FACTORS GOVERNING INDUCTION AND EXPRESSION
OF CELL MEDIATED IMMUNITY
AGAINST MODIFIED CELLS

by

ARNO MULLBACHER

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The experiments on specific adsorption (Chapter 3) were performed in collaboration with Dr. U. Kees and those on precursor frequencies (Chapter 9) with Dr. R. B. Ashman. The neutralisation tests described in Chapter 6 were performed by Dr. I. D. Marshall. All other experiments were my own original work and were carried out by me.

Arno Mullbacher
Department of Microbiology
John Curtin School of Medical Research,
Australian National University,
Canberra.

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ADCC</td>
<td>antibody dependent cellular cytotoxicity</td>
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<tr>
<td>ALS</td>
<td>anti lymphocyte serum</td>
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<tr>
<td>B cell</td>
<td>Bursa of Fabricius (or mammalian equivalent)-derived lymphocyte</td>
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<tr>
<td>BEB</td>
<td>Bebaru virus</td>
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<tr>
<td>CMI</td>
<td>cell-mediated immunity</td>
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<td>CML</td>
<td>cell-mediated lymphyolysis</td>
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<td>CMV</td>
<td>cytomegalo virus</td>
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<tr>
<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxid</td>
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<td>DNP</td>
<td>dinitrophenyl</td>
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<tr>
<td>DIN</td>
<td>delayed-type hypersensitivity</td>
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<td>EID</td>
<td>egg infectivity dose</td>
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<tr>
<td>F9</td>
<td>murine teratocarcinoma cells</td>
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<tr>
<td>F-15</td>
<td>Eagle's minimal essential medium</td>
</tr>
<tr>
<td>FcR</td>
<td>antibody cristallyzable fragment receptor</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>FLV</td>
<td>Friend leukemia virus</td>
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<tr>
<td>GVH</td>
<td>graft-versus-host reaction</td>
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<tr>
<td>H</td>
<td>histocompatibility</td>
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<tr>
<td>HLA</td>
<td>major histocompatibility complex in man</td>
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<td>H-2</td>
<td>major histocompatibility complex in the mouse</td>
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<td>HI</td>
<td>haemagglutination inhibition</td>
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<td>HSV</td>
<td>herpes simplex virus</td>
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<td>H-Y</td>
<td>male specific antigen</td>
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<td>i.c.</td>
<td>intracerebral</td>
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<td>i.p</td>
<td>intraperitoneal</td>
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<td>i.v.</td>
<td>intravenous</td>
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Ia  region associated
Ig  immunoglobulin
IR  immune response gene
JAP influenza virus strain A/JAP
L929 murine fibroblast cells (H-2K)
LCM lymphocytic choriomeningitis
LN  lymph node
Ly  lymphoid tissue alloantigen loci
2ME 2-mercaptoethanol
MEM Eagle's minimal essential medium
MHC major histocompatibility complex
MLR mixed lymphocyte reaction
ml millilitre (10^-3 litre)
µl microlitre (10^-6 litre)
MMTV mouse mammary tumor virus
MOI multiplicity of infection
MSV murine sarcoma virus
NEUT neutralisation
P815 mastocytoma cells (H-2d)
PFU plaque forming unit
PBS phosphate buffered saline
RLV Rausher leukemia virus
S.E. standard error of the means
s.c. subcutaneous
SFV Semliki forest virus
SIN Sindbis virus
SV40 simian virus 40
T cell thymus derived lymphocyte
Tc  cytotoxic T cell
TH  helper T cell
Thy-1  theta (θ) antigen
Tl  initiator T cell
TLa  thymus leukemia antigen
TNBS  trinitrobenzenesulphonlic acid
TNF  trinitropheryl
V region  antibody variable region
VSV  vesicular stomatitis virus
wk  week
WSN  influenza virus strain A/WSN
X  foreign antigen
AIMS AND SUMMARY

Murine cytotoxic thymus-derived lymphocytes (Tc cells) generated in a response to modified syngeneic cells show dual specificity. They recognize foreign antigens such as virus coded or determined antigens, haptens such as TNF as a result of chemical modification, or minor H antigens, in association with self H-2 antigen coded for in either the K, D or L region of the major histocompatibility complex (MHC). It was the aim of the experiments described in this thesis to define the specificity of Tc cells to self and to foreign antigens in more detail and possibly shed some more light on the elusive Tc cell receptor.

In Chapter 2, Tc cells generated against either allogeneic MHC antigens, TNF-modified self or virus-infected self were tested for crossreactivity at the level of target cell lysis. Alloreactive Tc cells crossreacted on syngeneic TNF-modified targets as well as TNF-modified and unmodified third party targets. Similarly, TNF-immune Tc cells showed crossreactivity on TNF-modified allogeneic and unmodified allogeneic targets. Neither TNF-immune nor allogeneic Tc cells lysed virus-infected targets more than uninfected. Target cells infected with TNF-modified Sendai virus were not lysed by TNF-immune Tc cells, but the converse did apply. Tc cells induced with TNF-modified Sendai virus lysed TNF-Sendai modified targets as well as TNF-modified targets. The same pattern was observed with a large number of virus-immune Tc cells. All crossreacted strongly on TNF-modified syngeneic cells. Cold target competition established that some clones of virus-immune Tc cells could recognize both TNP and virus modified self. The crossreactivity by virus-immune Tc cells at the effector level could not be observed at the level of induction as virus memory spleen cells could not be activated by TNF-modified stimulator cells. Thus, recognition by Tc cells at effector and induction
phase are different and different mechanisms of Tc cell recognition may exist in the various Tc cell populations.

