells. Magnification 130x, haematoxylin and eosin.

B A demonstration of the boundary between a necrotic area and blast cells adjacent to a small blood vessel in a 6 day old NLT reaction. Magnification 330x, haematoxylin and eosin.

C An area of dermis situated peripheral to the cellular infiltration of a 8 day old NLT reaction showin separation of collagen fibres by oedematous swelling. Magnification 330x, haematoxylin and eosin.

D An example of a severe 9 day old NLT reaction in which the central portion of the lesion and epidermis have undergone liquification necrosis. Magnification 12x, haematoxylin and eosin.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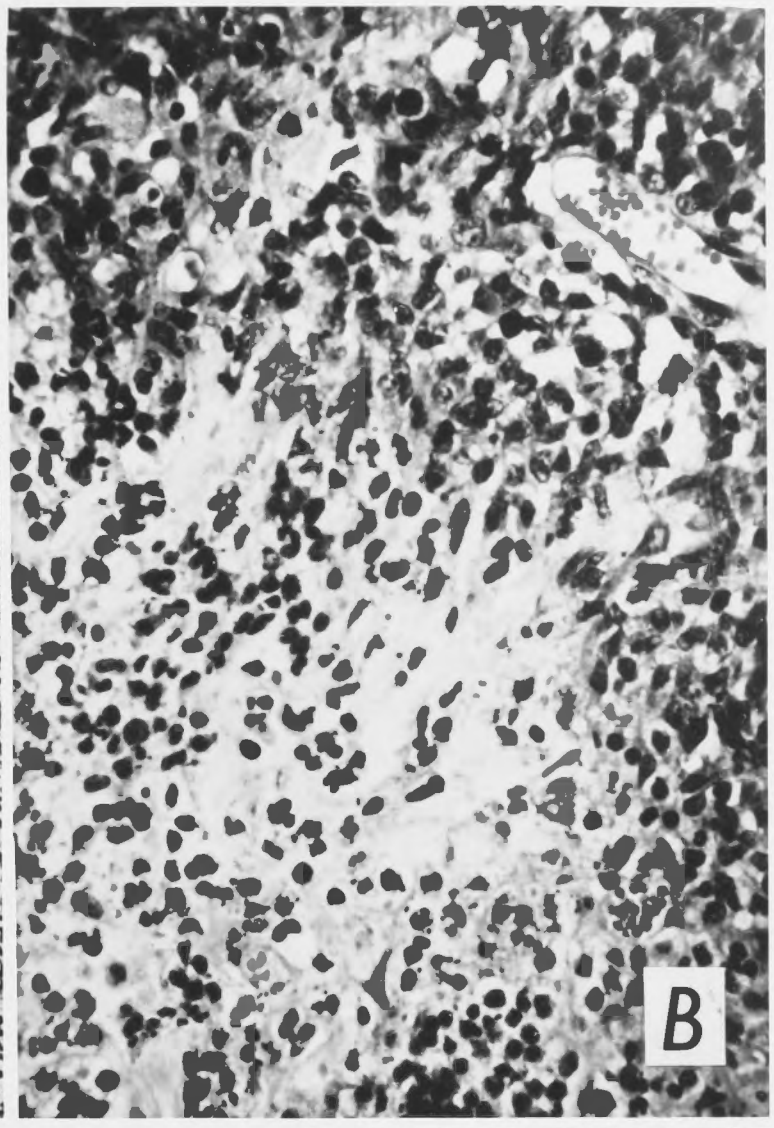
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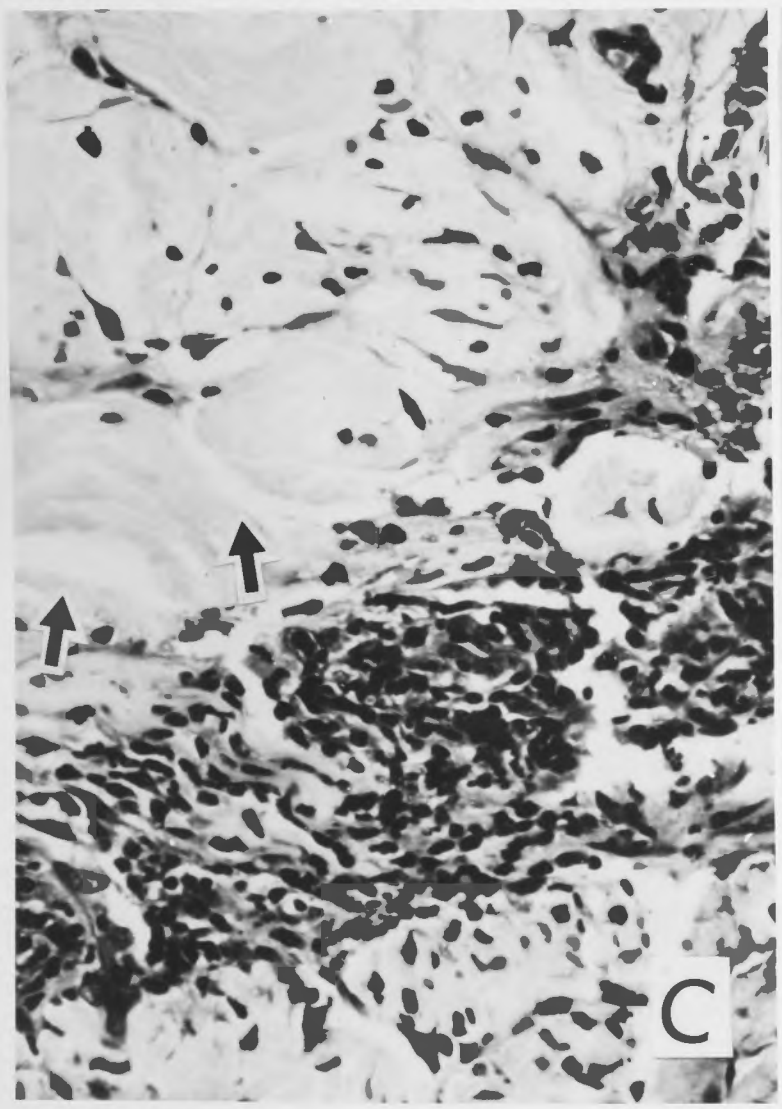
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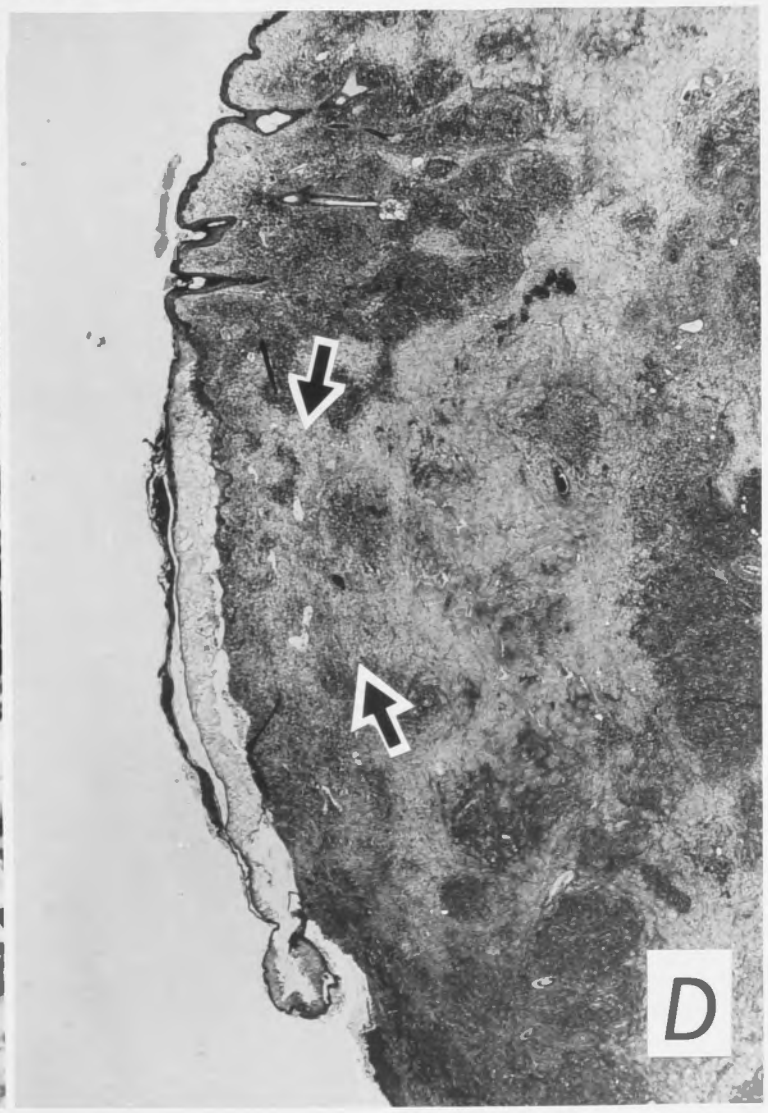
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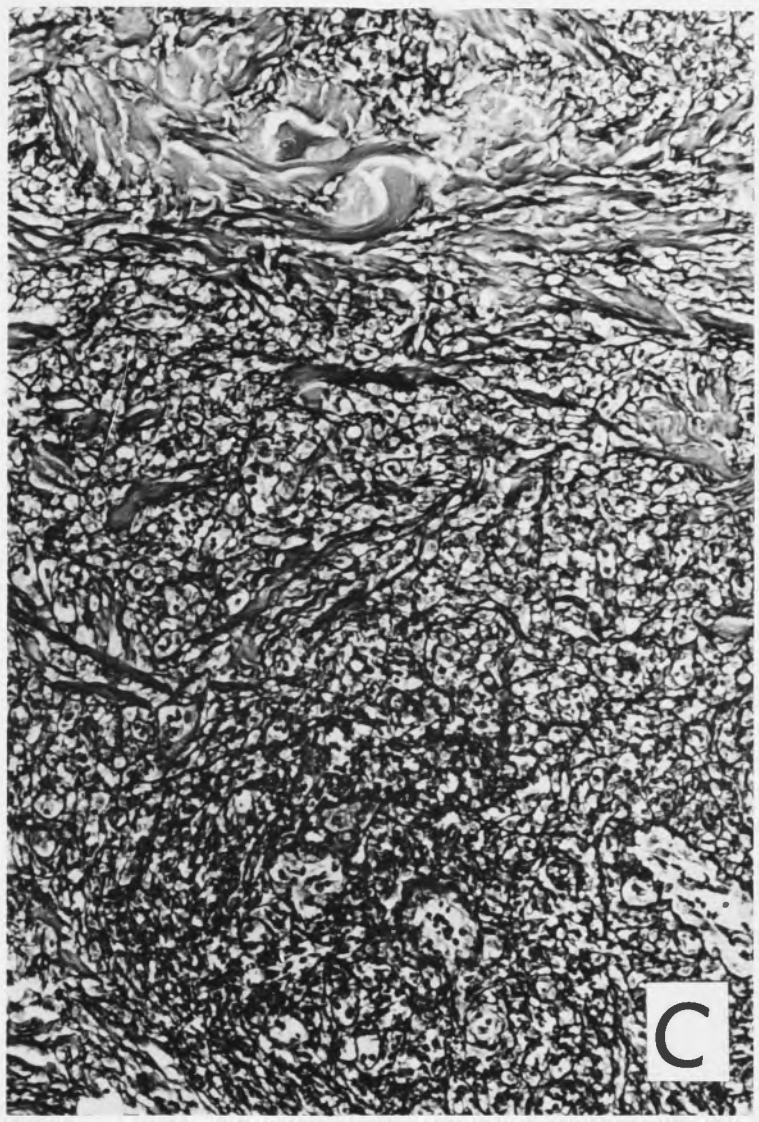
Figure 8.10

Further histopathological changes in NLT reactions in the sheep. These series of photomicrographs indicate the steady increase of a supporting framework of reticulin fibres within the cellular infiltration of NLT reactions with increasing time.

A NLT reaction after 24 hours.
B NLT reaction after 48 hours.
C NLT reaction after 120 hours.
D NLT reaction after 144 hours.

Magnification 130x Stain, untoned reticulin


A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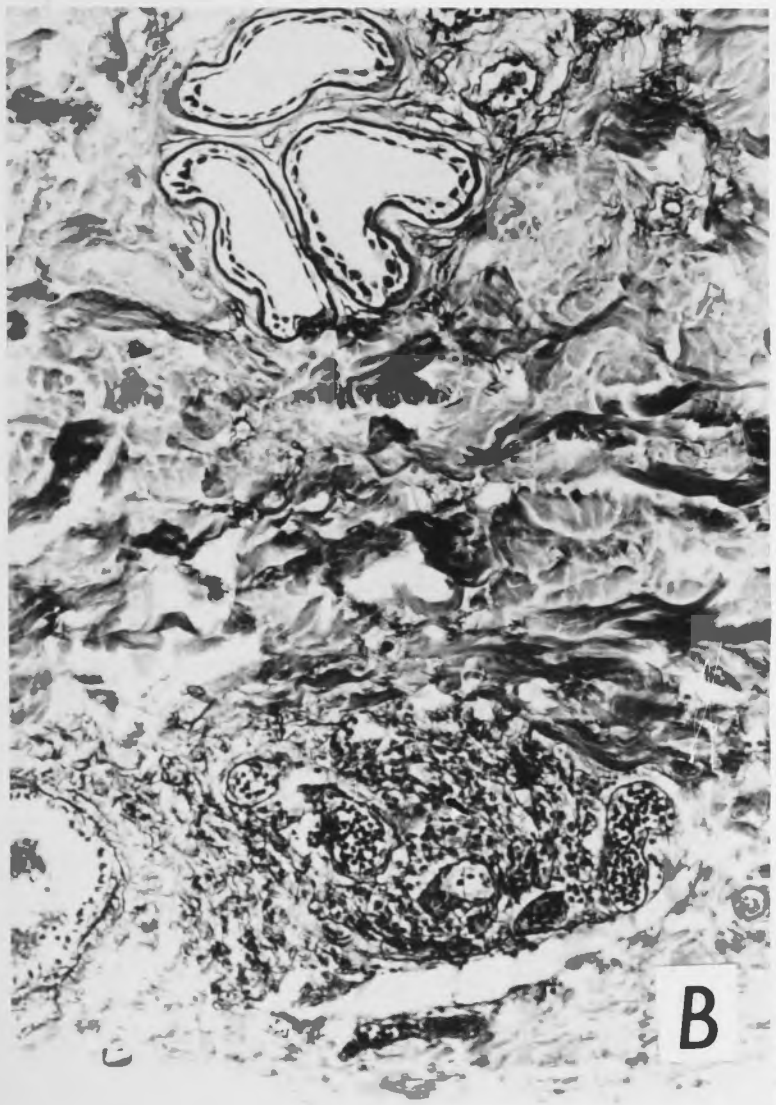
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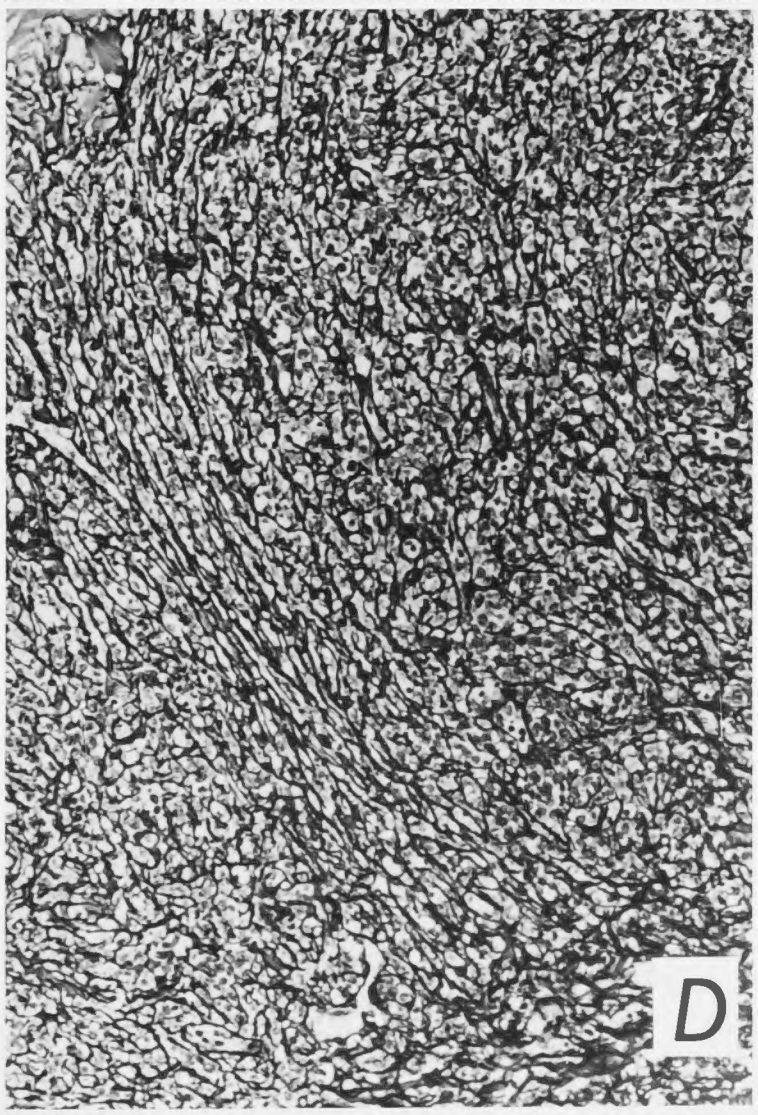
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Figure 8.11

A comparison of the cellular infiltration of the dermis during NLT reactions produced in sheep by the inoculation of 5 million goat lymphocytes or by 5 million bovine lymphocytes.

A NLT reaction produced by goat lymphocytes in a sheep after 2 days.

B After 3 days.

C After 4 days.

D After 5 days.

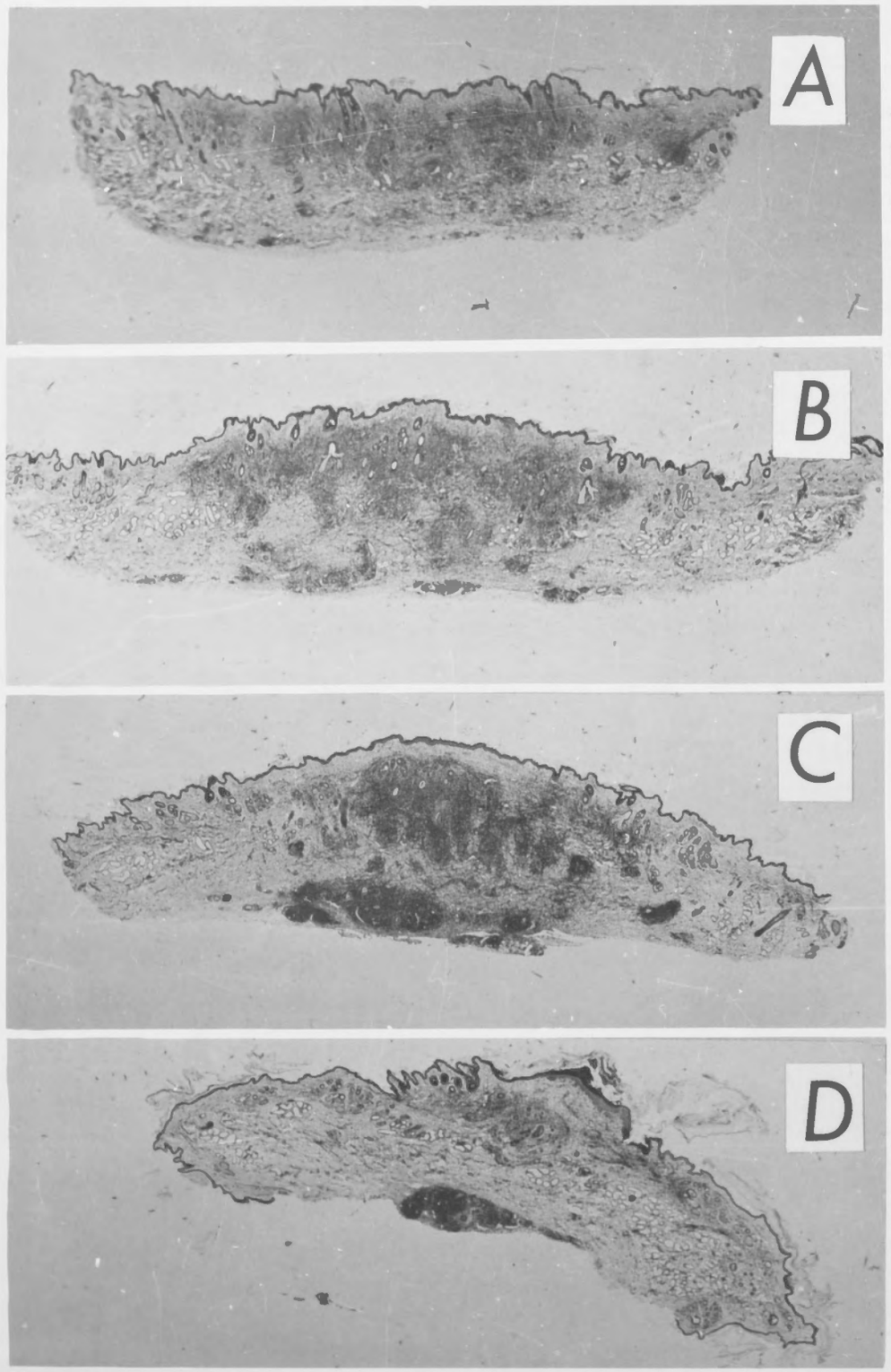
E After 6 days.

F NLT reaction produced by bovine lymphocytes in a sheep after 4 days.

G After 5 days.

H After 6 days.

Magnification 9x Stain, haematoxylin and eosin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B
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Figure 8.12

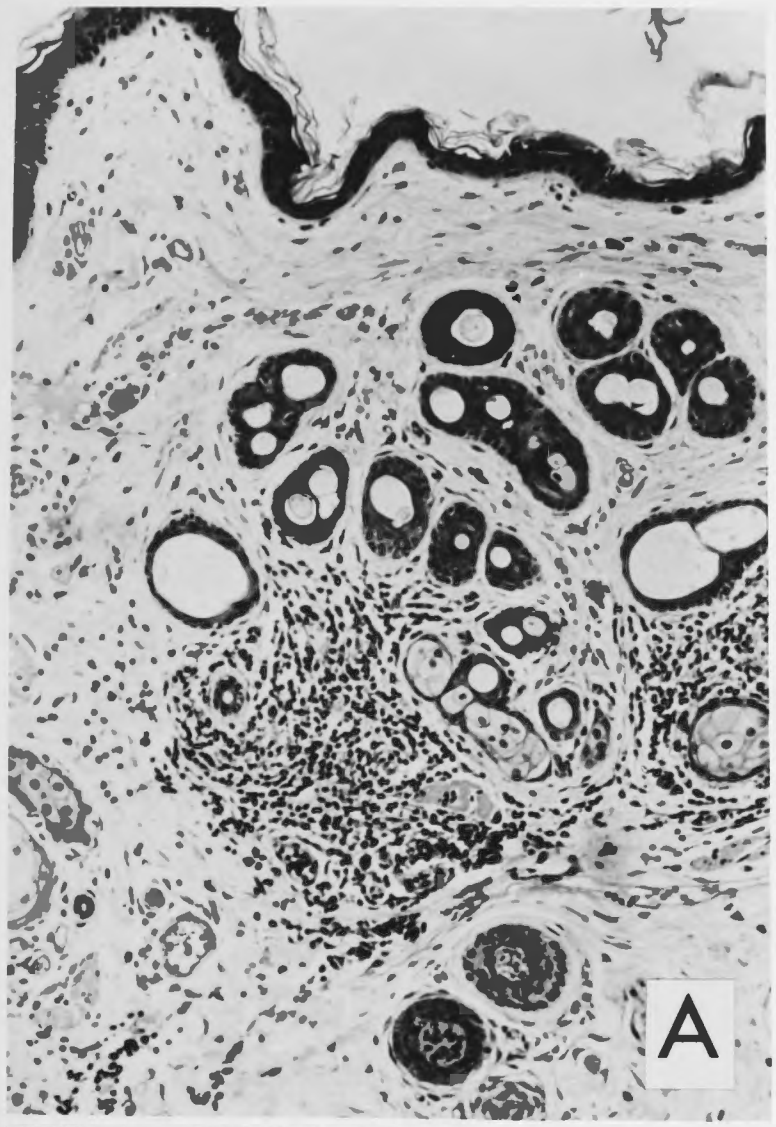
Histopathological changes occurring in NLT reactions produced by 5 million bovine lymphocytes inoculated intradermally into sheep.

A Local infiltration by mononuclear cells around cellular structure in the dermis during a 4 day old NLT reaction. Magnification 330x, haematoxylin and eosin.

B Demonstration of the increased infiltration of the dermis in a 5 day old NLT reaction as compared to a 4 day reaction. Magnification 130x, haematoxylin and eoxin.

C Higher power photomicrograph of a 5 day old NLT reaction showing the density of cellular infiltration and the few blast cells as compared to similar goat and sheep induced reactions. Magnification 330x, haematoxylin and eosin.

D A sebaceous gland situated adjacent to a collection of mononuclear cells showing no signs of invasion. Magnification 330x, haematoxylin and eosin.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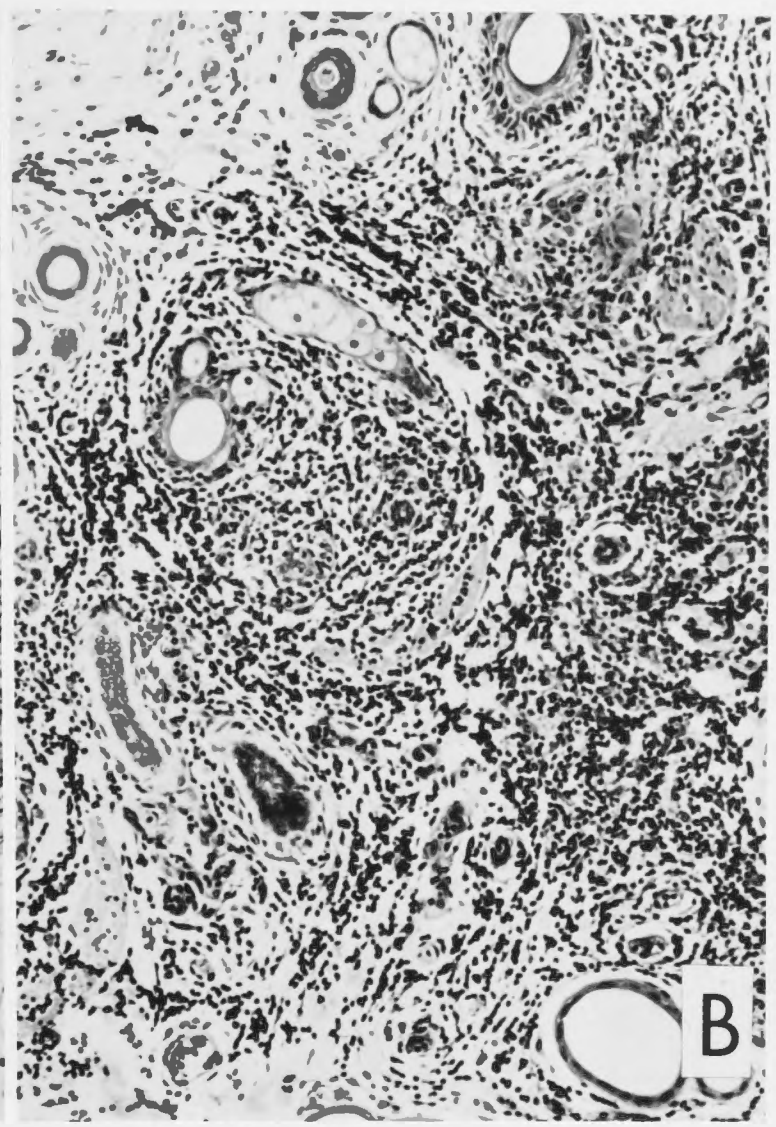
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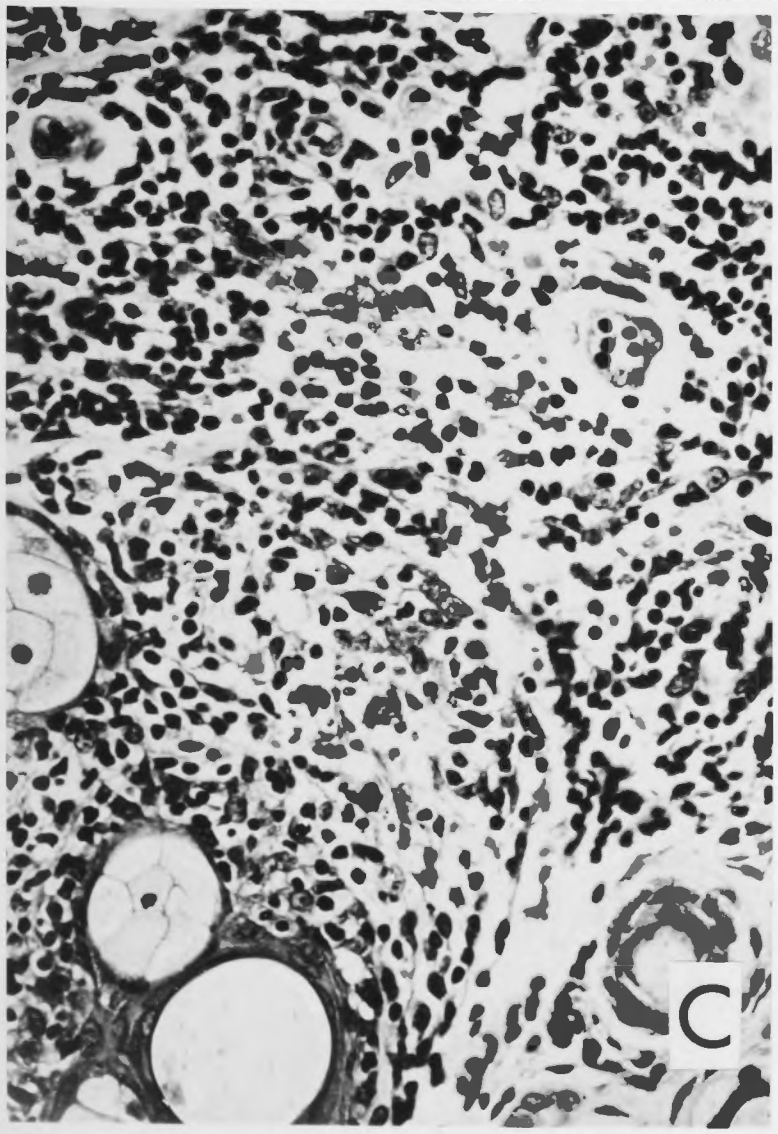
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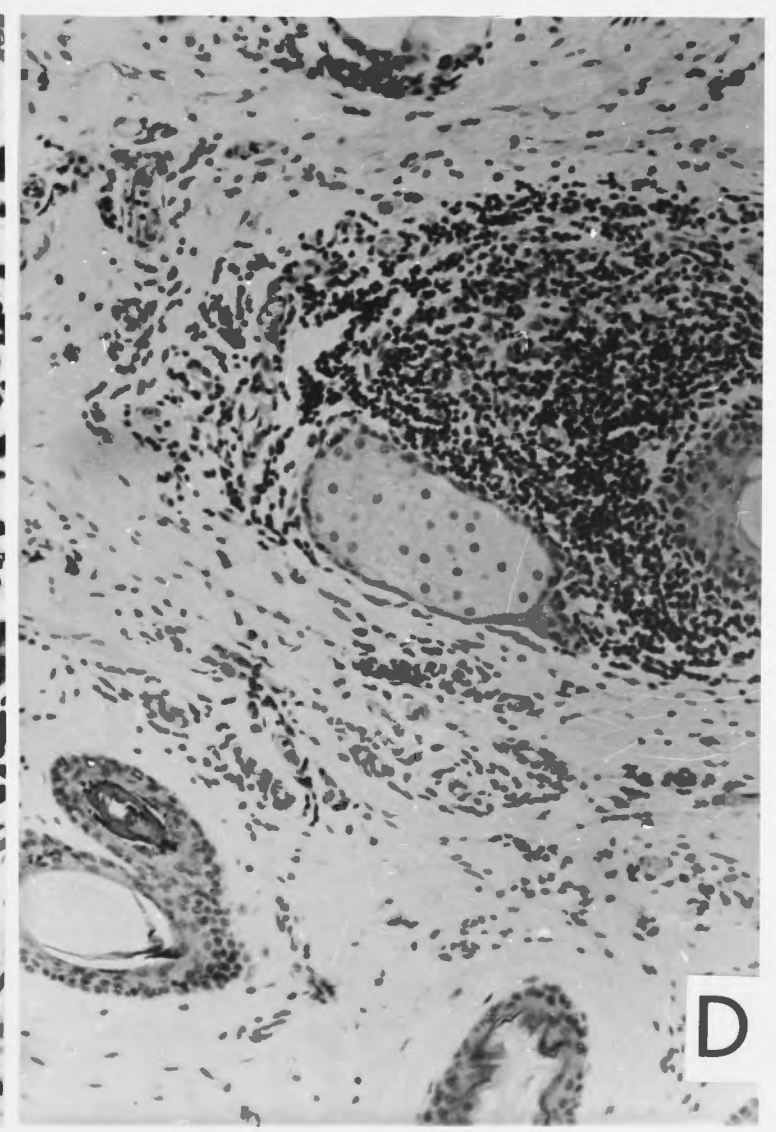
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Figure 8.13

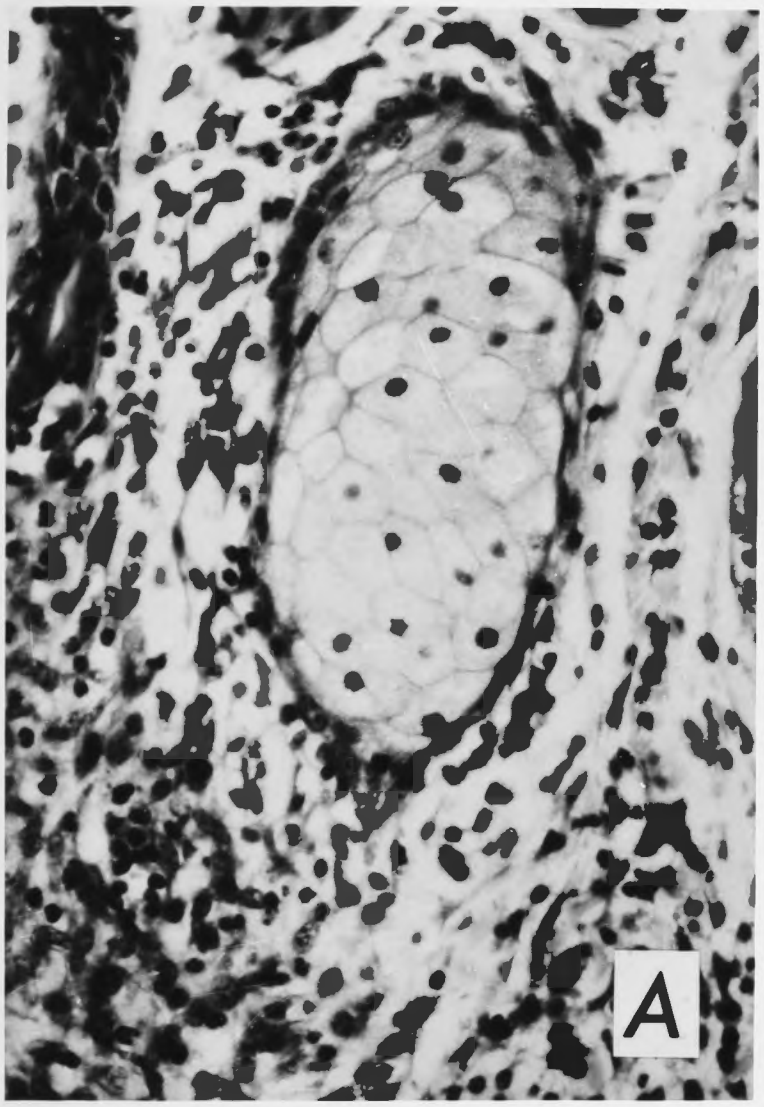
Histopathological changes occurring in NLT reactions produced by goat lymphocytes inoculated into the dermis of sheep.

A Collection of mononuclear cells surrounding a sebaceous gland in a 2 day old NLT reaction (goat-sheep). There does not appear to be any invasion of the cellular structures in the dermis. Magnification 130x, haematoxylin and eosin.

B Cellular infiltration of dermis of a sheep 3 days after inoculation of goat lymphocytes. Magnification 130x, haematoxylin and eosin.

C Higher magnification of wool follicle in B, showing invasion of mononuclear cells. Magnification 330x, haematoxylin and eosin.

D A collection of blast cells in a 5 day old NLT reaction produced by inoculation of goat cells into sheep. Magnification 130x, haematoxylin and eosin.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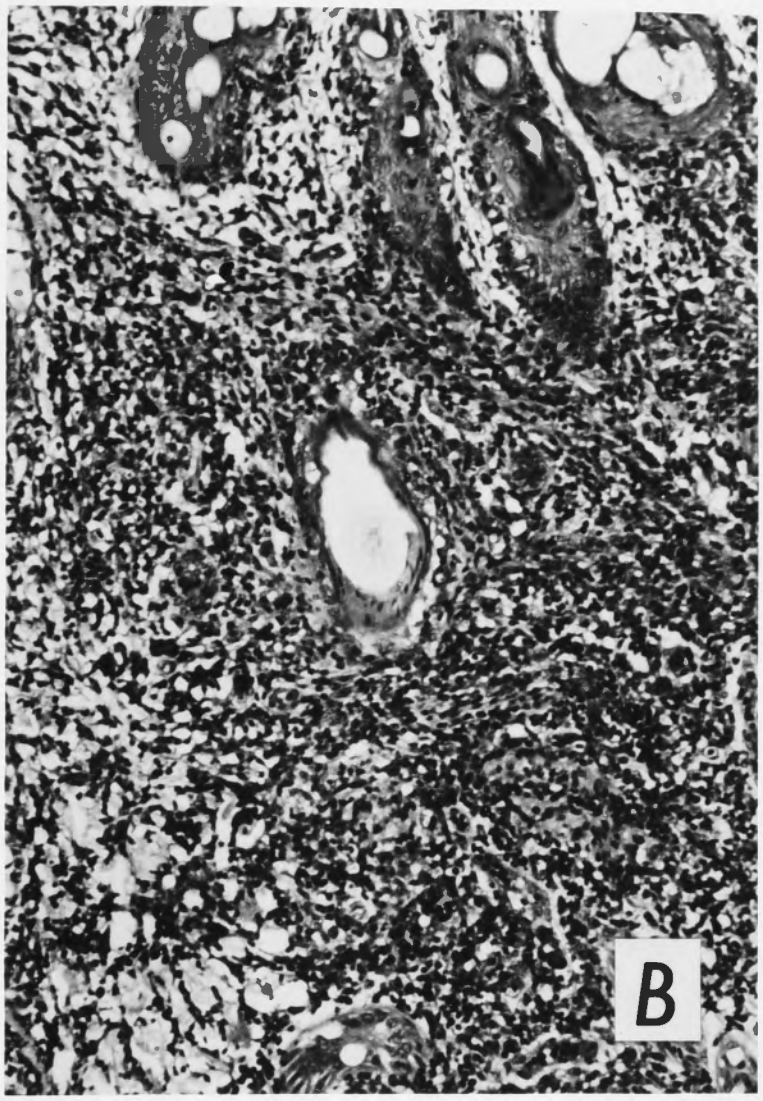
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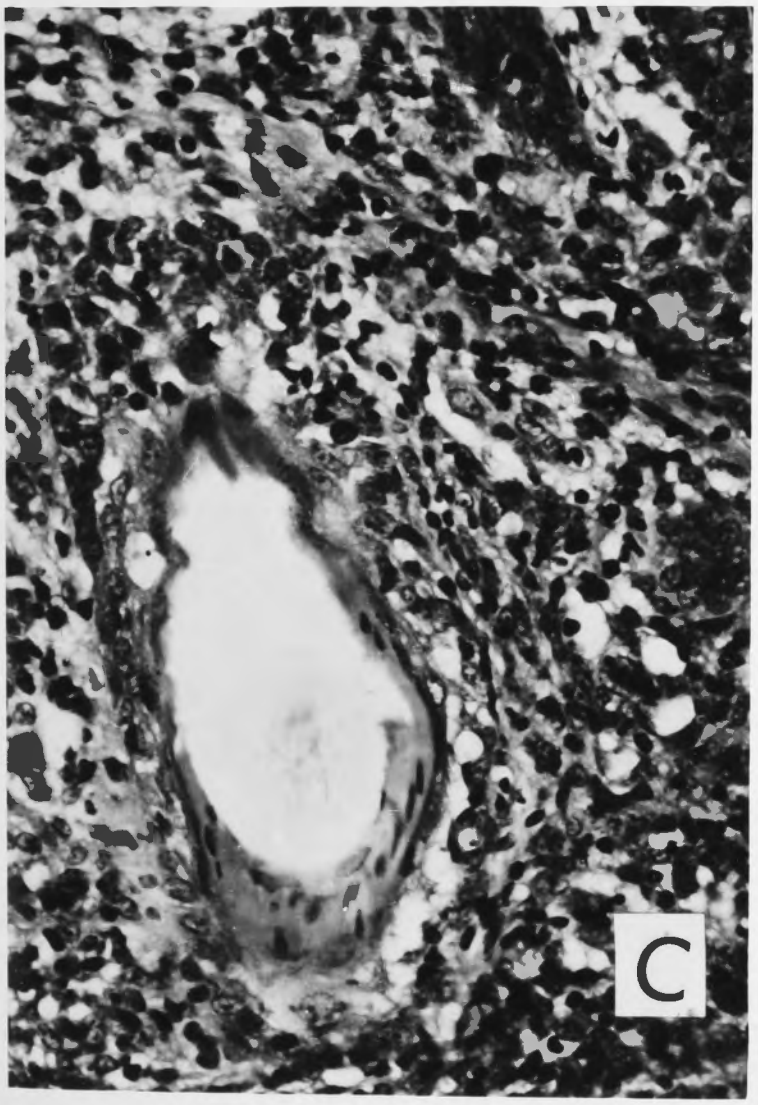
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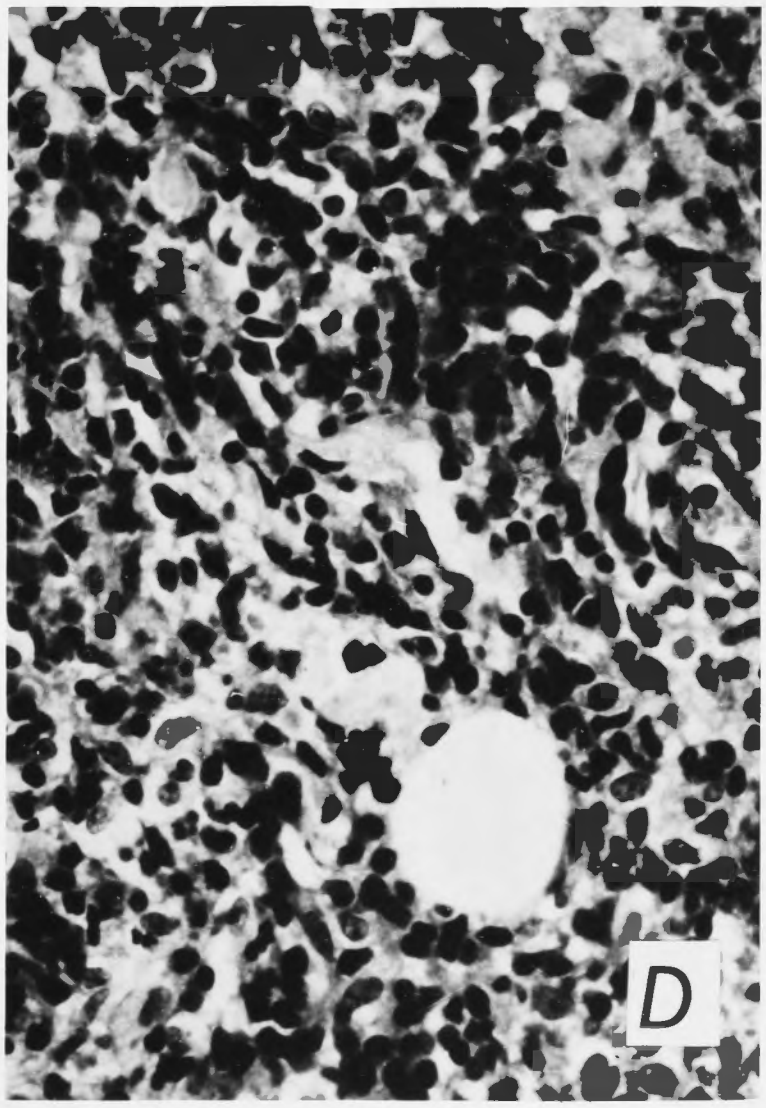
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Figure 8.14