Chapter 3 describes experiments using macrophage monolayers to specifically deplete populations of virus-immune and TNP-immune Tc cells. Results obtained showed conclusively that both self and foreign antigens have to be present to achieve specific depletion of H-2 restricted immune Tc cells. The results are discussed in the contexts of altered self and dual recognition, and favour the former.

In Chapter 4, the secondary in vitro Tc cell response to alphaviruses (Bebaru, Sindbis and Semliki Forest Virus) was characterised. The optimum of response was obtained at day 5-6 of culture and cytotoxic cells were of Ig-, δ+, Ly 2+3+ phenotype. The response was H-2 restricted and out of the haplotypes H-2k, H-2d, H-2s, H-2b and H-2q, only H-2k was a responder. Mapping studies located the response solely in the D end of H-2k. Non-responder strains showed high lysis on both infected and uninfected targets. This was tentatively mapped also to the D end of the H-2 complex. F₁ hybrids between responder and non-responder parents exhibited dominance of responsiveness, but no complementation occurred, that is, alphavirus-immune Tc cells of F₁ origin lysed only infected parental targets of responder type. Further analysis of the responder-, non-responder-mouse strains, using irradiation chimeras, are described in Chapter 5. It is shown that, when non-responder BALB/c (H-2d) stem cells are allowed to differentiate in a responder C3H.0H (H-2k) irradiated recipients, donor cells of H-2d phenotype can be stimulated to recognize H-2Dk plus alphavirus. The results are explained with a model which envisages ability of association of H-2K and D antigens with alphavirus antigens to be the cause of responder phenotype.

Chapter 6 investigates the specificity of alphavirus-immune Tc cells
between serologically distinct subgroups of viruses. Total crossreactivity was observed at both the induction and effector phase between Bebaru, Semliki Forest and Sindbis virus. This crossreactivity was substantiated by cold target competition experiments. Specific blocking of Tc cell lysis by antiviral antibodies suggested that Tc cells see the same viral antigens on the cell surface as the antigens recognized by viral neutralization tests, but with less specificity.

In Chapter 7, the ability of virus-immune sera to block target cell lysis by virus-immune Tc cells was investigated. The active component was characterised as immunoglobulin. It is shown that only fibroblast like cell lines are susceptible to virus antibody blocking in the ectromelia and influenza Tc cell systems. The blocking ability of serum is strain independent, but virus specific. After ectromelia infection, the kinetics of appearance of antiviral antibodies coincides with the decline of the primary Tc cell response in vivo. This might suggest an immuno-regulatory role of antiviral antibodies.

In Chapter 8, blocking experiments with monoclonal antibodies that bind to H-2K\(^k\) and H-2D\(^k\) antigens are described. Both alloreactive Tc cells and virus-immune Tc cells (ectromelia, Sendai and influenza) could be blocked when directed against K\(^k\) antigens. Tc cells directed against D\(^k\) plus virus gave different results. Both alloreactive and influenza-immune Tc cells could be significantly inhibited; in contrast, Bebaru-immune Tc cells were not significantly blocked. The results are discussed in the context of different antigens coded in the D region of the H-2 complex (D and L) being associated with different viruses.

Chapter 9 investigates the precursor frequencies in primary and secondary responses to modified self. Using a limiting dilution assay of spleen cells, the frequency of Tc cell precursors for both TNP and viruses
(Sendai, ectromelia and influenza) are about 3 to 10 fold lower ($10^{-5}$ to $10^{-6}$) than for precursors found in primed spleen cells. Secondary (memory) precursors to a given virus are present in spleen at about the same frequency as alloreactive Tc cells to a foreign haplotype.
CHAPTER 1

GENERAL INTRODUCTION

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1. **PREAMBLE**

The ascent of the immune system as we know it today is most likely the result of a continuous evolutionary struggle between the host and its parasites, may they be viral, bacterial, fungal, protozoal or of other origin. With the advent of a specific adaptive immune response, a mechanism of self recognition and self tolerance became obligatory. Such a self recognition system had probably evolved already, or was established in at least a rudimentary form, out of a necessity for diversity in sexual reproduction and, in phagocytic organisms, as a necessity for self-preservation.

The two elements, recognition of self and recognition of foreignness, have received very much attention recently with the discovery of major histocompatibility complex (MHC)-restricted responses and, in particular cytotoxic T cell (Tc) responses in which there is the recognition of self and foreignness by a single unit (cell) at a given time. It is this area of immunology with which the introduction and following chapters will deal.