A comparison of the degree of invasion of sheep sebaceous glands occurring in NLT reactions produced by sheep, goat and bovine lymphocytes with a normal gland.

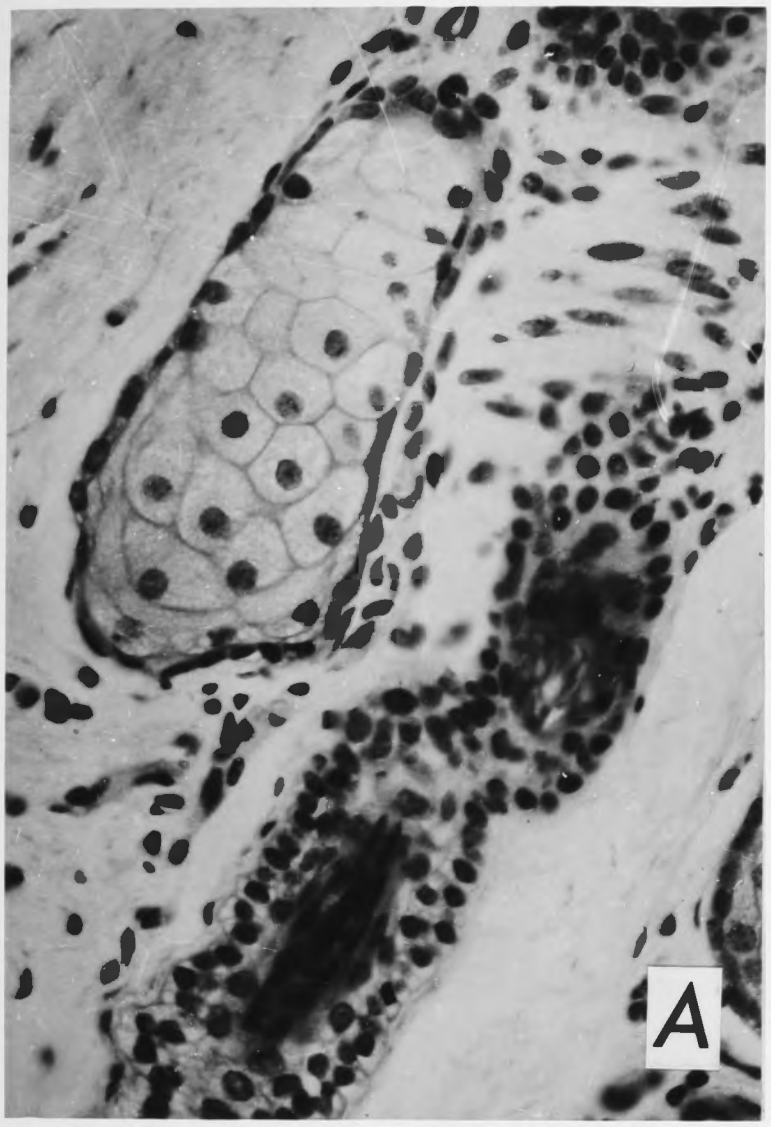
A Normal sheep sebaceous gland.

B Sebaceous gland showing no signs of invasion in a 6 day old bovine lymphocyte induced NLT reaction.

C Sebaceous gland showing some signs of invasion in a 6 day old goat lymphocyte induced NLT reaction.

D Extensive invasion of a sebaceous gland situated in a 6 day old sheep lymphocyte induced NLT reaction.

Magnification 330x Haematoxylin and eosin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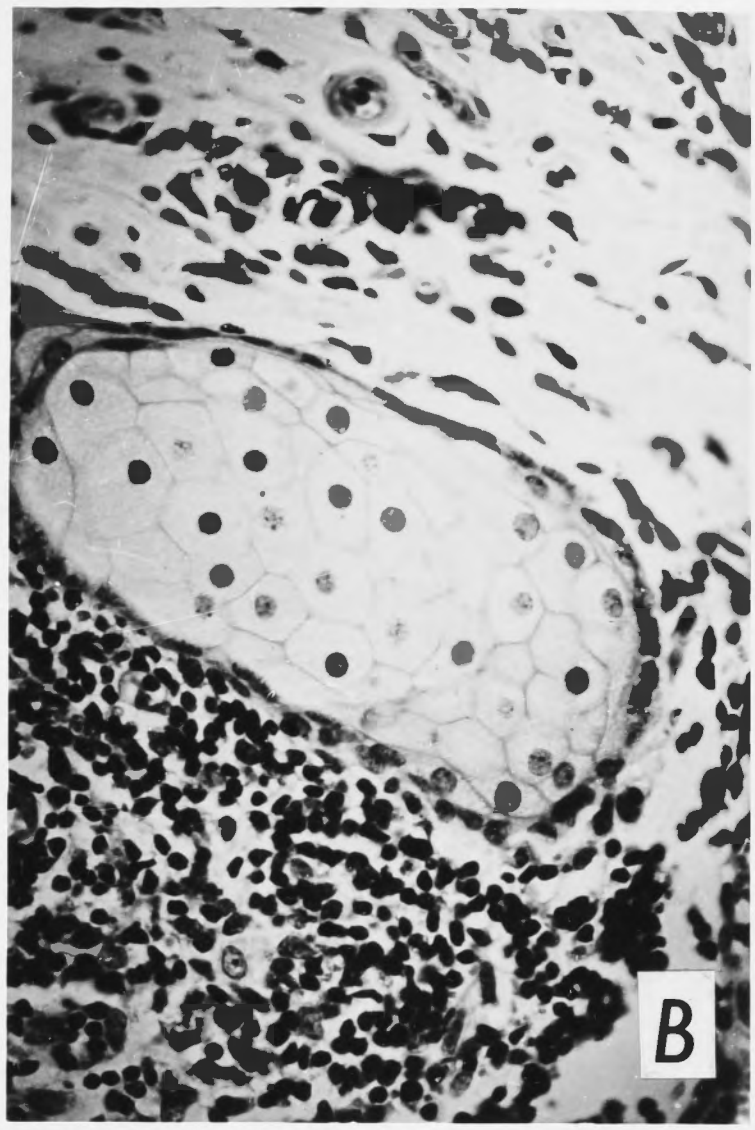
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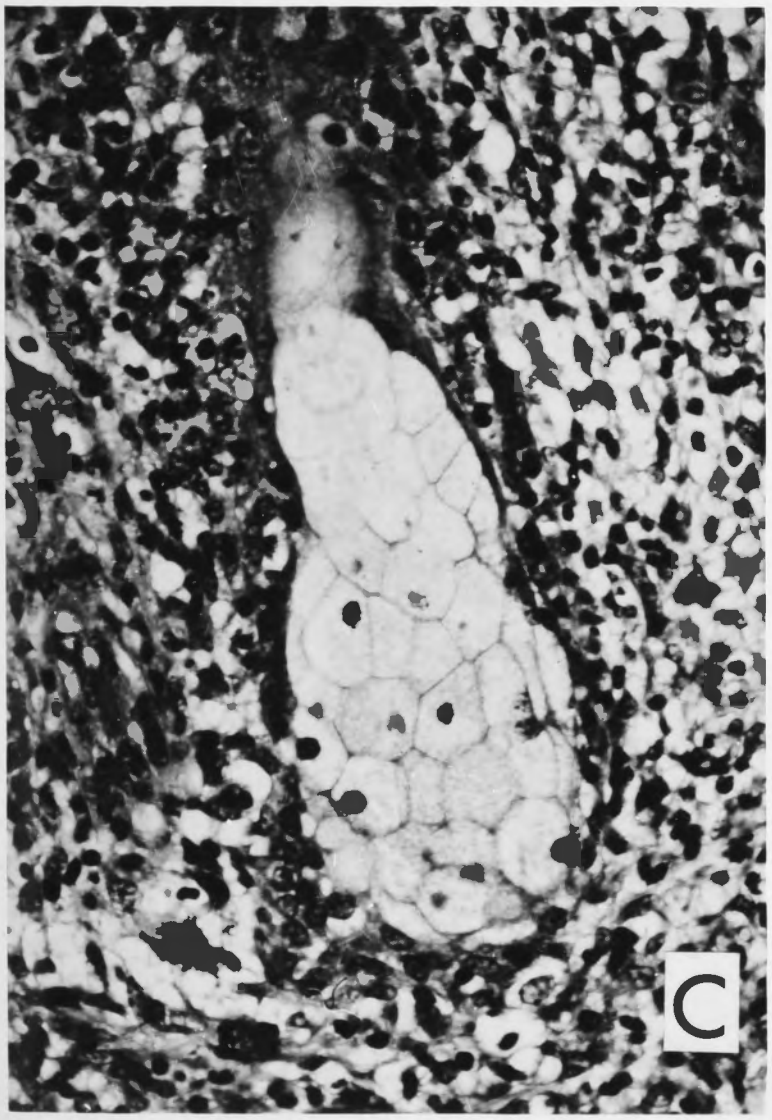
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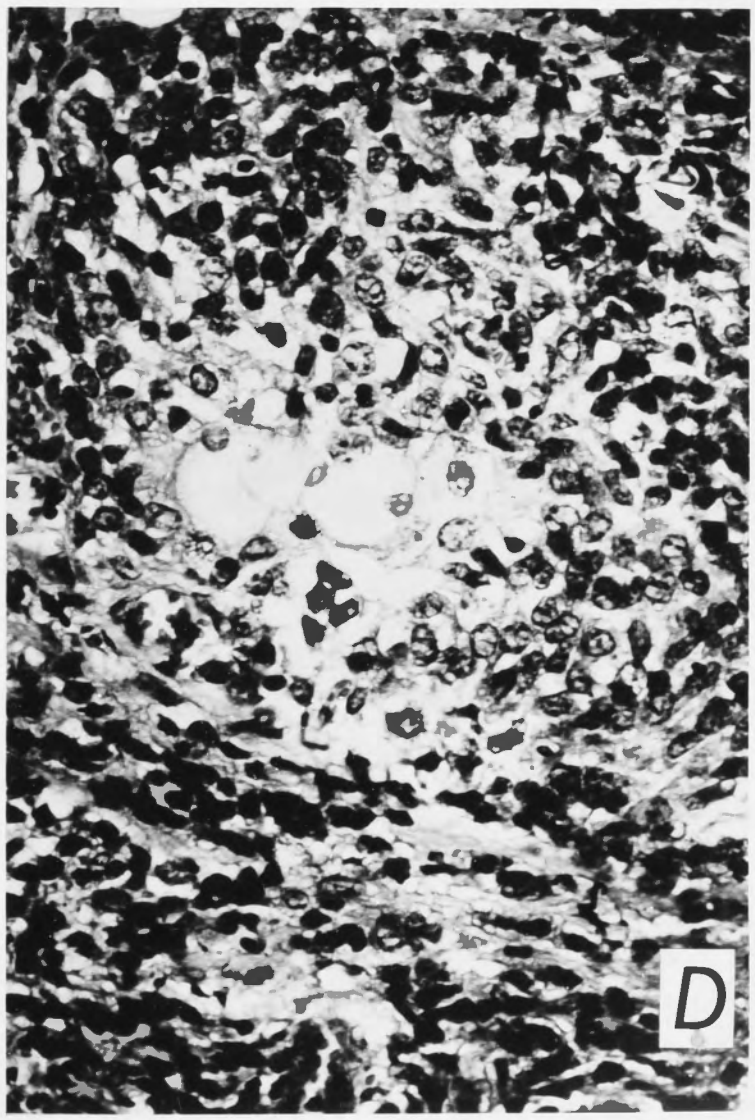
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lymphocytes as described in Chapter 2, were inoculated intradermally into a sheep, a transient skin reaction resulted, which was manifested as a small area of erythema, 1 to 2 cms in diameter, and a very slight increase in skin thickness. The histological examination of the above reactions showed only a mild infiltration of the dermis by small mononuclear cells during the 2 days that macroscopic reactions were detectable. The infiltrating mononuclear cell population differed from those seen in NLT reactions by the absence of the large reticulum cells, whilst the larger mononuclear cells that occurred in the reactions produced by immune chicken spleen cells were more regular in outline than the prominent reticulum cells of the NLT reaction. In the absence of the large blast cells, no detectable tissue damage or invasion of cellular structures were seen (Fig. 8.15).

Discussion

It is apparent that the NLT reaction in sheep presents a striking histological appearance, the most notable feature of which is the occurrence of rapidly increasing numbers of blast cells in the perivascular collections of mononuclear cells which accumulate at the reaction site. These large blast cells bear a close resemblance to the cells seen in the pocks of the "Simonsen" phenomenon and in the enlarged spleens of mice undergoing GVH reactions (Howard, 1961). The histological evidence presented in this chapter makes it clear that during the NLT reaction, tissue damage does not occur before the appearance of the large blast cells which invade and destroy surrounding tissues. Wakesman (1963) made a similar observation, in delayed type hypersensitivity reactions and during homograft destruction, when he noted that no tissue damage occurred until after blast cells had appeared in perivascular collections of mononuclear cells. In those dermal reactions produced by bovine lymphoid

Figure 8.15

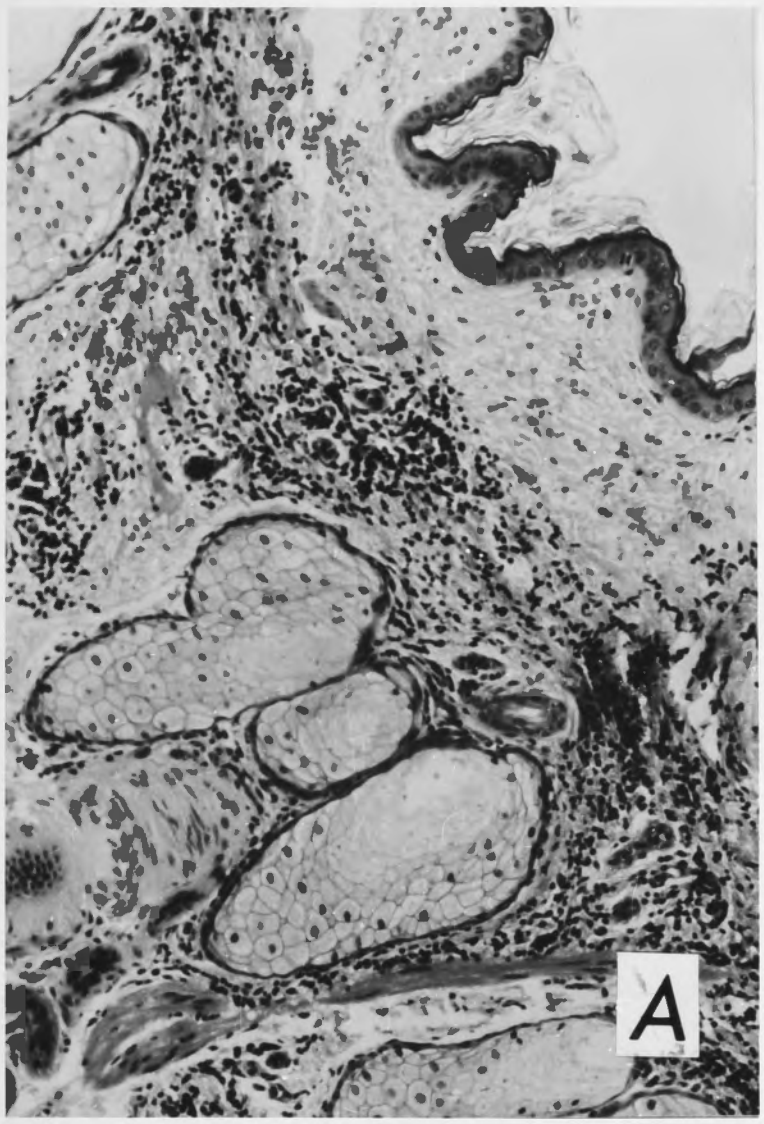
Histopathological changes occurring in an NLT reaction produced by 50 million immune chicken spleen cells in a sheep.

A Cellular infiltration of superficial layers of the dermis, 24 hours after inoculation of immune chicken spleen cells. Magnification 130x, haematoxylin and eosin.

B Infiltrating cells in a 24 hour reaction, contain few detectable blast cells. Magnification 330x, haematoxylin and eosin.

C Cellular infiltration in a reaction 48 hours after inoculation of immune chicken cells. Magnification 130x, haematoxylin and eosin.