As the field of T cell immunology, and with it H-2 restricted Tc cell responses, has taken on enormous proportions in data and publications, this introduction will be selective and some potentially relevant observations will not be dealt with.

2. **THE MAJOR HISTOCOMPATIBILITY COMPLEX**

2.1 **General**

Early transplantation research showed that duration of graft acceptance was related to genetic similarity between the donor and the recipient (Gorer 1937, Bittner 1936, Medawar 1944). Selective breeding in mice in connection with transplantation experiments led to the identification of a number of
genetic systems which are involved in allograft rejections and were termed histocompatibility systems (H-systems).

Subsequently, in all mammalian species, one genetic region has been found to be of paramount importance and is now referred to as the major histocompatibility complex or MHC. The MHC in the mouse is referred to as the H-2 system and its equivalent in man as the HLA system. Due to the availability of a large number of inbred strains of mice, the H-2 genetic region is better understood and characterised than any other MHC and will here be dealt with exclusively, unless otherwise stated.

The MHC has been found to be intimately associated with a large variety of immune functions, and genes mapping close to either side of the H-2 complex do show some similarities with structural components of H-2 and also mediate a series of essential steps in embryogenesis and spermatogenesis (Klein and Hammerberg 1977).

The MHC is highly pleiotropic and its effects are not restricted to immune responses, but include such diverse biological functions as ageing (Smith and Walford 1977), cell adhesion (Bartlett and Edidin 1978) and many more (Klein 1978).

2.2 Genetics of the H-2 system

The H-2 complex is located on chromosome seventeen and its estimated length could contain the coding capacity for at least 2000 average polypeptide chains (Klein 1975).

The H-2 genetic region has been subdivided into a number of genetically different but functionally similar segments by recombination. A stylized map illustrating the known regions in the H-2 complex is shown in fig. 1. The H-2 complex is preceded, to the left of H-2K, by the T/t region and mutations in this area prove lethal in the homozygous state and
affect tail length in the heterozygous (Klein and Hammerberg 1977, Levinson and McDevitt 1976).

The H-2K and H-2D region flank the H-2 complex. This region is genetically highly polymorphic, that is, a large number of alleles exist in the species (Klein 1974, Klein and Zaleska-Rutczynska 1977). Structural and serological analysis of the H-2K and H-2D coded antigens suggest that they have arisen by gene duplication and the homology observed with their equivalent antigens of other species, such as HLA-A and HLA-B in man, points to strong evolutionary conservation (Vitetta and Capra 1978, Terhorst et al 1976).

The I region is located in the H-2 complex between H-2K and Ss (David et al 1973). The region is currently divided into five sub-regions (IA, IB, IJ, IE and IC), and genes within it control immune responses to a variety of antigens (David 1976, Benacerraf and Germain 1978) and encode the Ia antigens.

The S region bordering the right of the I region and left of H-2G, controls serum proteins. The G region is not well understood genetically. A red blood cell and a weak transplantation antigen have been mapped in this region (Klein 1975).

The genetic region downstream of H-2, the T region, maps for at least 2 genes coding for cell surface antigens (Qa-2 and Tla) and some minor histocompatibility antigens (H-31 and H-32).

2.3 Structure and Function of MHC products and genes

It is not yet known if any of the marker loci identified by recombination are sites of the structural genes coding for proteins, or if these sites are merely regulatory genes. This problem awaits chromosome sequencing and matching with known peptide sequences of H-2 molecules.
We will assume here that the genes identified by genetic methods do correspond to structural genes.

The T/t region as stated above, is involved in embryogenesis and spermatogenesis. Serological testing identified a cell surface antigen (F9) on teratocarcinoma cells, cleavage embryos and sperm controlled by gene(s) mapping in this region.

The concentration of F9 antigen was shown to be inversely related to the amount of H-2K and H-2D antigens and diminished during differentiation (Jacob 1977).

The protein(s) precipitated with anti-F9 sera consisted of a glycoprotein of 44000 MW, together with a 12000 MW subunit. It has not yet been established if the F9 antigen is a structural analog of MHC products nor if the 12000 MW protein associated with it resembles β2-microglobulin (Vitetta et al 1975). The F-9 can be recognised by Tc cells as target and induction antigen (Wagner et al 1978).

The K and D antigens are both glycoproteins consisting of two chains, a heavy 44000 MW polypeptide and, associated with it, a 12000 MW light chain identified as β2-microglobulin. Sequence analysis of K and D antigens of a number of haplotypes(1) showed a large degree of homology between different alleles as well as between the K and D antigens themselves; but little homology was found with immunoglobulins (Silver and Hood 1976, Capra et al 1976, Coligan et al 1978, Vitetta and Capra 1978).

The K and D glycoproteins are cell surface molecules present in varying amounts in all tissues, with high concentrations on lymphocytes and relatively low ones on red blood cells, brain tissue and others.

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1. A particular combination of H-2 antigens controlled by a single chromosome.
The antigenicity of these molecules resides in the protein portions rather than the sugar residues. The major transplantation antigens are transmembrane proteins and probably are in communication with cytoskeletal structures (Walsh and Crumpton 1977). Individual K and D antigens bear antigenic determinants shared between alleles, the "public specificities" and antigenic determinants unique to a given allele the "private specificities" (Demant 1973, Shreffler and David 1975). Theories suggesting that the public and private specificities are carried on separate molecules have been discounted (Hauptfeld and Klein 1975).

Despite forty years of extensive analysis, the function of the major transplantation antigens has remained elusive. The most persuasive hypothesis so far suggests that H-2K and H-2D molecules act as markers for T cell recognition of self and as self tolerance signals encountered during ontogeny in the thymus (Bevan 1977, Zinkernagel et al 1978a). Tc cell activation apparently occurs when these K and/or D antigens are recognised together with foreign antigens such as virus-induced antigens or chemical modifications (Doherty et al 1976, Shearer et al 1975). An analogue of this syngeneic T cell activation takes place in the situation of skin graft rejection, graft versus host reaction, or in vitro MLR (Shreffler and David 1975, Klein 1976). In this case, foreign K and D antigens are potent T cell activators.

A recently discovered gene product mapping close to H-2D, the L antigen, is similar to K and D antigen, a glycoprotein of 44000 MW and associated with \( \alpha \)-2-microglobulin, but differs from the others as it

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1. MLR (Mixed lymphocyte reaction) will be used here in its original meaning, being the total of responses generated.

The I region codes for both the immune response (IR) genes and cell surface antigens (Ia antigens). The IA, IB and IC subregions determine immune responsiveness to a number of synthetic polypeptides and natural antigens (Benacerraf and Katz 1975, David 1976) and code for Ia antigens identified by a number of public specificities (McDevitt et al 1976). Helper factors also appear to be located in this region of H-2 and have been shown to carry Ia specificities (Munro and Taussig 1975). On the other hand, the IJ subregion is involved in T cell mediated suppressor activity (Tada et al 1976, Tada and Taniguchi 1976) for both allotype and antigen specific suppression. Analogous to the helper factor, a suppressor factor carries Ia specificities located in the IJ subregion.

The Ia antigens have been isolated from cell surfaces. They are composed of two glycosylated polypeptide chains of 35-38000 MW and 25-28000 respectively. The antigenicity of these cell surface Ia antigens resides in the peptide portion of the molecule (Cullen et al 1976).

Another group of Ia antigens is detected in serum, is dialyzable and appears to carry the antigenic specificities in the sugar moiety. Work by Parish et al (1978) suggests that these antigens are also found on cell surfaces and might be glycolipid in character rather than glycoprotein.

Cell surface Ia antigens are the major determinants of the H-2 complex involved in the proliferative response of MLR (Bach et al 1972(a), Meo et al 1973), stimulate graft versus host (GVH) reaction and can stimulate cell mediated lympholysis (CML) e.g. Tc cells (Klein and Park 1973, Klein et al 1977). In congenic strains of guinea pigs, differing only in the I region, Shevach's group has elegantly demonstrated that complexes of foreign antigen and self Ia antigen on macrophage surfaces stimulate T cell
responses (Thomas and Shevach 1977, Thomas et al. 1977).

I region genes are also involved in T–B cell co-operation (Erb and Feldmann 1975). Furthermore, delayed type hypersensitivity (DTH) is restricted by the I region (Miller et al. 1975).

The distribution of Ia antigens was originally thought to be restricted to certain tissue types, mainly lymphomyeloid cells and B cells in particular, but greater sensitivity in assay methods indicates a tissue distribution as wide as for the K and D antigens (David 1976). Even with the large amount of knowledge accumulated regarding immune response gene function and Ia antigen structure, it is not yet clear how the parts of the puzzle fit together.

The products of the S region of the H-2 complex differ from the other regions as they are primarily serum proteins. In man, several components of complement are associated with the region of HLA analogous to the S region in H-2 (Bodmer 1977). Two genes Qa-2 and TLa mapping downstream of H-2D have been shown to be responsible for all surface glycoproteins of MW 44000 in association with &2-microglobulin (Michaelson et al. 1977, Morello et al. 1977). These antigens may prove to be isozymes of the H-2K and D antigens expressed during differentiation of cells or in a transformed state of cells.