D Higher magnification of the infiltrating cells in C. Magnification 330x, haematoxylin and eosin.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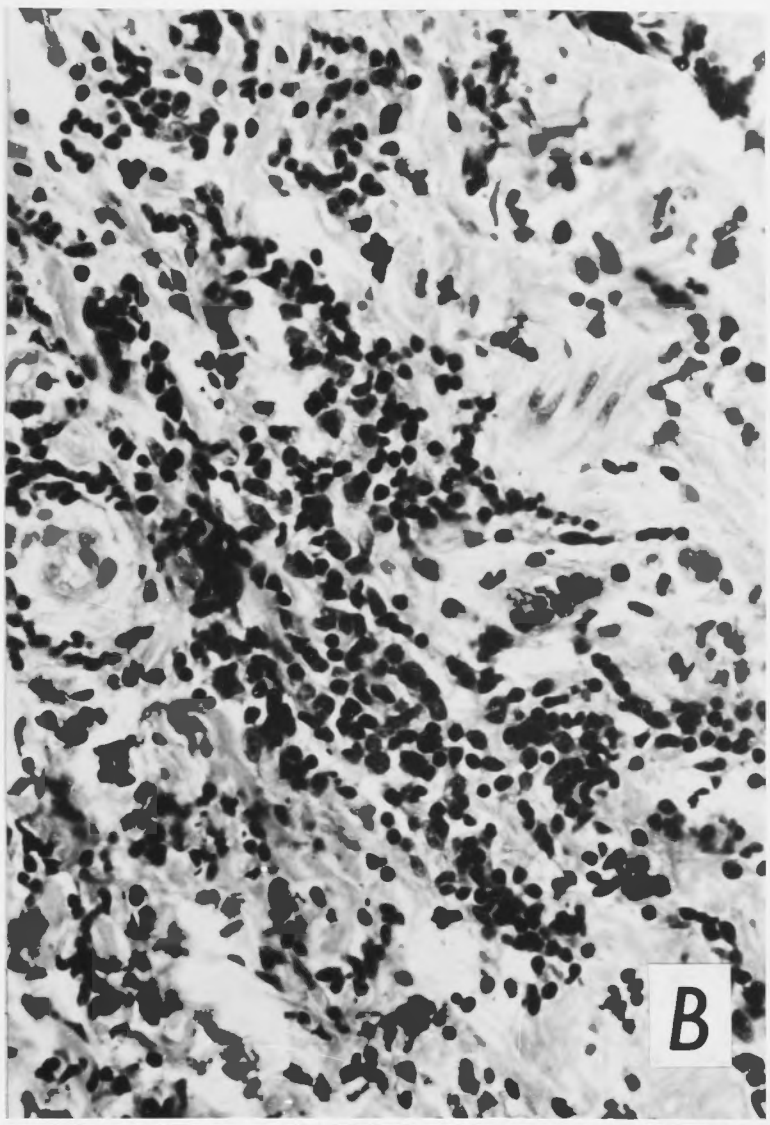
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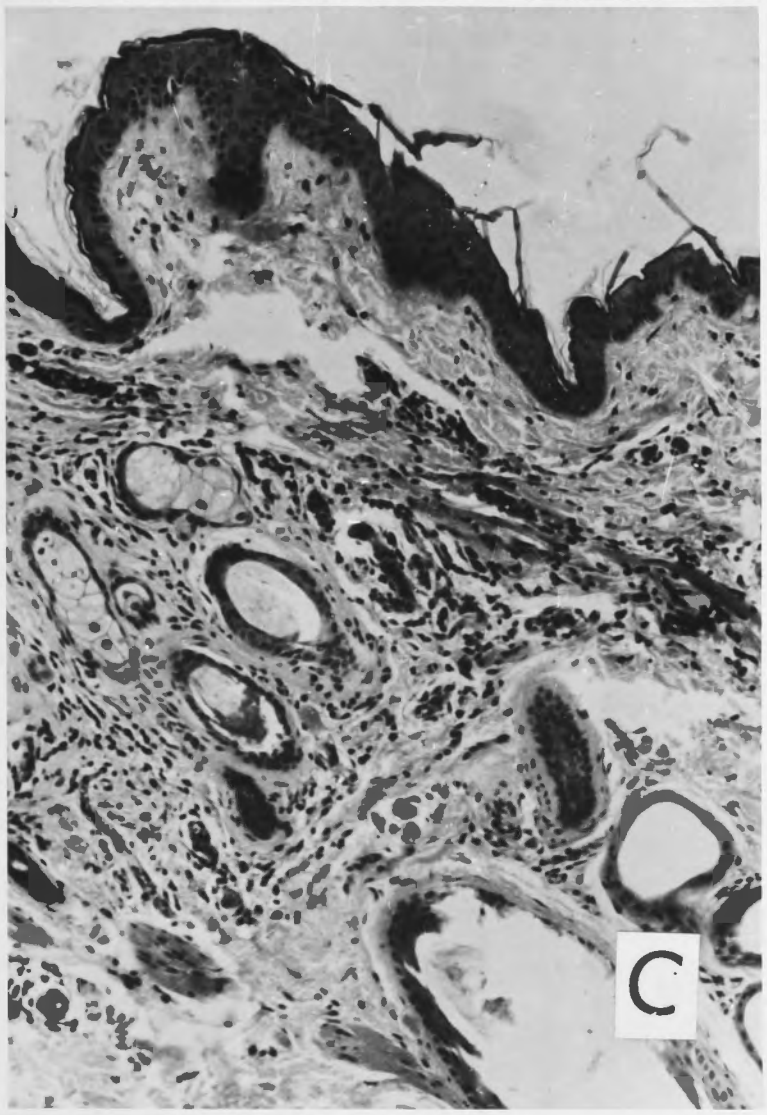
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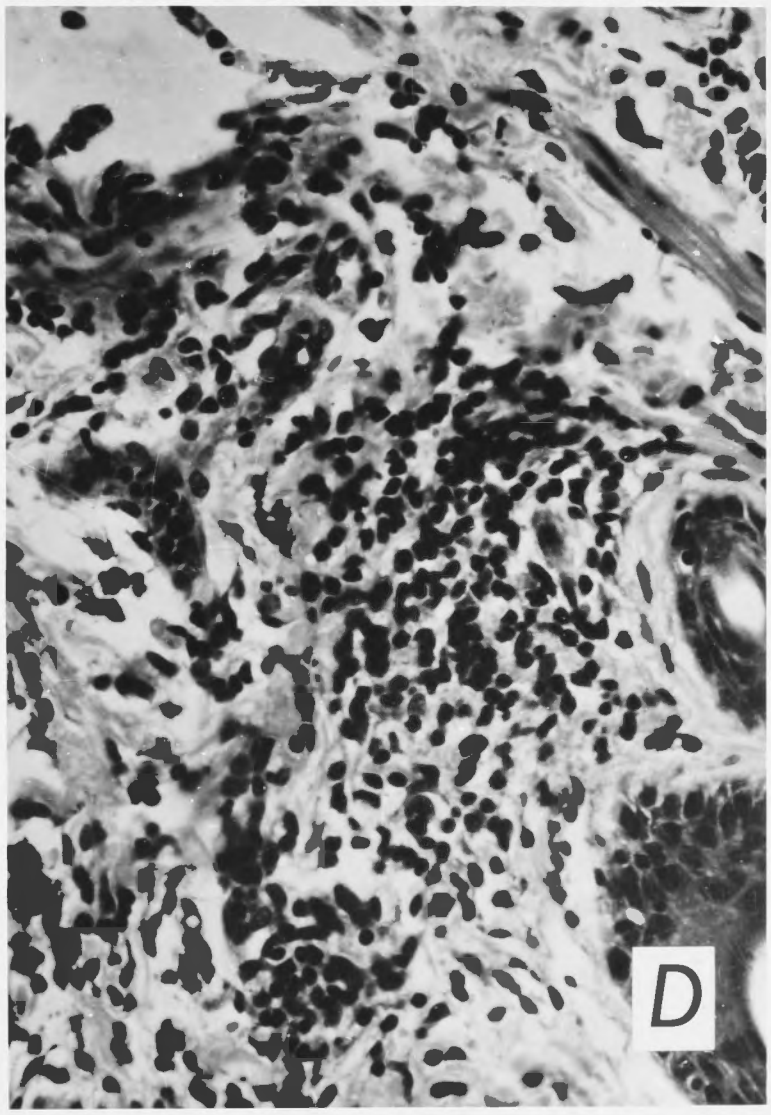
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cells, there were few blast cells formed and invasion of the cellular structures did not occur. Dermal reactions were not produced at the site of inoculation of xenogeneic lymphoid cells from guinea pigs and chickens. The failure of lymphoid cells from the latter donors to produce dermal reactions was not due to an inability to survive in the environment of the sheep, since spleen cells from immunized chickens can produce a dermal reaction. Further, it does not seem likely that the failure by normal guinea pigs and chicken lymphoid cells to react in the sheep, can be attributed to their destruction by the host, since previous studies with the NLT reaction in sheep indicated that inhibition of the injected allogeneic lymphocytes does not occur until at least 5 days after their inoculation. One possible explanation for the decrease in the dermal responses to xenogeneic lymphoid cells which occurs as the phylogenetic relationship between donor and recipient increase, would be a decline in the capacity of the grafted lymphocytes to interact with the hosts reticular tissue to produce the invasive blast cells. This is shown by the virtual absence of blast cells from reactions produced by immune chicken lymphoid cells and the lack of invasion of cellular structures situated within the lesion produced by such cells. Dvorak et al. (1963) noted the failure of immune lymphoid cells to invade the cellular structures of the dermis when they investigated the histology of dermal reactions produced in rabbits by the intradermal injections of lymphoid cells from immune donor rabbits. It is possible that this apparent incapacity of immune cells to produce blast cells of the type seen in GVH reactions with the associated proliferations of lymphoid cells may account for the observation by Warner (1964) that lymphoid cells from an immune parental strain produced smaller dermal reactions in their F_1 generation, than do cells from non-immune parental donors.

It may be concluded from the above observations that

the blast cells invade and destroy the surrounding tissue. This conclusion is supported by observations made on the destruction of grafted cardiac muscle from hearts perfused with splenic cells before grafting to the CAM of treated chicken embryos (see Chapter 4).

The nature of the stimulus that induces the formation of blast cells during an NLT reaction is not known but from the evidence presented in previous chapters it is likely to be the result of lymphocyte-lymphocyte interaction (see Chapters 4, 6 and 7). This supposition is supported by firstly, the appearance of the blast cells in the perivascular areas where the injected lymphocytes would be most likely to encounter migrating host lymphocytes, and secondly by the failure of blast cells to appear throughout the reaction site, as would be expected if the injected lymphoid cells were reacting against the structural cells of the dermis.

It will have been noted that after 5 or 6 days necrotic areas begin to appear in the lesion at the site of the NLT reaction. These necrotic areas are surrounded by large blast cells which are often assocated with collections of small round cells. At about the same time as the necrotic areas appear, the NLT reaction begins to regress. Previous experiments have shown that 6 or more days after the initial injection of allogeneic lymphocytes, the recipient develops some form of immunity which inhibits the production of NLT reactions from lymphocytes from the same donor. It is possible that the necrotic areas in 5 to 6 day old NLT reactions represent manifestations of this developing immunity of the host which is destroying the donor cells present in the NLT reaction.

Summary

The histological examination of daily biopsy specimens of an NLT reaction revealed a steady increase in the cellular

infiltration of the dermis up to 7 days. The infiltrating cells were found to consist of mononuclear cells and blast cells. Examination of the dermal reactions produced by xenogeneic (goat and bovine) lymphoid cells showed the presence of reduced numbers of blast cells as compared with the numbers present in NLT reactions. In xenogeneic lymphoid cell induced reactions the degree of tissue invasion was proportional to the numbers of blast cells present. Lymphoid cells from immune xenogeneic donors, although able to produce a dermal reaction, the lesion differed from an NLT reaction since it contained no blast cells and no evidence of cellular invasion. It was concluded that the presence of blast cells was necessary for the invasion and destruction of cellular structures in the dermis in reactions produced by injected lymphoid cells.

CHAPTER 9

THE DERMAL INFLAMMATORY REACTION INDUCED IN SHEEP BY AN RNA FRACTION EXTRACTED FROM ALLOGENEIC LYMPHOCYTES

Chapter 9 - The Dermal Inflammatory Reaction Induced in Sheep by an RNA Fraction Extracted from Allogeneic Lymphocytes

Introduction

Previous investigations of the mixed lymphocyte reaction *in vitro* and GVH type reactions in chicken embryos and sheep have led to the conclusion that antigens are probably not responsible for inducing the proliferative response of lymphoid cells that occurs during these reactions. Some indication of the nature of the cellular component of lymphocytes responsible for inducing the proliferative response in lymphoid cells during MLR comes from the work of Hashem and Rosen (1964). These workers observed that a ribonuclease sensitive ribosomal fraction of lymphocytes would induce "blast" cell formation when added to allogeneic lymphocytes *in vitro*. This observation, which was made in cultures of human leucocytes, indicated that cellular RNA might be involved in the initiation of the lymphoid cell proliferative response *in vitro*. If the transformation of lymphocytes into blast cells in culture is an *in vitro* manifestation of a GVH reaction then it is possible that RNA obtained from allogeneic lymphoid tissue would produce GVH reactions when injected into a suitable recipient providing the RNA was protected from degradation by tissue ribonuclease. It would be difficult to protect RNA from destruction by tissue ribonuclease once it had been injected systemically into an animal. However RNA would be protected if it were injected into a localized situation, such as the dermis, with a ribonuclease inhibitor. In addition, before the recipient's lymphoid cells could be influenced by the injected allogeneic RNA they must come in contact with it. A greater opportunity for contact between the injected RNA and the recipient's lymphoid cells might be provided by the induction of a migration of leucocytes into the area by injecting bentonite with the RNA. The bentonite,

TABLE 9.1

A comparison of the daily increases in skin thickness at the site of injection of allogeneic lymphocytes with those produced by RNA extracted from lymphocytes from the same donor.

Sheep No	Inoculation	*Increases in skin thickness in mms. Time in days after inoculation							
		1	2	3	4	5	6	7	8
1	cells(1)	0	1.2	1.4	2.4	3.0	4.1	5.8	6.0
	RNA(2)	1.9	2.5	2.5	2.4	2.6	2.5	2.6	3.0
2	cells	0.8	0.7	1.7	2.5	2.9	2.8	2.9	1.8
	RNA	2.9	2.1	2.0	1.6	1.5	1.6	2.0	2.0
3	cells	0.5	0.6	1.0	1.2	1.3	1.8	1.9	0.8
	RNA	0.8	1.4	0.5	0.6	0.8	0.5	0.9	1.0
4	cells	0.2	0.3	0.6	0.7	0.4	0.3	0.8	0.5
	RNA	2.2	1.8	1.5	1.5	1.2	1.4	2.1	2.0
5	cells	0.1	0.7	0.3	0.8	0.5	0.8	0.1	0.0
	RNA	0.8	0.5	0.0	0.5	0.0	-0.1	0.2	0.2
6	cells	0.6	0.6	0.8	1.3	2.1	2.0	3.7	4.3
	RNA	1.6	1.4	0.7	1.0	1.0	0.2	0.0	0.3
7	cells	0.3	0.2	0.4	0.0	0.5	0.8	0.3	0.5
	RNA	0.2	1.0	0.4	-0.5	-0.5	0.2	0.2	0.2
Donor	cells	0.4	0.4	0.2	0.2	0.2	0.2	0.2	0.2
	RNA	-0.3	-0.4	-0.2	-0.6	-0.6	-0.5	-0.1	-0.5

*Increases in skin thickness for RNA reactions are over and above a small basal bentonite reaction.

(1) 5×10^6 lymphocytes were injected into the 8 sheep recipients.

(2) 30γ RNA was injected into each recipient.

which besides being a powerful inhibitor of ribonuclease, also induces a mild local "foreign body" reaction (McCutcheon, 1961).

The experiments described in this chapter compare the responses produced in sheep by the intradermal injection of allogeneic lymphocytes with those produced by similar injections of RNA from different sources.

Experimental

Comparison of the Dermal Reactions Produced by Allogeneic Lymphocyte RNA with NLT Reactions Produced in Sheep

The dermal reactions resulting from the intradermal injections of allogeneic lymphocyte RNA were compared with NLT reactions produced by similar injections of 5 million lymphocytes from the same donor. The RNA used in these experiments was extracted from allogeneic lymphocytes by the method described in Chapter 2. After extraction, the RNA was dissolved in PBS to a final concentration of 300 γ per ml and 0.1 per cent bentonite added. The addition of bentonite before injection was found to be necessary since inoculations of RNA without bentonite failed to produce any increases in skin thickness. This failure of allogeneic RNA to produce any increases in skin thickness without bentonite is probably because of its inactivation by tissue ribonucleases. Inoculation of 30 γ of RNA in 0.1 ml of PBS containing 0.1 per cent bentonite were made intradermally into the thighs of 8 sheep, including the lymphocyte donor. Simultaneously, 0.1 ml of PBS containing 0.1 per cent of bentonite was inoculated intradermally into each sheep. The daily increases in skin thickness at the site of the RNA inoculation, from which any thickening due to the small basal reaction to bentonite had been subtracted, were compared with NLT reactions resulting from the injection of 5 million allogeneic lymphocytes. It can be seen from the results, recorded in Table 9.1, that the increases in skin thickness produced by

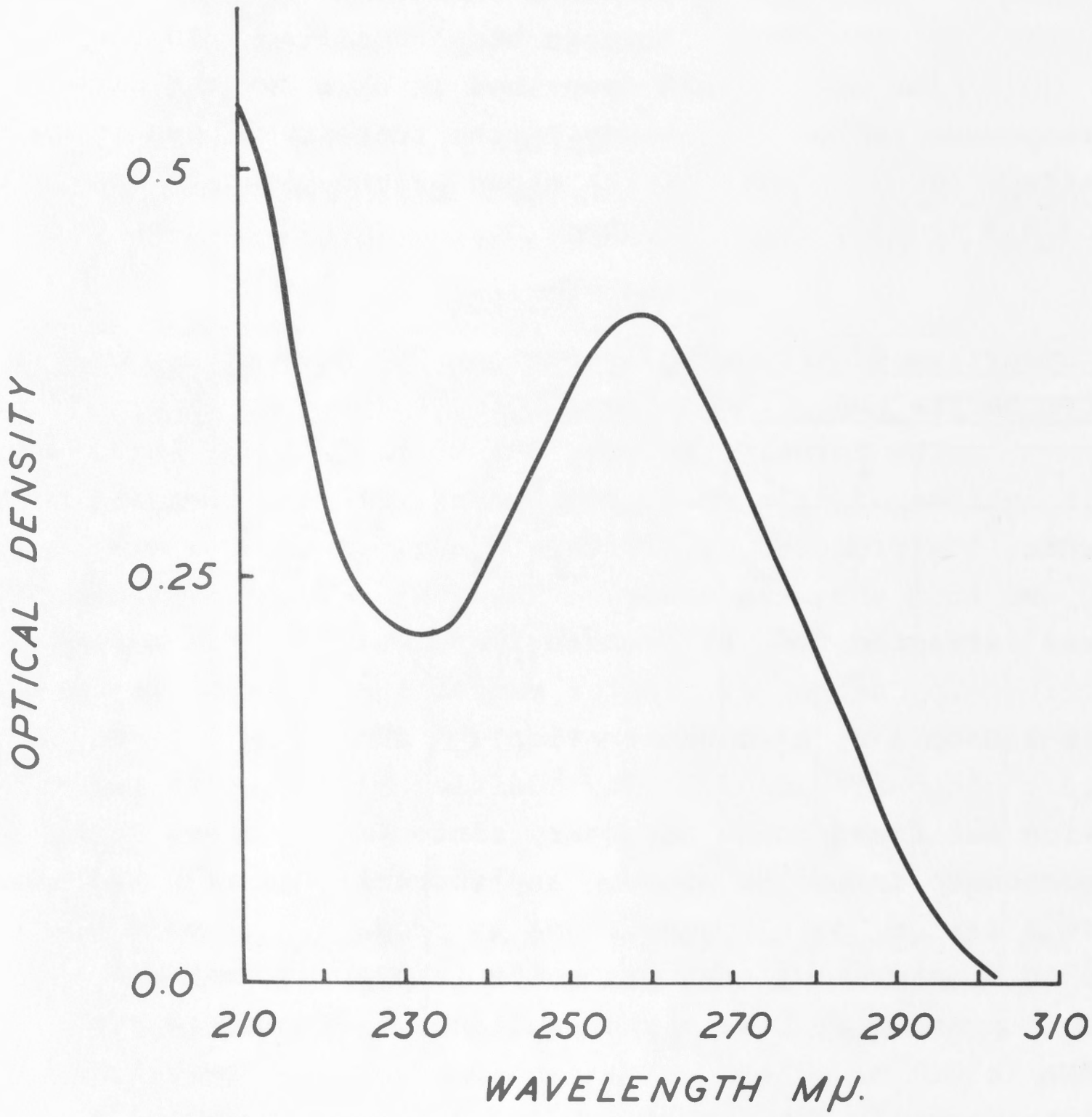


Figure 9.1

Optical density spectrum of an RNA preparation from sheep lymphocytes.

RNA are closely paralleled by those resulting from the injection of lymphocytes obtained from the same donor. In the donor no significant reactions were detected at either the site of the inoculation of the RNA or where the cells had been inoculated. It should be noted that in this group of sheep there was one (sheep 4) in which a significant RNA reaction occurred when there had been no reaction to allogeneic cells. This apparently anomalous result will be discussed later.

Although the method used for the extraction of the lymphoid cells is one developed specifically for obtaining RNA from cells, it is possible that the RNA preparation may be contaminated by other materials which account for its capacity to produce dermal reactions in sheep.

Determination of the Purity of the RNA Preparation

The material obtained from allogeneic lymphocytes by phenol extraction, as described previously, had an ultra violet absorption spectrum characteristic of RNA (Fig. 9.1). The same preparation contained less than 10 γ of protein per ml as determined by the microbiuret test. It has been shown that when solutions of RNA are centrifuged to equilibrium in concentrated cesium sulphate, they band at a buoyant density of 1.63 (Hearst and Vinograd, 1961). The buoyant density of the material contained in the lymphocyte extract was determined by layering 0.1 ml of such an extract, containing 120 γ of RNA, on top of 4 mls of a 66 per cent solution of cesium sulphate in glass distilled water. After overlayering with 0.2 ml of paraffin oil, the tubes were centrifuged at 38,000 RPM in a Spinco SW39 swinging bucket rotor for 60 hours. After centrifugation, the density and optical density at a wavelength of 260 mμ for a 1 cm light path were determined for 0.2 ml fractions of the resultant gradient. Figure 9.2 demonstrates that the material extracted from lymphocytes bands at a buoyant density of 1.62 when centrifuged to equilibrium in cesium sulphate.

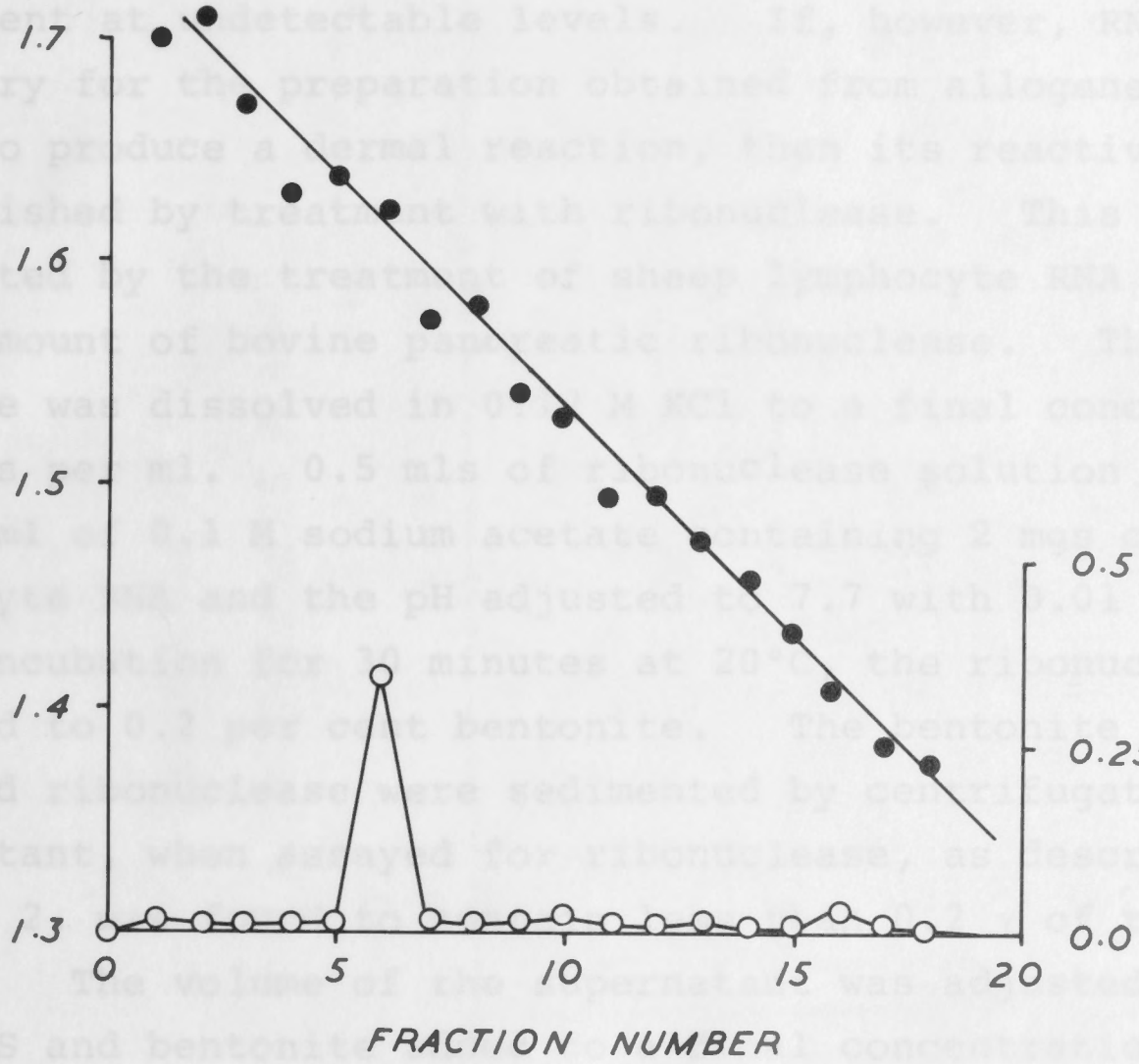


Figure 9.2

The optical density at 260 mμ for a 1 cm light path and density of fractions of a cesium sulphate gradient to which an RNA containing extract had been added. The RNA and cesium sulphate had been centrifuged for 60 hours at 38,000 RPM in a SW39 Spinco rotor. Optical density, o; buoyant density, ●.

However, this information does not allow one to conclude with certainty that the reactivity ascribed to the lymphocyte extract is due to RNA, since other contaminating materials may be present at undetectable levels. If, however, RNA is necessary for the preparation obtained from allogeneic lymphocytes to produce a dermal reaction, then its reactivity should be abolished by treatment with ribonuclease. This proposition was tested by the treatment of sheep lymphocyte RNA with an equal amount of bovine pancreatic ribonuclease. The ribonuclease was dissolved in 0.12 M KCl to a final concentration of 2 mgs per ml. 0.5 mls of ribonuclease solution was mixed with 1 ml of 0.1 M sodium acetate containing 2 mgs of sheep lymphocyte RNA and the pH adjusted to 7.7 with 0.01 N NaOH. After incubation for 30 minutes at 20°C, the ribonuclease was adsorbed to 0.2 per cent bentonite. The bentonite and the adsorbed ribonuclease were sedimented by centrifugation and the supernatant, when assayed for ribonuclease, as described in Chapter 2, was found to contain less than 0.2 γ of ribonuclease per ml. The volume of the supernatant was adjusted to 2 mls with PBS and bentonite added to a final concentration of 0.1 per cent. The dermal reactions produced in sheep by 0.1 mls of the ribonuclease treated RNA were compared with those occurring at the sites of inoculation of 100 γ RNA from the same lymphocyte extract. The results of this experiment are shown in Figure 9.3 which indicate that after treatment of RNA obtained from allogeneic lymphocytes with ribonuclease, all activity was abolished.

Although the above results indicate that RNA is vitally involved in the production of a dermal response in sheep by the material obtained from allogeneic lymphocytes, it is possible that the RNA is acting in a role similar to that described for macrophage RNA by Askonas and Rhodes (1965). They found that RNA from mouse peritoneal cells, in association with antigen

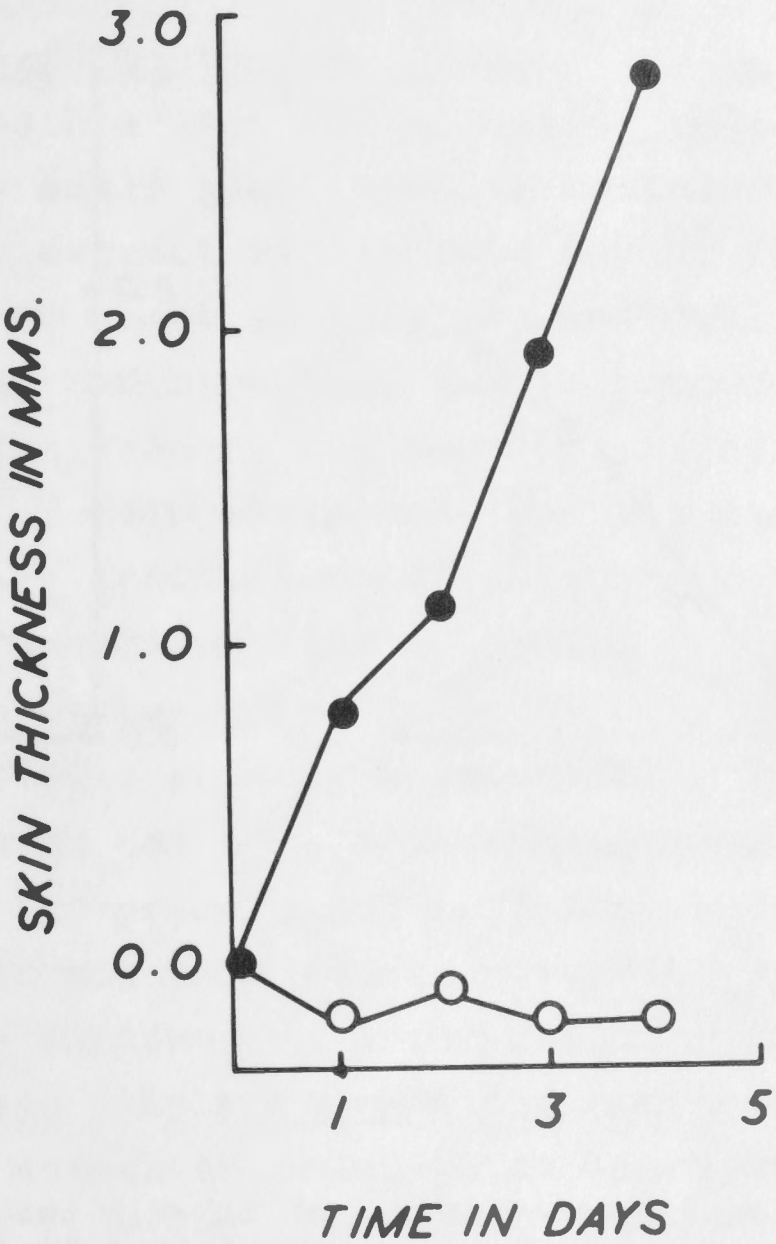


Figure 9.3

Comparison of increases in skin thickness resulting from the intradermal inoculation of untreated allogeneic lymphocytes RNA (filled circles) and ribonuclease treated RNA (open circles).

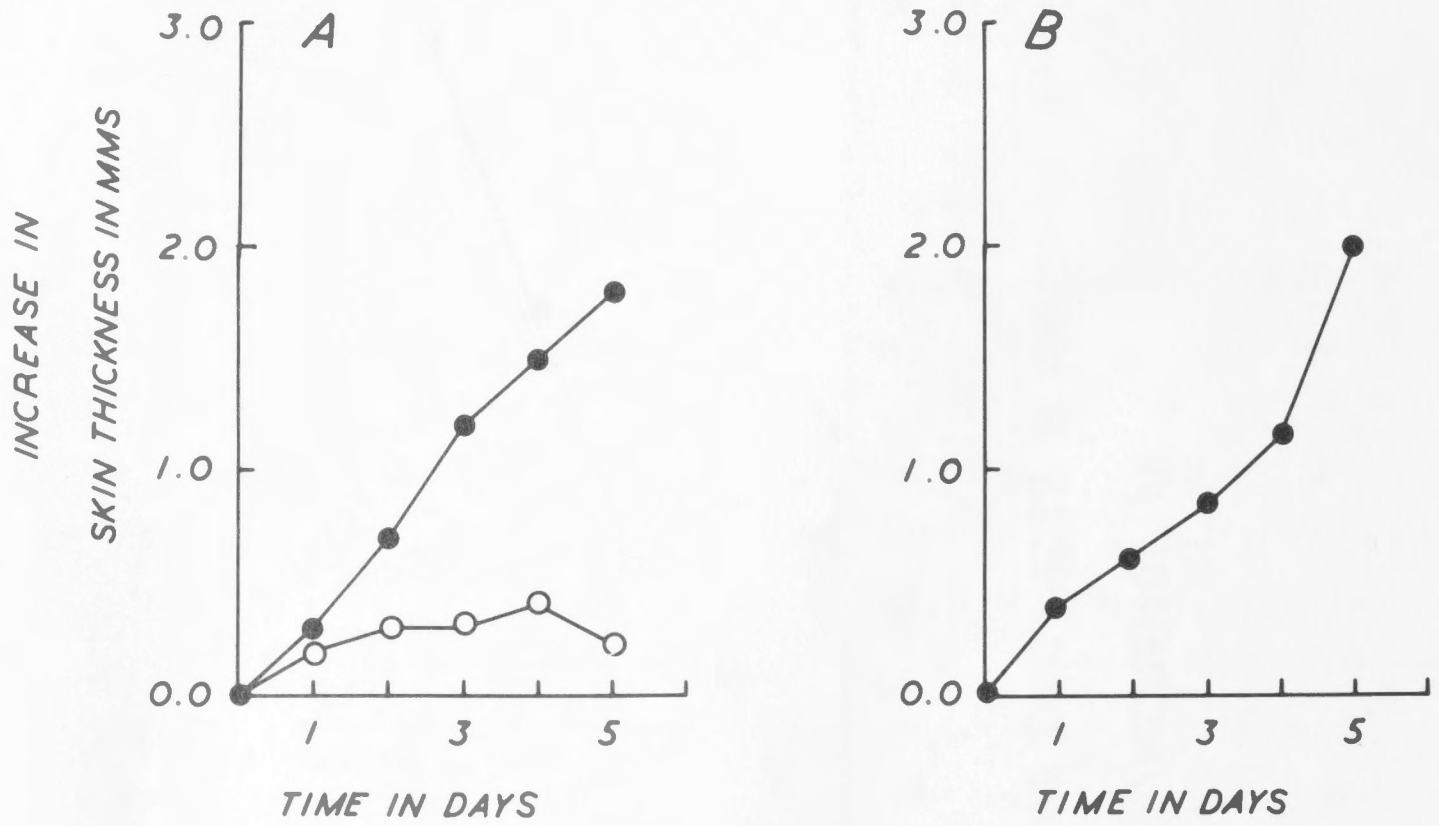


Figure 9.4

A. Comparison of increases in skin thickness resulting from a primary intradermal inoculation of allogeneic lymphocytes (filled circles) and that produced by reinoculation with lymphocytes from the same donor 16 days later after primary inoculation (open circles).

B. Increases in skin thickness resulting from the intradermal inoculation of allogeneic lymphocytes into a sheep that had previously been inoculated with lymphocyte RNA from the same donor.

had an adjuvant-like action resulting in the immunization of the recipients.

The Effect on a Subsequent NLT Reaction of a Previous Inoculation of Lymphocyte RNA from the Same Donor

It is possible that the allogeneic RNA is acting as an adjuvant for very small quantities of cellular antigen present in the lymphocyte extract and in this manner the recipient is being sensitized to react against any residual antigen at the site where the RNA had been injected to produce the dermal response. To test whether the host was being sensitized by the dermal injection of allogeneic RNA, use was made of the observation that a primary inoculation of allogeneic lymphocytes specifically inhibits the production of an NLT reaction by a second injection of lymphocytes taken from the same donor. Figure 9.4A shows the results of a primary and secondary inoculation of a recipient sheep with the same allogeneic lymphocytes. The time interval between the primary and secondary inoculations was 16 days. No significant skin reaction results from the secondary inoculation. In contrast, a prior inoculation of 30 γ of reactive allogeneic lymphocyte RNA did not abolish the ability of the recipient animal to react to an inoculation of 5 million lymphocytes taken from the same animal (Fig. 9.4B). It may be concluded from these observations that an intradermal inoculation of allogeneic lymphocyte RNA does not lead to the sensitization of the recipient to the antigens of the donor.

The Activity of RNA Obtained from Different Sources

It has already been demonstrated in Chapter 5 that allogeneic tissue cells and most xenogeneic lymphoid cells are unable to produce dermal reactions similar to those produced by allogeneic lymphocytes. Figure 9.5A compares the dermal reactions produced when 10 million allogeneic spleen and liver cells and 10 million chicken spleen cells were each inoculated separately into a recipient sheep. It can be seen from the results

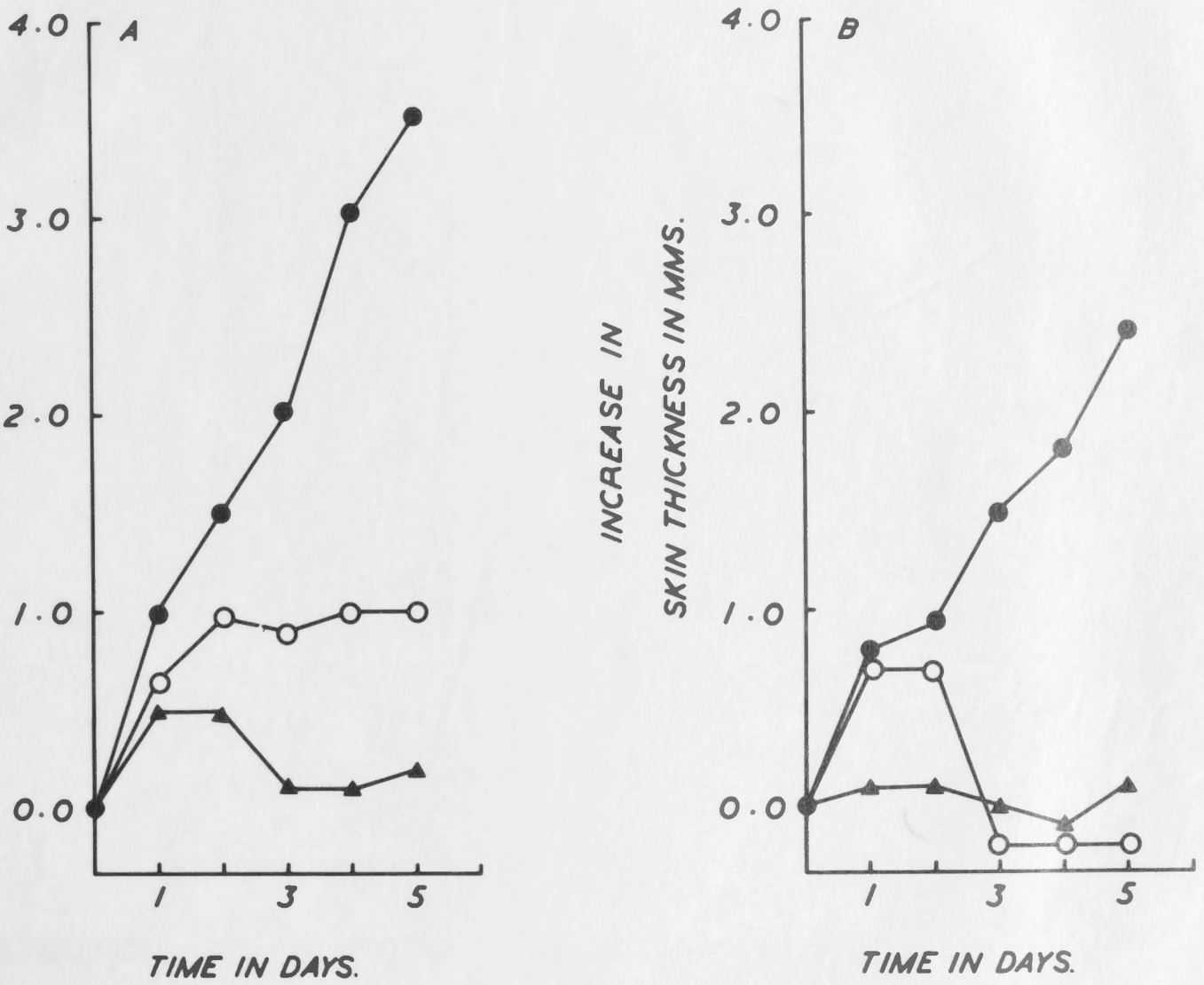


Figure 9.5

A. Comparison of increases in skin thickness resulting from the intradermal inoculation of allogeneic spleen cells (filled circles), allogeneic liver cells (open circles) and xenogeneic spleen cells (triangles).