2.4 Mutants of the H-2 complex

H-2 mutants which came to prominence in the last few years might prove to be a useful tool in further probing the mysteries of the MHC (Klein 1978, McKenzie et al. 1977a). The first mutants were discovered by skin-graft rejection by two independent groups, Egorov and Blandora; and Bailey and Kohn, and were mapped by complementation tests (Klein 1976). The H-2K region has an extremely high mutation rate for a eucaryotic chromosome.
The use of skin-graft rejection as a selection method favours discovery of mutations in the K and/or D region of the H-2 complex, perhaps more than I regions. Of twenty-two H-2 mutations described to date, twelve occurred in the K region (with most in the H-2b haplotype) and three in the D or L region (Klein 1978). Biochemical analysis of two such K^b mutants (H-2K^ba, H-2K^bf) by peptide mapping and sequence analysis revealed the mutation in the protein portion (Brown and Nathenson 1977). It is thought likely that these are point mutations. A number of these K^b mutations are serologically silent, that is they are not recognised by B cells, but provide T cell recognisable differences, as indicated by reciprocal skin-graft rejection and MLR's (i.e. they are of gain and loss type) (Forman and Klein 1975, Cornelis et al. 1977). Analysis of these mutants in H-2 restricted syngeneic systems is dealt with later. Two types of mutant in the D region have been characterised, H-2d^a is a mutant which has gross structural differences in carbohydrate as well as in the protein moiety and is also serologically detectable and again of the gain and loss type (Brown et al. 1978, Klein 1978). A mutant in the D region of the H-2d haplotype (H-2D^db) led initially to the mapping of the L region. (McKenzie et al. 1977b) and is of the loss type only. That is, wild type does not reject mutant skin-grafts nor are wild type T cells activated in MLR by mutant stimulator cells, but the mutant is stimulated by wild type. Genetic immunological and biochemical analysis supports a loss of H-2L antigens (Hansen et al. 1977).

So far, no mutation has been discovered which maps in the I region, a region in which mutations could be detected by skin-graft tests, and no explanation for this asymmetry of mutability in the H-2 gene complex has been advanced.
3. **ONTogeny of t CELLS**

3.1 **Thymus, prothymocytes and intrathymic maturation**

Lymphocyte populations have conventionally been divided into two parts on physiological criteria, namely: cells derived from the Bursa of Fabricius in birds or its mammalian equivalent (the B cell line), and cells which are processed through the thymus (T cells) (Raff 1971). The former will not be dealt with in this introduction.

The thymus is a primary lymphoid organ (Auerbach 1960) and is derived from the lining of the third branchial pouch which buds off and migrates into the thorax during early foetal life (about days 9-10 of gestation in the mouse). In the adult, the thymus consists of many lobules, each containing an outer cortex and inner medulla. The thymus is the inductive micro-environment for the differentiation of T lymphocytes and the inductive stimulus (or stimuli) appears to be a property of its epithelial cells (Mandel and Russell 1971). Removal of the murine thymus at birth affects immunocompetence severely, but there is little effect after thymectomy later in life (Miller 1961, Good 1972, Boyse and Old 1976). In the mouse, T cell precursors first enter the thymus on about the tenth day of gestation (Strutman 1976). These precursors or "pre thymocytes" are first found in the yolk sac, later in foetal liver and postnataley in bone marrow and spleen (Owen and Ritter 1969, Owen and Raff 1973). MHC antigen distribution on the thymic epithelium, tested by immunofluorescence technique showed uneven distribution. K and D antigen concentrations were low in cortical areas and high in the medullary sections of the thymus. Ia antigens, on the other hand, were found rather diffusely distributed throughout the thymus, with some localised high density areas in the cortex (Weissman personal communication). Whether differential
MHC antigen distribution reflects locations of specialised differentiation for functional T cell subsets remains to be seen.

In vitro induction assays devised by Komuro (Komuro and Boyse 1973) to investigate prothymocyte to thymocyte differentiation led to the discovery of a number of agents which were capable of inducing thymocyte specific cell surface antigens (Scheid et al 1973). Out of a number of such inducers, thymopoietin, a fully sequenced polypeptide of MW 5560 daltons, which has been isolated from thymic extracts, appears to be the biologically meaningful one (Schlesinger and Golstein 1975). However, no functionally active T cell has yet been induced from prothymocytes by any of these agents and the field is still rather controversial. Nevertheless, the ontogeny of T cells is probably better understood today than that of any other cell type. The reasons for this are the large number of cell surface markers that the T cell lineage expresses exclusively or selectively at various stages of differentiation, and the availability of assay systems to test for functional populations corresponding with particular surface phenotypes. The main alloantigens used as markers include the following: the Gix antigen is expressed on thymocytes of only a limited number of mouse strains and its antigenic properties are either very similar or identical to the group specific antigen, gp 70 of mouse leukemia virus. Strains of mice capable of expressing Gix experience a higher incidence of leukemias than Gix- (negative) strains. Gix expression has been correlated with a number of C type virus related functions (Tung et al 1975, Stockert et al 1971, Boyse 1977). The TL antigen or thymus leukemia antigen is coded by Tla in the T region close to the H-2 gene complex. As with the Gix antigen, its phenotypic expression is strain dependent. This antigen has been described in the section on the MHC. The most widely used T cell markers
are the Thy-1 antigens. The Thy-1 (or θ, theta) locus is carried on the mouse chromosome 9 and codes for a cell surface antigen expressed on T lymphocytes, brain and skin (Reif and Allen 1964). It appears to exist in two alleles whose antigens are referred to as Thy 1.1 and Thy 1.2. The Ly or lymphocyte antigens are surface molecules of unknown function. They have proven of great importance in delineating T cell subpopulations. Ly antigens found on T lymphocytes at some or all stages of development are Ly 1, 2, 3, 5, 6 and 7, and two alleles have been recognised for most of them, e.g. Ly 1.1 or Ly 1.2 (Boyse et al. 1968, Komuro et al. 1975, McKenzie et al. 1977, Woody et al. 1977). One enzyme marker, namely terminal deoxynucleotidyl transferase (TdT) has also been associated with lymphocytes and T cells in particular (Kung et al. 1975). Its role in lymphocytes is still uncertain. The enzyme polymerises deoxynucleotides in vitro without the need of a template, which confers on it the role of a possible somatic mutator (Baltimore 1974). TdT is absent in mature peripheral T cells. With the help of these cell surface markers or enzymes, the development of haemopoietic stem cells into mature functional peripheral T cell sub classes has been studied.