B. Comparison of increases in skin thickness resulting from the injection of ribonucleic acid extracted from allogeneic spleen cells (filled circles), allogeneic liver RNA (open circles) and xenogeneic spleen RNA (triangles).

Figure 9.6

A comparison of the dermal responses produced by injections of allogeneic RNA from various tissues 4 days after inoculation.

Upper left	Bentonite control.
Lower left	Liver RNA 120 γ
Upper right	Spleen RNA 120 γ
Lower right	Spleen RNA 60 γ

obtained in this experiment that allogeneic lymphoid cells produce a very strong dermal reaction. In marked contrast, the chicken spleen cells did not cause a significant reaction, although the sheep liver cells produced a small but significant dermal response. When 120 γ of RNA extracted from the liver and spleen of a sheep and from chicken spleen were each injected separately into the skin of the same recipient sheep, the resultant dermal reactions paralleled those produced by cells. Figures 9.5B and 9.6 show that the RNA isolated from the allogeneic spleen cells produced a strong reaction, while that from chicken spleen was inactive. The RNA extracted from allogeneic liver produced a reaction of dubious significance.

The Size of Allogeneic Lymphocyte RNA Capable of Producing Dermal Responses in the Sheep

It is well known that although the macromolecules of RNA within mammalian cells occur in a wide range of sizes, the great majority can be divided into three broad groups, ribosomal, transfer, and messenger RNA. The use of linear sucrose gradients allows the separation of smaller transfer RNA from the larger ribosomal RNA, although messenger RNA may contaminate all fractions. If the mechanism of the dermal response to allogeneic lymphocyte RNA is to be understood then it it necessary to know whether the activity is restricted to any particular fraction.

The size of the active RNA fraction was determined by centrifuging the RNA extracted from allogeneic lymphocytes on a 30 mls linear gradient from 5 to 20 per cent sucrose in 0.005 M Tris-HCl buffer containing 0.04 M EDTA. 3 mls of PBS containing 2.8 mgs RNA were layered on to the top and then centrifuged for 16 hours at 23,500 RPM in an SW25 swinging bucket rotor on an ultracentrifuge (Beckman Spinco). After centrifuging, the bottom of the centrifuge tube was punctured and 0.8 ml fractions were collected. The optical density at a wavelength

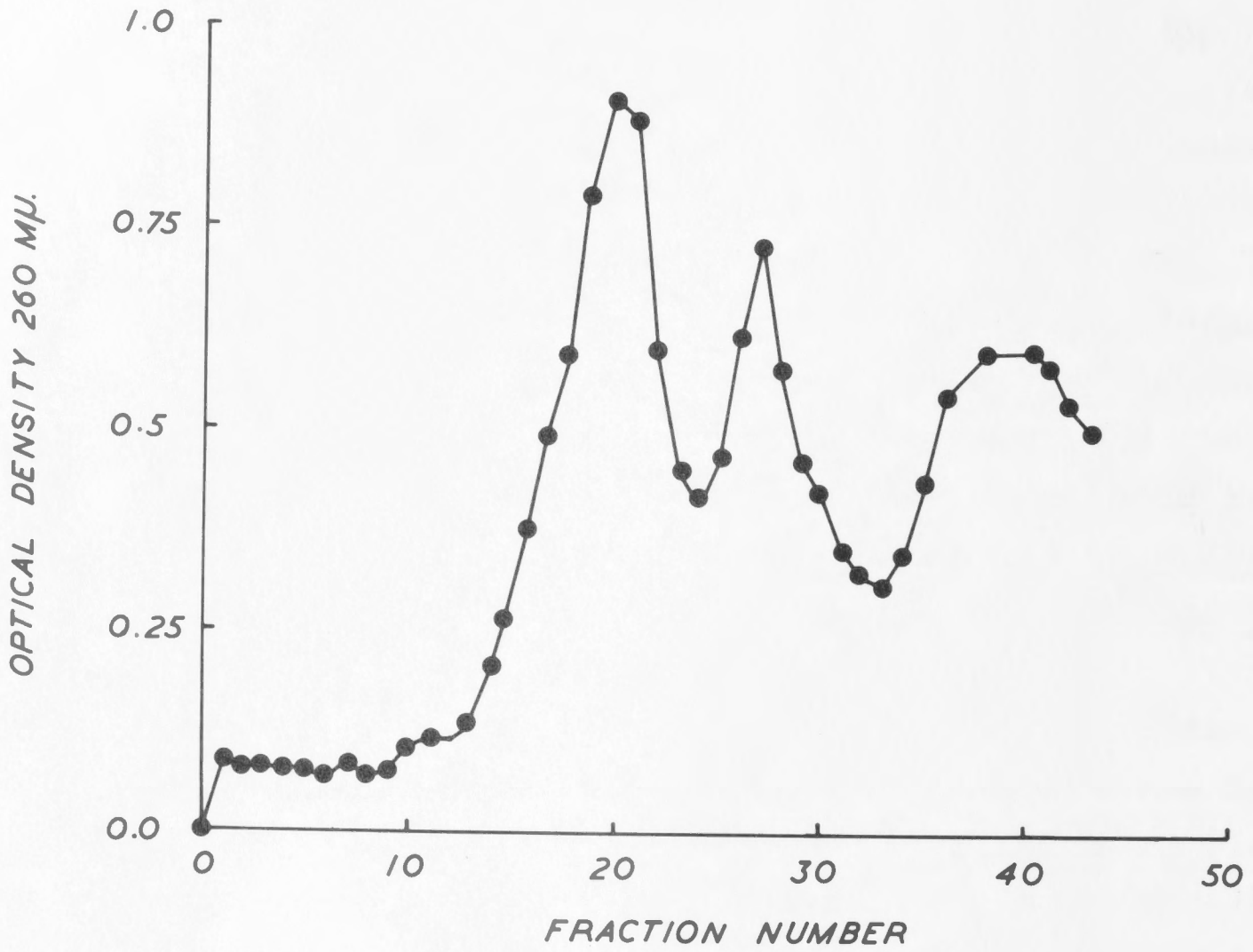


Figure 9.7

Sucrose density gradient analysis of sheep lymphocyte RNA. 2.8 mgs of RNA in a final volume of 3 mls were layered onto a 5 to 20 per cent (w/v) linear sucrose gradient and centrifuged for 16 hours at 23,500 RPM in a Spinco SW25 rotor.

of 260 mμ for each fraction was determined. The results are presented in Figure 9.7 and show that although some RNA is present in all fractions of the gradient, 60 per cent is concentrated in three peaks. The RNA in fractions numbered 20, 26 and 38, which are representative of the three peaks, was adjusted to a concentration of 400 γ per ml in PBS and 0.1 per cent bentonite added before 0.1 ml was inoculated into a sheep. Simultaneously an equal quantity of the RNA containing material before fractionation was inoculated with bentonite into an adjacent area.

Figure 9.8 compares the daily increases in skin thickness produced by each fraction with the original material. A significant increase in skin thickness results with the RNA in fraction 26 but not with the material in the other two fractions.

Histopathological Changes Produced by an Intradermal Injection of RNA and Bentonite

The increases in skin thickness following an intradermal injection of RNA and bentonite occur more rapidly than those produced by an injection of allogeneic lymphocytes. Figure 9.9 A-F shows that much of this increased thickness was due to an increased cellularity up to 5 days. After 5 days the central area of the lesion becomes necrotic with subsequent liquefaction of the tissues. A very similar sequence of events is seen in the control inoculation of bentonite. The bentonite produces an infiltration of polymorphonuclear neutrophiles during the first 24 hours after injection (Fig. 9.10A). At this stage of the reaction there is no detectable histological difference between the bentonite controls and those sites that received RNA and bentonite (Fig. 9.10B).

During the next 3 days the bentonite becomes largely localized by an increasing number of surrounding fibroblasts and histiocytes and is finally walled off (Fig. 9.9F). When the areas surrounding the central bentonite lesion in those

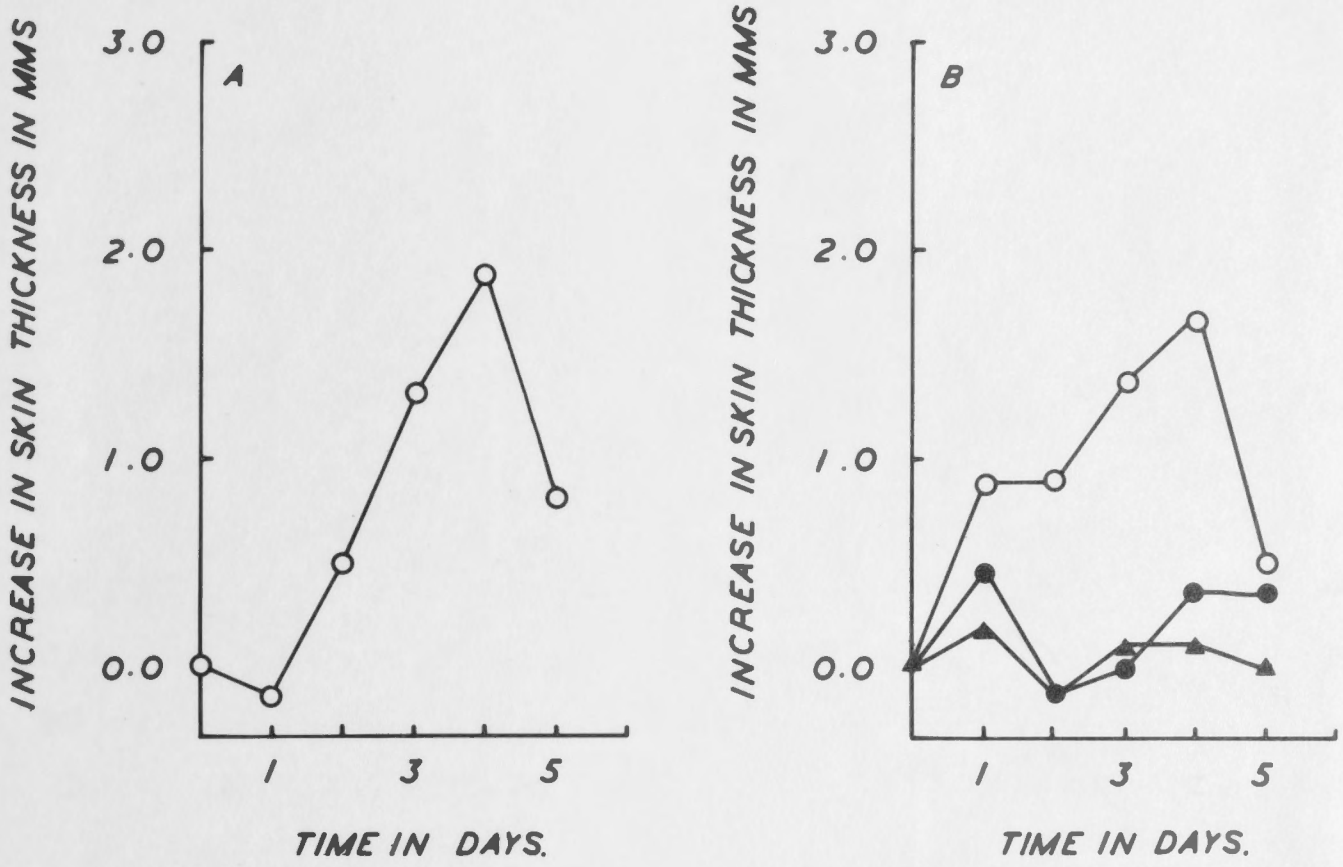


Figure 9.8

A. Increases in skin thickness at daily intervals with unfractionated RNA extracted from allogeneic lymphocytes.

B. Comparison of mean daily increases in skin thickness of RNA fractionated on a linear sucrose gradient as in Fig. 9.6. Fraction number 20 is represented by filled circles and fractions 26 and 38 by open circles and filled triangles, respectively.

Figure 9.9

A comparison of the histopathological changes produced in sheep by intradermal injections of 0.1 mls of PBS containing 30 γ allogeneic lymphocyte RNA and 0.1 per cent bentonite with those occurring after the injection of 0.1 per cent bentonite.

A RNA and bentonite after 24 hours.

B RNA and bentonite after 72 hours.

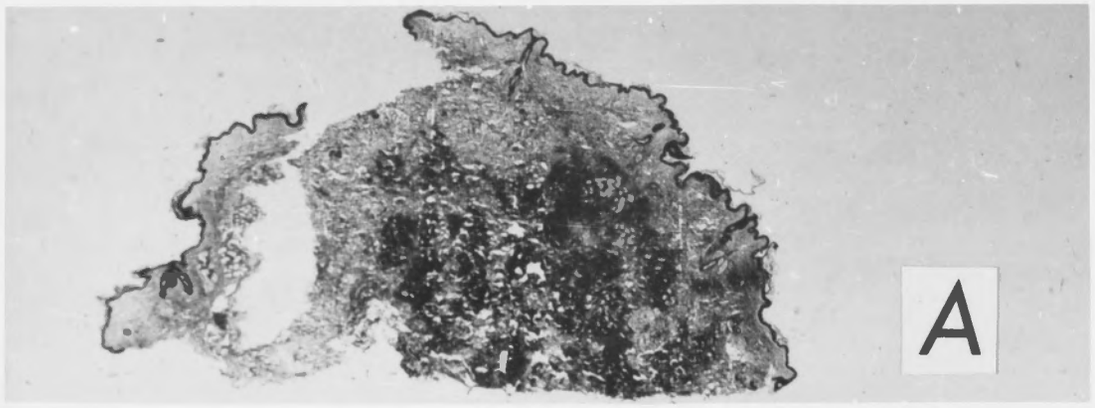
C RNA and bentonite after 96 hours.

D Bentonite after 24 hours.

E Bentonite after 72 hours.

F Bentonite after 14 days.

Magnification 10x Stain, haematoxylin and eosin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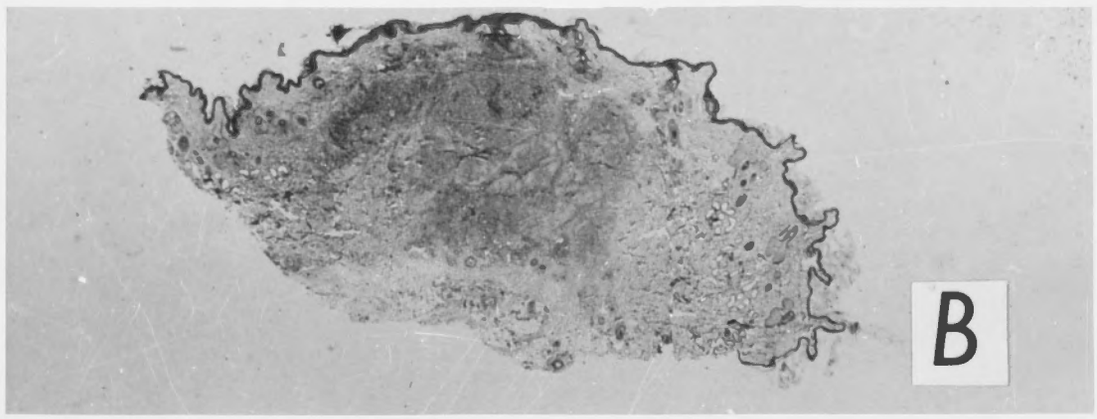
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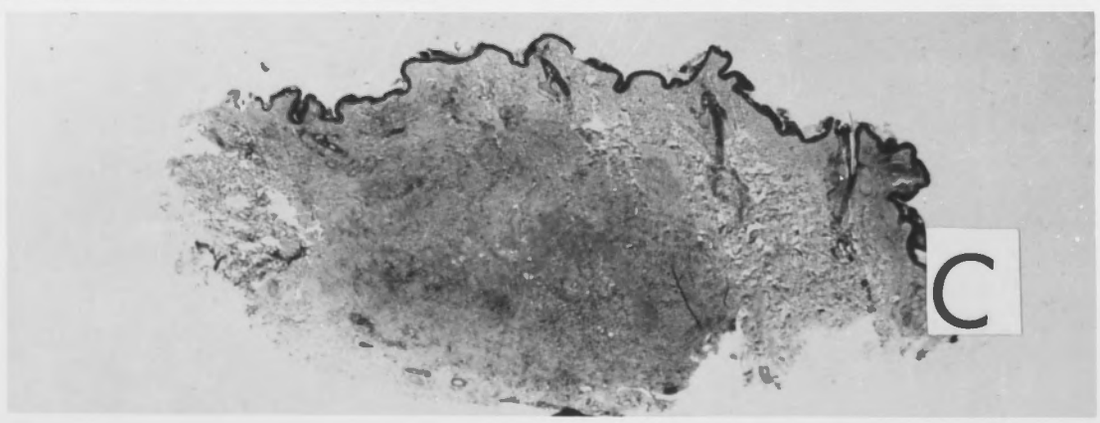
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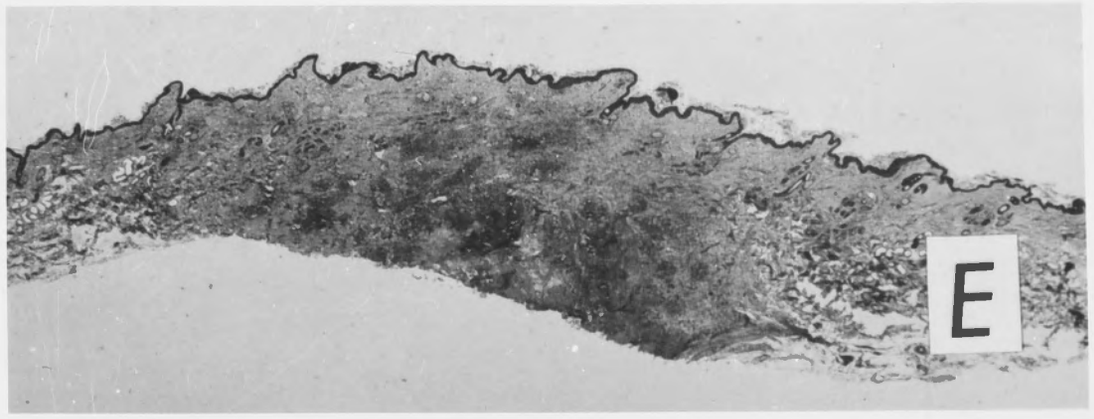
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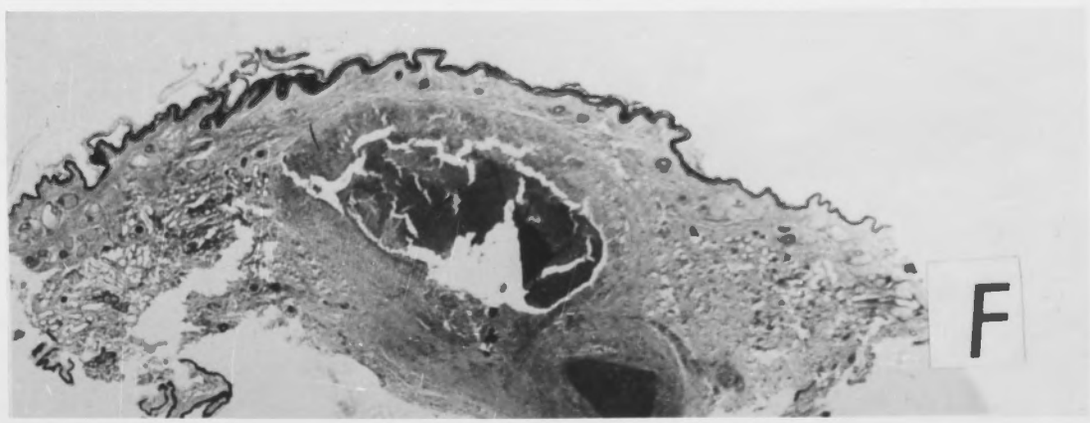
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Figure 9.10

A comparison of the histopathological changes produced by an intradermal inoculation of 0.1 mls of PBS containing 30 γ allogeneic lymphocyte RNA and 0.1 per cent bentonite with those occurring after the injection of 0.1 per cent bentonite.

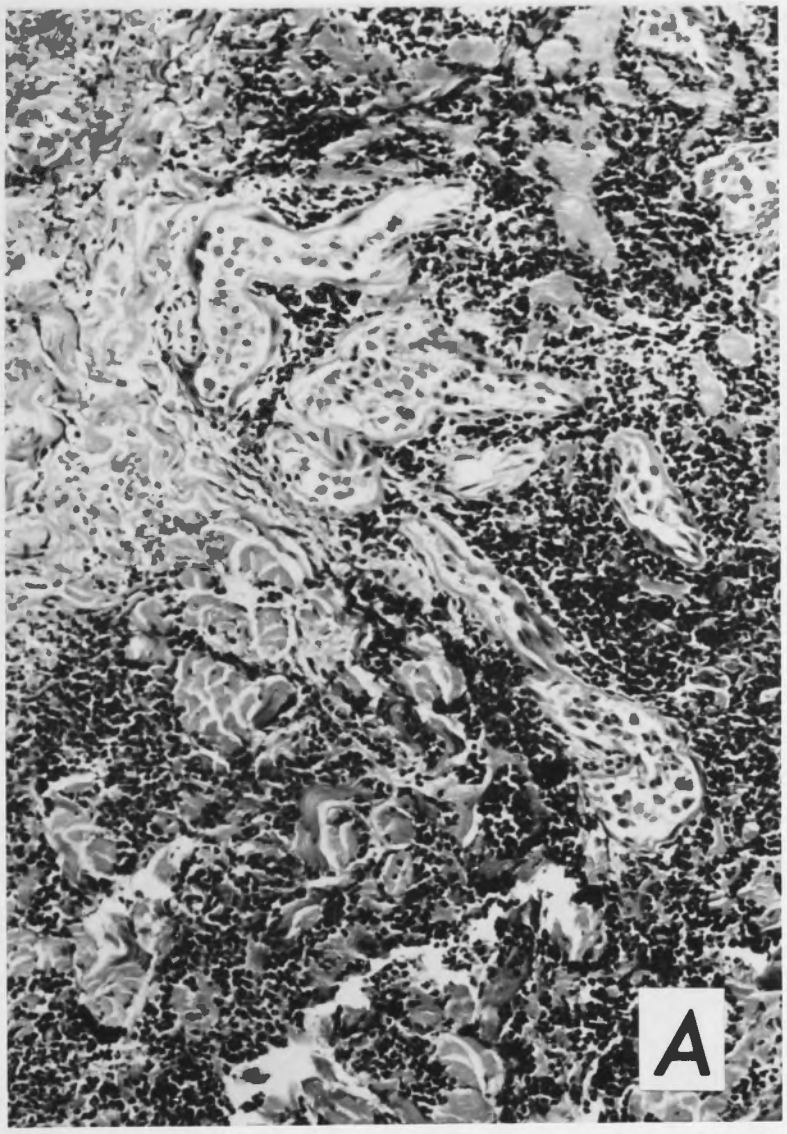
A RNA and bentonite 24 hours after injection. Magnification 130x.

B Bentonite 24 hours after injection. Magnification 130x.

C RNA and bentonite 24 hours after injection. Magnification 330x.

D RNA and bentonite 72 hours after injection showing the large blast cells present. Magnification 330x.

Stain, haematoxylin and eosin.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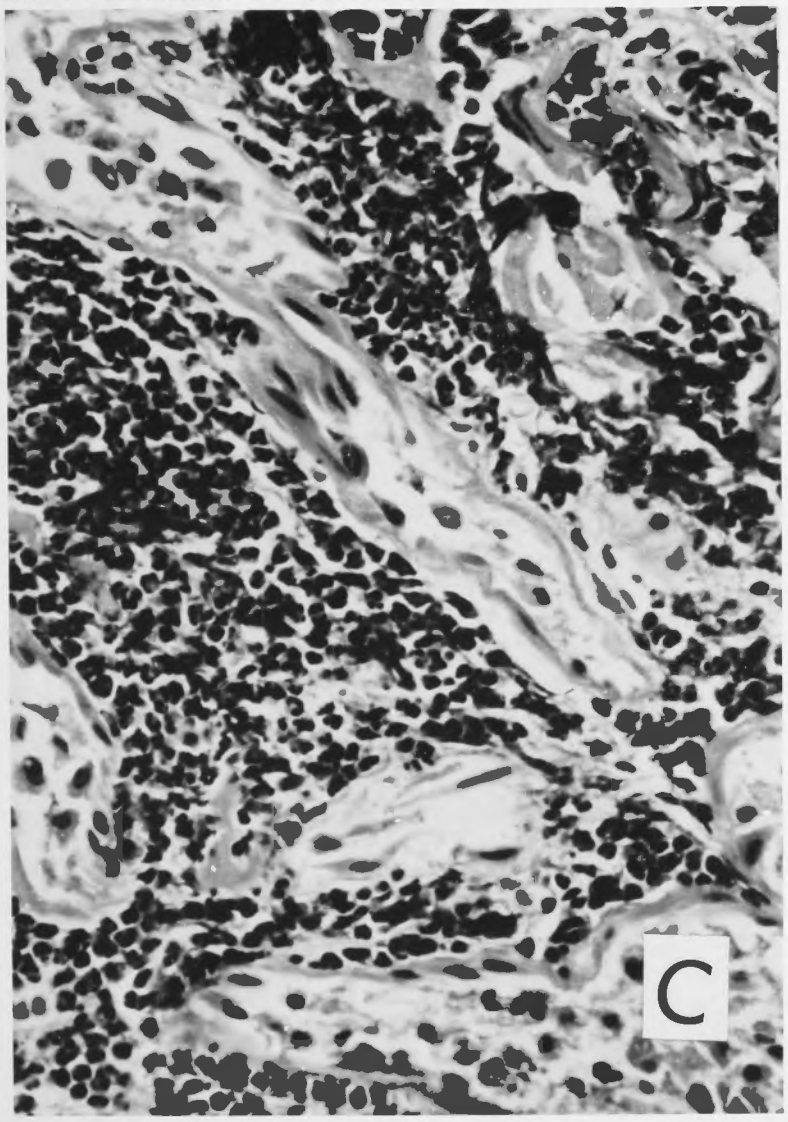
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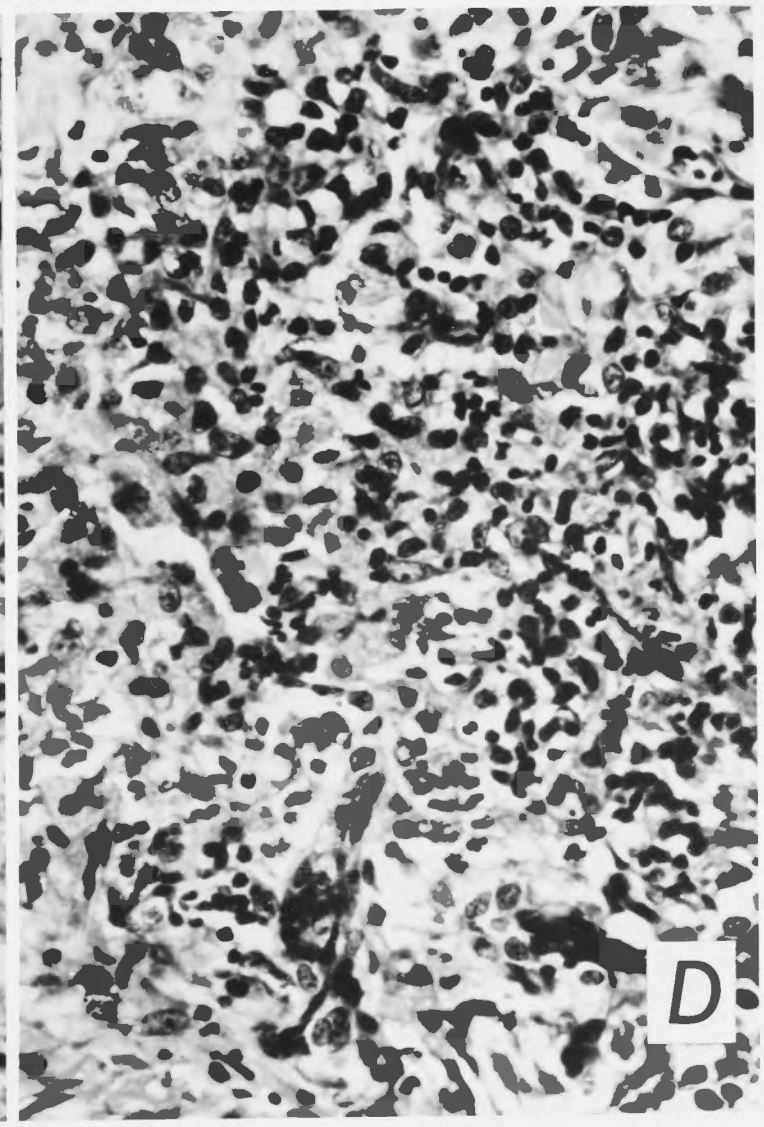
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Figure 9.11

A comparison of the types of cells invading cellular structures in the dermis of a 4 day old reaction produced by allogeneic lymphocyte RNA with those seen in an NLT reaction induced with allogeneic lymphocytes.

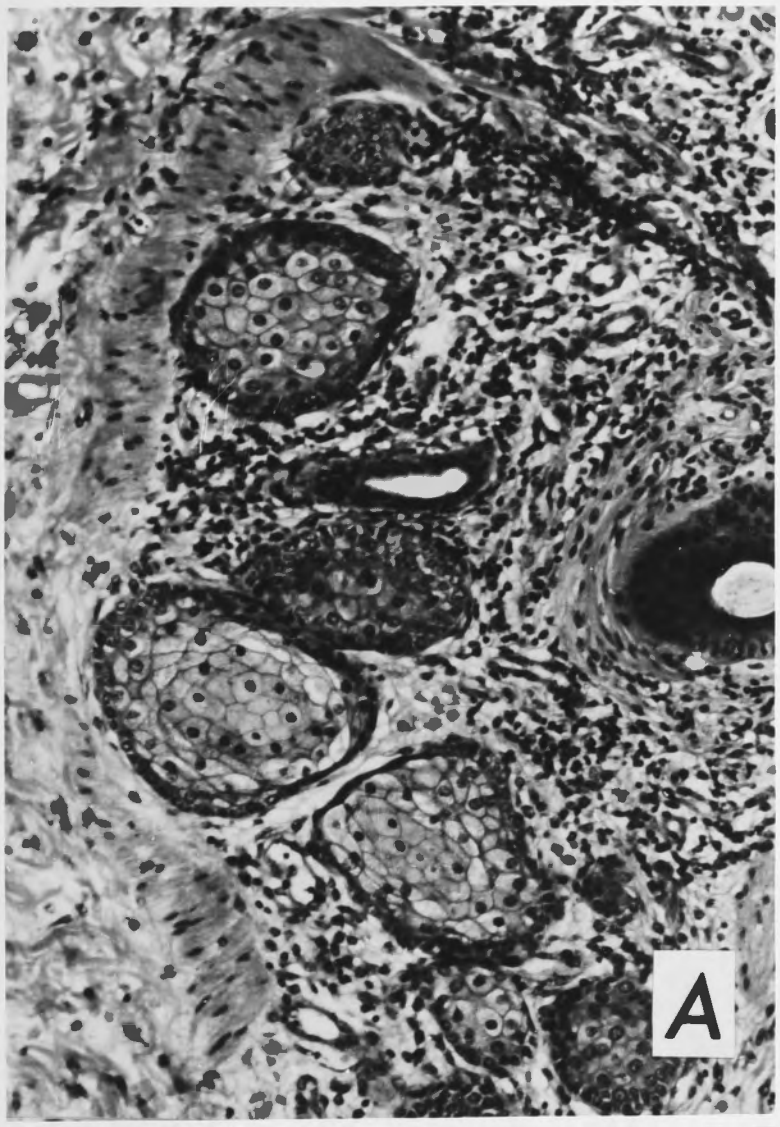
A Infiltration of mononuclear cells around cellular structures in the dermis of a 4 day old RNA induced reaction. Magnification 130x.

B Cellular infiltration around cellular structures in the dermis of a 4 day old control injection of bentonite. Magnification 130x.

C Invasion of a sebaceous gland with mononuclear cells in a 4 day old RNA induced reaction. Magnification 330x.

D Invasion of a sebaceous gland with mononuclear cells in a 4 day old NLT reaction induced by allogeneic lymphocytes. Magnification 330x.

Stain, haematoxylin and eosin.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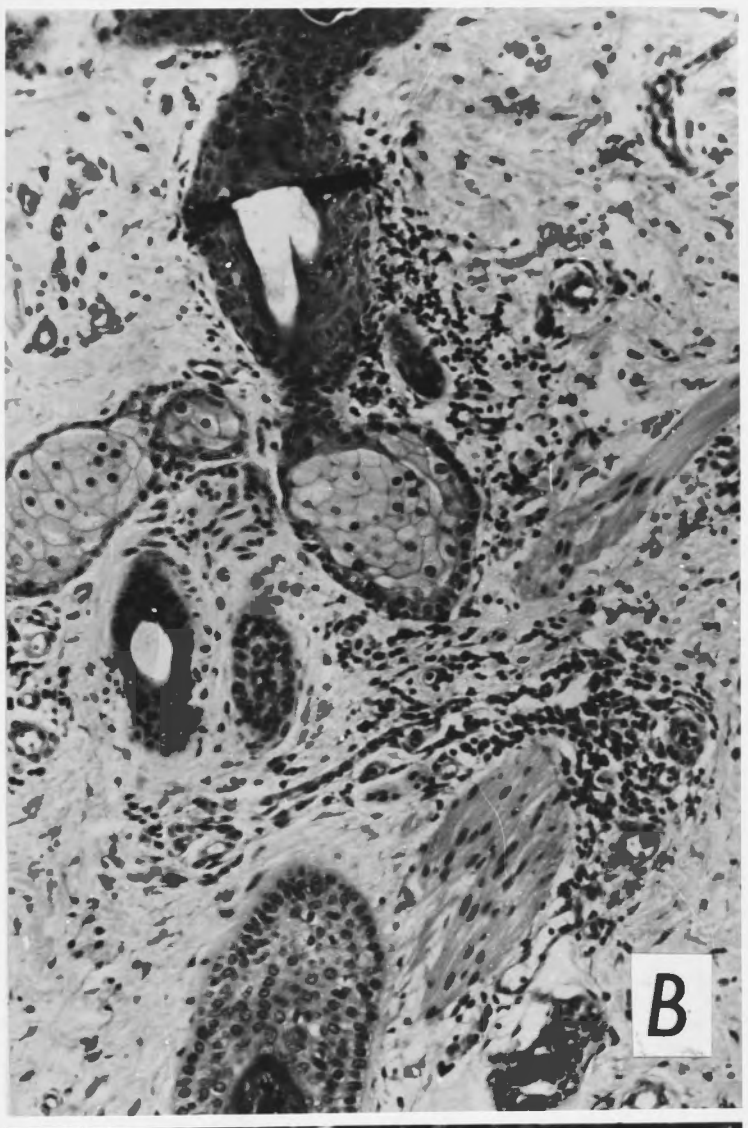
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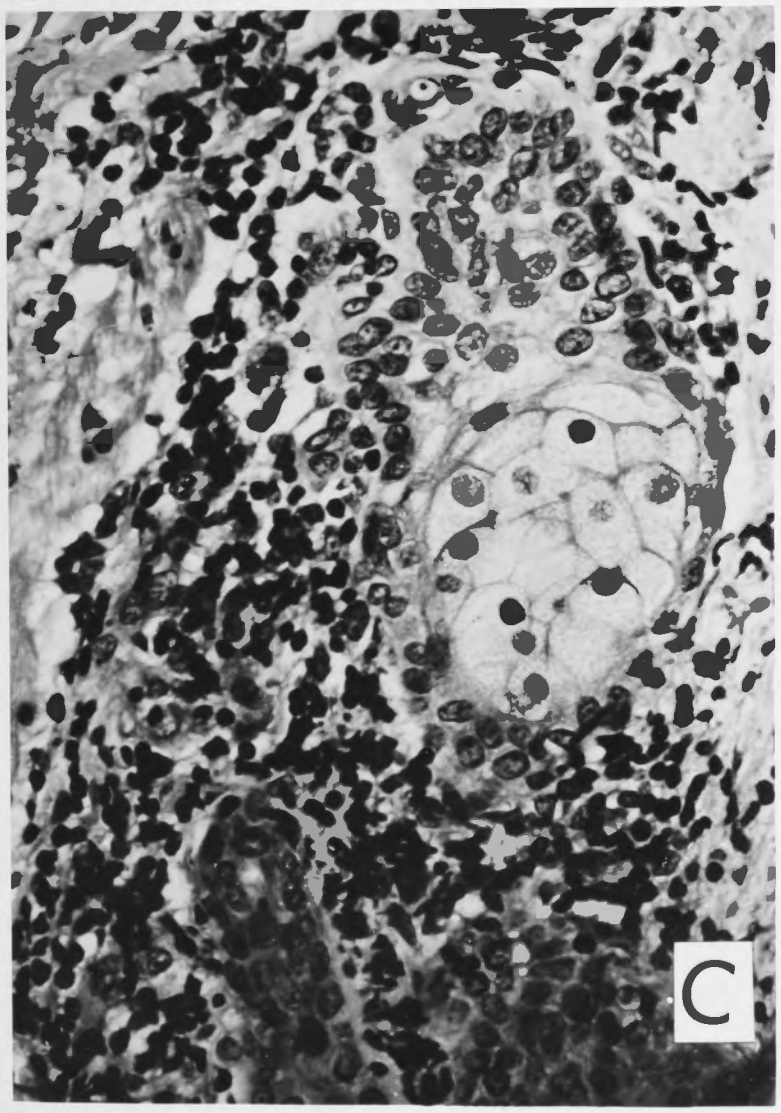
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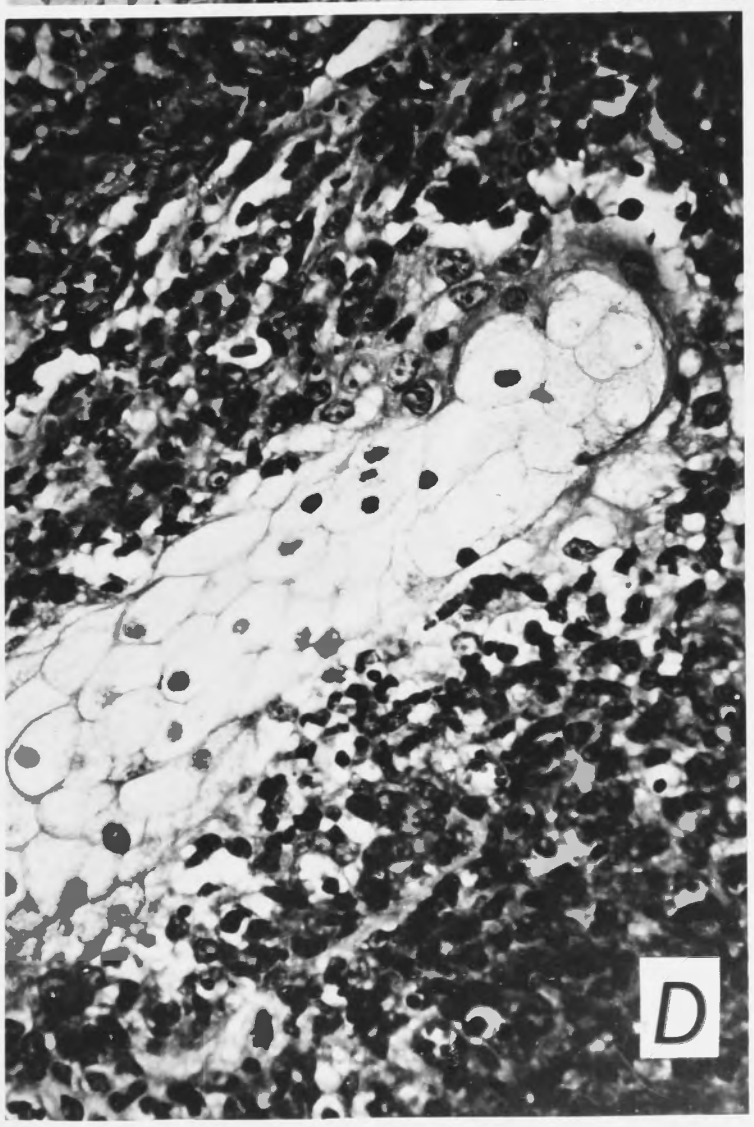
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reactions produced by RNA and bentonite are examined, there are seen perivascular collections of infiltrating mononuclear cells which for the first day are heavily contaminated with polymorphonuclear neutrophiles (Fig. 9.10A-C). Over the following 2 days there is a steady increase in the numbers of large blast cells in such areas (Fig. 9.10D) and any cellular structures in these areas are invaded by these cells. Figure 9.11A-D compares the types of invading cells found in these peripheral areas of the RNA reactions with those seen in NLT reactions. The cells found in both the RNA and NLT reactions are largely mononuclear cells with similar morphological appearance and characteristic invasive capacities. No evidence of invasion of cellular structures of the dermis or collections of similar blast cells were seen in any of the control bentonite sections examined.