During embryogenesis the stem cells originate in the yolk sac and initially migrate into foetal liver. With the later development of bone marrow and spleen, haemopoietic stem cell populations appear in these organs. These stem cells are pluripotent and give rise, after the appropriate stimulus(1), to any of the mature cells of the haemopoietic system. The prothymocytes of bone marrow or spleen are phenotypically Ly−, TL−, Thy 1− and Gix−. They can already be distinguished from pluripotent stem cells by their ability to be induced by thymopoietin (Komuro et al. 1975) to express surface antigens characteristic of immature thymocytes, TL+, Gix+, Thy 1+, Ly 1+, 2+, 3+ and Ly 5+. Prothymocytes are therefore already
pre-committed for migration to the thymus. Removal of prothymocytes from bone marrow cells delays the appearance of mature T cells significantly. One distinction that can be made between prothymocytes of bone marrow and of spleen is that the former are positive for TdT. The appearance of this marker is at present the earliest distinguishable property of cells that eventually can undergo thymus-dependent development (Silverstone et al. 1976). TdT activity is lost before the final maturation of the T cell to a circulatory lymphocyte (Barton et al. 1976). An unresolved question is by what mechanism are prothymocytes produced? Is their appearance regulated by antigens or hormones, and what homeostatic factors of the immune system are involved?

The recruitment of prothymocytes from bone marrow or spleen in the adult, or from foetal liver into the thymus has been reviewed recently by Stutman (1977). Using chromosome markers of the T6-T6 type, the kinetics of T cell precursor immigration and T cell emigration was studied. Twenty days after injection of haemopoietic stem cells, 10-25% of the dividing cells in the thymus were of donor type. These cells made their first appearance in the peripheral lymphoid organs thirty days after transfer. Similar kinetics of recruitment into the thymus, and subsequent export, have been obtained with experiments using radiolotope tracers.

Differentiation of prothymocytes into thymocytes as indicated by alloantigen expressions (TL, Gix, Thy 1, etc.) has been achieved both in vivo and in vitro by thymus organ cultures (Mandel et al. 1972, Kamarck and Gottlieb 1977, Ritter 1978). The stimulus for this differentiation step is most likely hormonal, like thymopoietin. Cell division or DNA synthesis appears to be unnecessary, but RNA and protein synthesis is obligatory (Boyse and Old 1976). These thymocytes are immuno-incompetent when tested for cytotoxicity, DTH, helper function or activation by lectines. To become
The thymus is an organ which, in the young animal, supports an enormous amount of cell proliferation. The number of thymus cells produced would suffice to replace all peripheral T cells six times daily in the mouse, but efflux from the thymus as measured by radioactive tracers is only around 25% and suggests that 50-70% of all newly formed thymocytes die in situ (Joel et al 1977, Feinendegen et al 1973). Experiments increasing the thymic epithelium by additional grafts have substantiated the notion of high intrathymic death as the grafted lobes functioned normally but no substantial increase in the peripheral T cell pool was observed (Matsuyama et al 1966).

The intrathymic lymphocytes can be subdivided into a number of distinct subgroups by a number of means. Shortman et al (1975) separated thymocytes mainly by their physical properties such as size, density and electrophoretic mobility. Separation by size led to two populations. A large to medium size class of cells comprised 10% of the total population. They were found mainly in the cortical region and were undergoing DNA replication and mitosis, giving rise to a class of small thymocytes which comprised the remaining 90% of the thymic population (Borum 1973, Shortman 1977). These two populations were heterogeneous with respect to other properties such as Thy-1 antigen concentration, cortisone and radiation-sensitivity, and TL antigen expression. Based on these findings, Shortman (1977) developed a dual pathway of intrathymic maturation. Large cells give rise on the one hand to high Thy-1, TL⁺, cortisone–and radiation-sensitive small lymphocytes destined for intra thymic death, and, on the other hand, give rise to low Thy-1, TL⁻ cortisone–resistant, high H-2 antigen-bearing immunocompetent T cells destined for peripheral export. But the notion that only mature T cells emigrate from the thymus is still
controversial, as Stutman (1977) argues strongly for post thymic precursors which differentiate further in the periphery. In spite of the massive research and literature, no definitive picture of T cell differentiation and traffic in the thymus has emerged, and opinions range from single to multiple pathways of intra thymic lymphocyte maturation. Neither has any generally accepted theory been brought forward that satisfactorily explains the need for this massive cell proliferation and its associated cell death in the thymus. Some kind of selection process based on the distinction between self and non-self, leading to the elimination of "forbidden" clones as originally proposed by Jerne (1971) is still an attractive working hypothesis.

3.2 Peripheral T cell subpopulations

T lymphocytes can be separated into various subsets according to different criteria. These may be characterised by means of biophysical procedures, or may be subdivided according to physiological differences revealed by their stage of post thymic maturity, tissue distribution differential migration, life span and susceptibility to the effects of adult thymectomy or antilymphocyte serum. They can also be characterised according to the Ly surface antigens. Finally, a classification based on the distinct functions they subserve can be made: helper cells, suppressor cells, cytotoxic cells and cells responsible for delayed type hypersensitivity (Transplantation Rev. 1975, Cantor and Boyse 1976b, Raff and Cantor 1971, Droege 1976). Historically, the peripheral T cells have been subdivided into T1 and T2 cells (Raff and Cantor 1971). Where T1 cells were defined by their short half life and insensitivity to anti-lymphocyte serum (ALS) treatment, the T2 population on the other hand was characterised by longevity, little affected by adult thymectomy and highly sensitive
to ALS treatment. It is thought that T1 cells are a precursor of T2 cells and conversion from one to the other might be facilitated by an encounter with antigen. This may occur during ontogeny, and give rise to the allo-reactive Tc cell pool, or later in life in the periphery for helper T cells (Aroneo 1977, Raff and Cantor 1971). The more conventional and more useful subdivision applied today relies mainly on functional criteria and allo-antigenic markers, especially by the Ly series. The precursor to all functional peripheral T cells is most likely the TL"Ly 123+ TdT" thymocyte. An Ly 123+ subclass of T cells is detectable in the spleen before either Lyl+23- or Ly 1-23+ cells appear (Cantor and Boyse 1975a)\(^1\). The pathway of differentiation of the probable Ly 123 precursor to give the Lyl helper T cell and/or the Ly 23 effector T cell is not yet clear (Cantor and Boyse 1977).

The abbreviations used for T cell subpopulations will follow the convention applied by Snell (1978) and will be the following: Initiator (T_I), Helper (T_H), Delayed hypersensitivity (T_DH), Suppressor (T_s) and cytotoxic T cell (T_c).

The T_I cell is the least well defined of the peripheral T cell subclasses (Cohen and Livnat 1976). They are found mainly in the spleen and, to a lesser extent, in the thymus, but are absent from lymph node. When stimulated in vitro by sensitisation on allogeneic fibroblast monolayers and transferred back into isogeneic animals, they lodge in lymph nodes and recruit Tc cells with GVH potential for the primary antigen. Stimulation occurs in vitro within 4-6h, requires RNA and protein synthesis but not

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1. T cells of Ly 123+ phenotype will be referred to as Ly 123 cells. T cells of Ly 1+23- phenotype will be referred to as Ly 1 cells and T cells of Lyl-23+ phenotype as Ly 23 cells.
DNA synthesis. They are sensitive to anti Thy-1 plus complement but relatively insensitive to both ALS treatment and irradiation. Adult thymectomy reduces their numbers markedly which implies a short life span (Cohen and Livnat 1976). These properties are reminiscent of those described for the T1 subpopulation mentioned above and it appears that T1 could be a subgroup of those. Both T1 and T1 cells show a higher Thy-1 antigen concentration than other peripheral T cells. A proposed function of T1 cells is that of a surveillance role, responsible for the detection of invading immunogens. Their short priming time would suit such a role.

It is not yet established if any additional T cell subset(s) besides alloreactive T cells are recruited by T1 cells. No definitive typing with Ly sera has yet been performed on T1 cells. Adult thymectomy reduces both Ly 123 cells and T1 cells, which might indicate that T1 cells are Ly 123.

Approximately 30% of peripheral T cells are of the Ly 1 phenotype, a characteristic of T1 cells. They are long lived and belong presumably to the T2 pool of cells with low Thy-1 antigen concentration and high ALS sensitivity. They are also Ia+ with the possible exception of T1 in DTH responses (Murphy et al 1977). Ly 1 cells can be generated from Ly 123 cells by polyclonal activation with Concanavalin A (Con A) (Cantor and Boyse 1977). The Ly 1 phenotype appears to be stable, and does not seem to revert to the Ly 123 phenotype nor do they give rise to Ly 23+s cells. This has been concluded from parking experiments in "B" mice (lethally irradiated, thymectomized mice restored with syngeneic T cell deficient bone marrow cells) where Ly 1 T cell populations, syngeneic to the recipient mouse were capable of conferring helper function for up to six months post-reconstitution (Huber et al 1976b) but no Ly 23 cells emerged. This suggests that Ly 1 cells are the final product of a differentiation pathway.