Discussion

Intradermal injections of an RNA containing preparation extracted from allogeneic lymphocytes produced dermal responses in the recipient sheep which were similar in size and some histological features to the NLT reactions produced by injections of lymphocytes from the same donor. It was found that the lymphocyte RNA, although reactive in allogeneic recipients, did not produce reactions when injected into the lymphocyte donor. This correlation between the sizes of the dermal reactions produced by lymphoid cell RNA with the sizes of the reactions produced by lymphocytes obtained from the same donor as the RNA, was even more clearly shown by the finding that xenogeneic (chicken) RNA, like chicken lymphoid cells, was totally inactive when inoculated into the sheep. These findings support the hypothesis that the NLT reaction may result from the transfer of RNA or an RNA containing material between the lymphoid cells of donor and recipient. The capacity to produce dermal reactions comparable with those produced by the intradermal injection

of lymphocytes from the same donor was found to be restricted to RNA containing preparations of allogeneic lymphoid cells. Not all the RNA present in the material obtained from allogeneic lymphocytes has the capacity to induce dermal inflammatory reactions in sheep. This was shown by the recovery of the capacity of allogeneic lymphoid cell RNA to produce dermal reactions in one fraction of RNA fractionated on a linear sucrose gradient and the absence of this activity from the other fractions tested. This observation suggests that only RNA molecules of a certain size have the capacity to induce dermal inflammatory reactions in sheep.

It will have been noticed that one of the 8 recipient sheep in the experiment in which the activities of allogeneic lymphocytes and lymphocyte RNA were being compared, showed a significant RNA reaction when there had been no reaction to allogeneic cells. This observation can be explained either by the possibility that the injection of cells was made subcutaneously, where it failed to produce a reaction or else the recipient, which was a multiparous ewe, may have been immunized against cells that cross react with the injected cells. It has been shown that the prior inoculation of lymphocytes specifically inhibits any reaction to a second injection of the same lymphocytes, but does not affect the reaction produced by the RNA extracted from these lymphocytes.

Observations on the dermal reactions produced by RNA have been made by other workers (Willoughby et al., 1964; Inderbitzen et al., 1964, 1965), who have described an increase in vascular permeability and leucocyte migration following the intradermal inoculation of RNA into rat skin. This phenomenon lacks the specificity of the reaction described in this chapter, in so far as both allogeneic and xenogeneic RNA produce a change in vascular permeability. On the other hand, a number of workers claim to be able to induce the rejection of skin isografts

following their treatment with allogeneic RNA (Guttman et al., 1964; Burrows et al., 1966). These workers suggested that the role of the RNA was one of a messenger RNA which was responsible for the production of structural proteins in the treated grafts which were foreign to the recipient animal. It seems unlikely that the dermal reactions which resulted from the inoculation of lymphoid tissue RNA can be explained by such a mechanism. In the first place liver RNA was inactive in this system and in the second place the inoculation of reactive RNA did not render the animal immune to the effects of a subsequent injection of lymphocytes.

Several unsuccessful attempts have been made to produce dermal inflammatory responses by the intradermal injection of allogeneic lymphoid RNA (Mannick and Egdahl, 1962; Burrow et al., 1966). Burrows et al. (1966) claim that RNA extracted from allogeneic spleen cells will induce a dermal reaction when inoculated into mice that have previously rejected a skin graft from the same donor, although when the same RNA was injected into a normal allogeneic mouse no reaction occurred. These findings are in agreement with observations made in the sheep, where RNA unprotected by bentonite, fails to produce more than an occasional trace reaction.

The histology of reactions produced by RNA is confounded by the foreign body reaction induced by the bentonite, that is injected to protect the RNA from tissue ribonuclease. However, the significant changes occur around the periphery of the foreign body reaction where formation of large blast cells results in the invasion of cellular structures of the dermis in a comparable manner to that seen in NLT reactions. It is tempting to conclude that these large "blast" cells have been produced in response to the allogeneic RNA, and like those occurring in NLT reactions, are attacking the cellular structures.

The results of the experiments reported in this chapter

support the hypothesis that the NLT reaction results not from an immunological attack by the grafted lymphocytes against the antigens of the recipient but rather from the exchange of RNA between the inoculated lymphocytes and the lymphoid cells of the host.

Summary

When an RNA containing preparation, extracted from allogeneic lymphocytes, was injected into the skin of sheep with bentonite, the resultant dermal reactions were similar in size and histological changes to those produced by the injection of allogeneic lymphocytes. The capacity to produce these dermal reactions while present in the extracts from allogeneic lymphoid cells was absent from extracts of allogeneic liver and chicken spleen cells. Treatment of an allogeneic lymphoid cell extract with ribonuclease before inoculation into the skin of a sheep abolished its capacity to produce the dermal response. It was concluded from these observations that the ability of the preparations obtained from allogeneic lymphoid cells to produce dermal reactions was due to the presence of RNA. When the RNA preparation was fractionated on the basis of molecular size, the activity was found to reside mainly in one fraction. Injections of allogeneic RNA did not prevent subsequent injections of lymphocytes, obtained from the same donor, producing NLT reactions. It was concluded from this observation that the injected RNA was not acting as an adjuvant for any transplantation antigens that may contaminate the lymphoid cell extracts. At the same time the RNA cannot be acting as a messenger RNA resulting in the production of structural proteins within the cells at the injection site. The histological changes indicate the production of "blast" cells in the periphery of the lesion and the invasion of the cellular structures of the dermis. These results led to the conclusion that the stimulus

for the production of NLT reactions was the exchange of RNA between host and recipient lymphoid cells.

CHAPTER 10

GENERAL SUMMARY AND CONCLUSIONS

Chapter 10 - General Summary and Conclusions

There is a strong body of circumstantial evidence to support the proposition that reactions of the GVH type are immunological events (Simonsen, 1962). This evidence has led to the generally accepted view that the manifestations of the GVH reaction are initiated by the interaction of lymphocytes with antigen. Previous studies (Payne and Jaffe, 1962), and those reported in this work, have shown that the degree of GVH reactivity manifested by an injection of foreign lymphoid cells into the chicken embryo, was correlated with the phylogenetic relationship between the donor and recipient. It was found that manifestations of GVH reaction were produced by lymphoid cells from closely related species (red jungle fowl, turkey), but not by distantly related species (pigeon, duck, goose) and unrelated species (sheep). It has been postulated by Simonsen (1962), that the failure of lymphoid cells from distantly related and unrelated donors to produce a reaction is due either to the inability of the injected cells to survive or that the foreign environment inhibits the normal functioning of the grafted cells. It is apparent from the experimental findings that grafts of sheep lymph node and pigeon spleen cells are able to survive in the chicken embryo. Such cells are capable of mounting a reaction against the chicken embryo, but only if they are taken from an animal that has been immunized with chicken tissues. It follows from these findings that the normal lymphoid cells, obtained from a species of animal unrelated to the chicken, are either not stimulated by the antigen they encounter in the chicken embryo, or else the environment in which they have been placed is unsuitable for the transformation of the stimulated cells into fully functional effector cells.

The possibility that the environment of the chicken embryo is unsuitable for the transplanted adult lymphoid cells, from unrelated or distantly related donors, to be primarily stimulated can be discounted. This follows the demonstration that pigeon spleen cells are capable of recognizing and destroying grafts of embryonic pigeon bone on the CAM of chicken embryos. One may conclude, therefore, that lymphoid cells fail to initiate a GVH reaction in a xenogeneic environment because they are not primarily stimulated by the antigens they contact in this situation. Similarly, the failure of syngeneic lymphoid cells to bring about the destruction of xenografts, carried by the chicken embryo, can only mean that these cells are not primarily stimulated by xenogeneic antigens, even when the cells are in a syngeneic environment.

It is possible that the GVH reaction represents a secondary response, produced by 'naturally' immune cells, reacting against the antigens of the host. Some credence is given to such a possibility by the demonstration that pigeon bone grafts in the chicken embryo are destroyed, by adult chicken lymphoid cells obtained from an immunized donor. However, a comparison of the pathological effects that result from the inoculation of chicken embryos with adult allogeneic lymphocytes with those produced by equivalent doses of immune duck cells, showed it to be unlikely that the GVH reaction was the manifestation of a reaction of immune allogeneic lymphoid cells. The inoculations of allogeneic lymphoid cells produced tumour-like lesions in the spleen of recipient embryos, such changes were not seen in embryos that had received immune duck cells. Ramseier and Billingham (1966) have shown that local GVH reactions result from the interaction of lymphocytes with

allogeneic reticular tissue. When the interaction of lymphocytes with allogeneic reticular tissue is studied in vitro, as in the mixed lymphocyte reaction, the stimulus was found to allogeneic lymphoid cells. Mixtures of xenogeneic (rat-sheep) lymphocytes failed to stimulate each other, supporting the above conclusion that the lymphocyte-lymphocyte interaction can not be a primary immunological event. The mixed lymphocyte reaction is inhibited, following the irradiation of one of the cellular components of the mixture, 24 hours before mixing with a population of normal allogeneic lymphocytes. This treatment of the lymphocytes did not destroy their antigenicity, as measured by their ability to produce delayed type hypersensitivity reactions in guinea pigs. These observations make it clear that GVH and mixed lymphocyte reactions are unlikely to represent a secondary response by naturally immune cells to allogeneic tissue antigens.

It has been shown that during a GVH reaction, allogeneic lymphoid cells involve a proliferation of reticular cells in the spleen of the recipient, which can not be attributed to antigenic stimulation (Nisbet and Simonsen, 1967). The experiments with immune duck lymphoid cells showed that this proliferation resulted from an interaction of allogeneic lymphocytes with cellular elements of the recipient's spleen. The importance of reticular elements of the spleen and other grafted tissues in initiating such a proliferative response, and its effect on graft destruction, was demonstrated in the case of heart muscle grafts. Heart muscle, obtained from allogeneic embryos, in contrast to other allogeneic tissues, is not damaged when transplanted to 'treated' embryos, except when reticular elements have been added to the heart before grafting. The allogeneic

lymphoid cells, after interacting with one another, proliferate in the cardiac muscle graft invading and destroying surrounding tissues.

The specificity of the GVH reaction and the NLT reaction indicate that the material, which initiates the response in interacting lymphocytes, must reflect the genetic constitution of the cell. In an investigation of dermal reactions in sheep, produced by lymphoid cells and cellular extracts, it was shown that the intradermal injection of RNA, obtained from allogeneic lymphoid cells produces a skin reaction, similar to that produced by viable allogeneic lymphoid cells. Injection of lymphocyte RNA into the donor of the cells, from which the RNA was extracted, failed to induce dermal reactions, although, the same extract was reactive in allogeneic recipients. Similarly, inoculations of RNA extracted from lymphoid cells obtained from xenogeneic donors, like whole lymphocytes from the same source, do not produce dermal reactions. When the RNA induced dermal reactions are compared histologically with NLT reactions, both reactions are seen to contain large blast cells capable of invading surrounding tissues. It is suggested that these cells are produced in response to allogeneic lymphoid cell RNA.

It is very likely that, interactions occur between the lymphocytes present in allogeneic tissue grafts, at the time of grafting, and the recipient's lymphocytes. The importance of the invasive blast cells, that would be formed following lymphocyte-lymphocyte interaction, in the destruction of allografts is not known, but it seems likely from the observations on the cardiac muscle grafts in the chicken embryo and from the histology of the NLT reaction, that such cells must contribute extensively to the early destruction of any allograft.

To maintain that lymphoid cells are incapable of reacting against the antigens of the graft would not be valid. Quite clearly, the acquisition by adult chicken leucocytes of the ability to destroy pigeon bone grafts, in chicken embryos following immunization, represents the reaction of lymphoid cells against antigen.

Tissue grafts are usually rejected when there is a genetic difference and hence an antigenic difference between recipient and donor (Simonsen, 1965). Lymphocytes have been shown to be primarily involved in the production of antibodies (Gowans and McGregor, 1965). There seems to be no reason to suppose that the production of antibodies against antigens present on alien tissues should be any different from that against any other antigen. This being the case antibody production would occur in the draining lymph node and perhaps locally in the allograft as well.

It may be concluded that the reaction by lymphocytes with alien tissues probably has three distinct components, all contributing to the destruction of the foreign material. These three components are :-

(1) The interaction of allogeneic lymphocytes with one another, resulting in the production of invasive blast cells. This interaction does not seem to be mediated by cellular antigens but rather by the exchange of RNA or RNA containing material between cells.

(2) The interaction of immune lymphocytes with the antigens of the tissues, leading to their destruction. This is the type of reaction seen late in the destruction of allograft and perhaps in delayed type hypersensitivity.

(3) The production of antibodies against the antigen of the graft.

Appendix I

Statistical analysis

Routine statistical procedures were used as described by Quenouille (1950).

The mean ($\bar{x}$) of a series of observations x_1, x_2, x_3 .. .. x_n is given by $\bar{x} = \frac{\Sigma x}{n}$ where "n" is the number of observations and Σx is the sum of the observations.

The variance (σ^2) is taken as the total of the squared deviations divided by one less than the number of observations and is given by $\sigma^2 = \frac{\Sigma (x-\bar{x})^2}{n-1}$.

The standard deviation (σ) of a single observation is given as the square root of its variance. $\sigma = \frac{\Sigma (x-\bar{x})^2}{n-1}$

The standard error of the mean (S.E.$\bar{x}$) of "n" observations was calculated as the square root of the variance of the distribution of the mean ($\bar{x}$). $(S.E.\bar{x}) = \frac{\sigma^2}{n}$

Transformation of data and testing of homogeneity of variance

The transformation of data and the testing of homogeneity was carried out according to the method described by Finney (1952). The data was transformed into logarithms and the calculated estimates of variance tested for homogeneity. The test may be stated as follows. If the several estimates of the variance within groups are denoted as Si^2, the corresponding degrees of freedom being f_i, this test was

$$\chi^2_{[k-1]} = 2.3026 \left\{ f \log S^2 - \Sigma (f_i \log S_i^2) \right\} \qquad (1)$$

where k is the number of estimates whose heterogeneity is under test, S^2 and f relate to a pooled variance estimate, so that

$$f = \Sigma(f_i) \quad (2)$$

$$S^2 = \Sigma(f_i S_i^2) \quad (3)$$

To test whether the variances of the transformed data in Tables I and II were homogeneous, values from the tables were substituted into expression (1). It was found that the values for χ^2 were 34.12 and 14.4 from Table I and II, respectively. These values are in close agreement ($P > 0.5$) with what might be expected by chance. This shows that the variances of the transformed data may be considered as homogeneous and the pooled estimates of variance can be used to calculate the standard error. The standard errors from the pooled estimates of variance were found to be 0.10 and 0.12, respectively. These were expressed as a percentage and the arithmetical errors of the data calculated.

TABLE I

Variance estimates for transformed data on the effect of irradiation of lymphocytes from one sheep on the incorporation of ^{3}H-thymidine uptake by lymphocytes from an allogeneic sheep in a MLR

Mixture	CPM[1]	Log mean CPM[2]	Si^2[3]	Log Si^2	Fi[4]	($FiSi^2$)	(Fi logSi^2)
Sheep 1 + Sheep 2	1616 2821 3658 4534	3.4696	0.0378	$\bar{2}$.5775	4	0.1512	-5.6900
Sheep 2 Control	424 160 399 141	2.3959	0.0643	$\bar{2}$.8082	4	0.2772	-4.7672
Sheep 1 Control	147 160 202 49	2.0924	0.0752	$\bar{2}$.8762	4	0.3008	-4.4952
Sheep 2 Control 24 hrs.	333 454	2.5898	0.0096	$\bar{3}$.9823	2	0.0192	-4.0354
Sheep 1 Control 24 hrs.	161 134	2.1686	0.0037	$\bar{3}$.5682	2	0.0074	-4.8636
Sheep 1 Control 1 Kr[5]	38 34	1.5501	0.0009	$\bar{4}$.9542	2	0.0018	-6.0916
Sheep 1 6 Kr	27 45	1.5423	0.0247	$\bar{2}$.3927	2	0.0494	-3.2146
Sheep 2 1 Kr	37 47	1.6230	0.0051	$\bar{3}$.7076	2	0.0102	-4.5848
Sheep 2 6 Kr	41 49	1.6514	0.0030	$\bar{3}$.4771	2	0.006	-5.0458
Sheep 2 + Sheep 1 1 Kr	216 179	2.2948	0.0032	$\bar{3}$.5051	2	0.0064	-4.9894
Sheep 2 + Sheep 1 6 Kr	68 103	1.9221	0.0167	$\bar{2}$.2227	2	0.0334	-3.5546
Sheep 1 + Sheep 2 1 Kr	895 557	2.8489	0.0218	$\bar{2}$.3385	2	0.0436	-3.3230
Sheep 1 + Sheep 2 6 Kr	133 195	2.2077	0.0140	$\bar{2}$.1461	2	0.0280	-3.7078
Sheep 2 + Sheep 1 1 Kr, 24 hrs.	96 85	1.9582	0.0018	$\bar{3}$.2553	2	0.0036	-5.4894
Sheep 2 + Sheep 1 6 Kr, 24 hrs.	146 115	2.1135	0.0055	$\bar{3}$.7404	2	0.0110	-4.5192
Sheep 1 + Sheep 2 1 Kr, 24 hrs.	79 91	1.9297	0.0017	$\bar{3}$.2304	2	0.0034	-5.5392
Sheep 1 + Sheep 2 6 Kr, 24 hrs.	74 78	1.8799	0.0006	$\bar{4}$.7782	2	0.0012	-6.4430

(1) Counts per minute

(2) Mean of counts per minute after log transformation

(3) Variance of counts per minute after log transformation

(4) Number of determinations

(5) Kr = 1000 rads of γ-ray irradiation

TABLE II

Variance estimates for transformed data on ^{3}H-thymidine uptake by mixtures of allogeneic and xenogeneic lymphocyte mixtures.

Mixture	CPM[1]	Log mean CPM[2]	Si^2[3]	Log Si^2	Fi[4]	($FiSi^2$)	(Fi $LogSi^2$)
Sheep 1 + Sheep 2	847 2181 715 1999	3.1052	0.0625	$\bar{2}.7959$	4	0.25	-4.8164
Rat 1 + Sheep 1	346 1100 407 327	2.6763	0.0611	$\bar{2}.7860$	4	0.2444	-4.8560
Rat 1 Control	416 75 308 82	2.2241	0.1483	$\bar{1}.1726$	4	0.5942	-3.3096
Sheep 1 Control	258 332 286 226	2.4358	0.0049	$\bar{3}.6902$	4	0.0196	-9.2392
Sheep 2 Control	196 214 153 226	2.2907	0.0056	$\bar{3}.7482$	4	0.0224	-9.0072

(1) Counts per minute

(2) Mean of counts per minute after log transformation

(3) Variance of counts per minute after log transformation

(4) Number of determinations

TABLE III

Variation in measurements of skin thickness with skin calipers

Recipient	Site	Skin thickness in mms	Standard Deviation ±
1	1	2.0, 2.0, 2.1, 2.0, 2.2, 2.0, 1.9, 1.9	0.1
	2	2.0, 2.0, 2.1, 2.0, 2.0, 2.1, 2.0, 1.9	0.1
	3	1.7, 1.7, 1.6, 1.8, 1.7, 1.7, 1.8, 1.8	0.1
	4	2.2, 2.2, 2.1, 2.2, 2.2, 2.1, 2.0, 2.0	0.1
2	1	1.5, 1.5, 1.5, 1.6, 1.5, 1.5, 1.5, 1.5	0.1
	2	1.4, 1.6, 1.6, 1.6, 1.5, 1.5, 1.4, 1.5	0.1
	3	1.9, 2.0, 2.0, 1.9, 1.9, 2.0, 1.8, 1.9	0.1
	4	1.8, 1.9, 1.8, 1.8, 1.8, 1.8, 1.8, 1.8	0.1
3	1	3.6, 3.5, 3.7, 3.8, 3.8, 3.5, 3.6, 3.6	0.2
	2	4.2, 4.2, 4.1, 4.0, 4.1, 4.1, 3.8, 4.2	0.2
	3	3.8, 3.6, 3.5, 3.7, 3.7, 3.4, 3.5, 3.4	0.2
	4	2.2, 2.3, 2.2, 2.2, 2.1, 2.2, 2.2, 2.3	0.2
4	1	3.5, 3.7, 3.6, 3.5, 3.5, 3.4, 3.6, 3.4	0.2
	2	3.5, 3.4, 3.2, 3.3, 3.2, 3.4, 3.2, 3.4	0.2
	3	2.5, 2.6, 2.7, 2.5, 2.5, 2.4, 2.5, 2.4	0.2
	4	3.2, 3.1, 3.2, 3.1, 3.1, 3.2, 3.0, 3.2	0.2

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