T1 cells are a subpopulation of T cells that play an obligatory
accessory function in most immune reactions. They synergise with B cells in the production of antibodies, except in the case of T cell independent antigens, they are the main proliferative cell population in MLR's (Bach et al 1972, Festenstein 1974, Cantor and Boyse 1975a), and are involved in Tc cell responses to modified self (Pang et al 1976, Zinkernagel et al 1978b). They are phenotypically similar to $T^{\text{DTH}}$, but functionally different (Ramshaw et al 1976) and may play a role in the generation of Ts cells (Feldman et al 1977). The functional test applied to determine helper function is the in vitro antibody response to heterologous red cells; e.g. putative $T^{H}_{H}$ cells are added to purified B cells, together with the heterologous red cells and anti-red cell plaque forming B cells are measured (Cantor et al 1976). Such collaborative responses have been shown to necessitate H-2 compatible T and B cells (Sprent and von Boehmer 1976, Sprent 1978a, Sprent 1978b, Katz et al 1973) but evidence to the contrary, e.g. collaboration between allogeneic T and B cells has been reported (von Boehmer and Sprent 1976, Heber-Katz and Wilson 1975). It is not yet clear why these discrepancies exist. Regarding antigen recognition of $T^{H}_{H}$ cells, Sprent (1978b) has elegantly shown that self antigens coded in the H-2 region are necessary for maturation of $T^{H}_{H}$ in the anti-sheep red blood cell (SRBC) response, supporting earlier evidence by Erb and Feldman (1975) and Yano et al (1977) which implied a role by macrophages for antigen presentation in conjunction with Ia antigens. Ia antigens coded in the IA and IC subregions of the H-2 complex are at least part of both non-specific (Armerding et al 1974) and specific (Taussig et al 1975) helper factors, and secretion of Ia antigen by a Ly-1 T cell has been documented (McKenzie and Parish 1976). A further subdivision of $T^{H}_{H}$ has been proposed, depending on the allotype of B cells with which they synergise (Herzenberg et al 1976). This might lead to a much more extensive subdivision of $T^{H}_{H}$ cells than is
anticipated at present. A further subpopulation of Ly 1 cells has been indicated by the findings that at least a subgroup of Ly 1 cells possess Fc receptors (FcR) and cells lacking them (FcR⁻) correspond to TH involved in antibody responses, whereas the FcR⁺ Ly 1 cells are accessory to T cells of the Ly 23 phenotype (Stout et al. 1976, 1977). The helper function of Ly 1 cells in the generation of Tc cells or Ts cells is very much less well understood than help in the antibody response. As it appears at the moment, T helper cells have primarily a regulator role to play, be it as amplifiers or for inductive signals. Cantor et al. (1978) have recently identified a subpopulation of TH cells that induces feedback inhibition in the formation of antibody B cells.

The T_DTH cell is phenotypically also a Ly 1 cell (Huber et al. 1976, Vadas et al. 1976), but differs functionally from TH cells. Both populations T_DTH and TH are producers of lymphokines. Transfer of DTH requires homology between donor and recipient at the IA region of H-2 if proteins or polypeptides are used as sensitising agents; I region or K and/or D region homology is necessary in the case of contact chemicals (Miller 1978).

Ts cells have now been demonstrated for both humoral and cell mediated systems (Pierce and Kapp 1976) and are intricately involved in the complex phenomenon of immune regulation (Yamamoto et al. 1977, Hamaoka et al. 1977). The phenotype of cells mediating suppressive effects is Ly 23, Thy-1⁺ and they belong to the long lived recirculating pool, highly sensitive to ALS, but they are little affected by adult thymectomy. Ly 23 Ts cells share the same Ly phenotype as Tc cells (see later). 5-10% of all peripheral T cells fall into the Ly 23 category. There was some contention that Ts and Tc cells are one and the same cell, but while Tc cells can have a suppressive effect Pang and Blanden (1976a), this is not generally the case (Al-Adra and Pilarski 1978). Suppression of humoral responses operates in some systems
via soluble factors while Tc cells operate via membrane contact. Ts cells can be lysed with appropriate anti-Ia sera plus complement, but Ia antigens have not been detected by this method on Tc cells. The Ia antigens expressed by Ts cells are coded for in the I-J subregion of H-2 (Beverley et al 1976). Soluble factors that are able to suppress antibody responses specific for the inducing antigen also have I-a antigens associated with them and are the products of Ly 23 cells (Tada et al 1976, Feldman et al 1977). The precursors of effector Ts cells have identical Ly phenotype to effector Ts cells, but they can be differentiated from the effector cells by an apparent lack of I-J coded I-a antigens. Memory or secondary Ts cells, able to give rise to an enhanced suppressive response after further stimulation with antigen have been reported recently (Loblay et al 1978). These memory Ts cells are phenotypically indistinguishable from primary Ts cells. Present evidence indicates that Ly 23 cells are preprogrammed for the Ts or Tc subsets of effector T cells, as stimulation of purified Ly 23 cells with Con A, or stimulation in an allogeneic in vitro system generated Ts or Tc cells, but never T cells (Cantor and Boyse 1977, Jandinski et al 1976). Ts similar to Tc are apparently restricted to the D and/or K end of the H-2 complex (Simpson and Beverley 1977).

The earliest precursor for Ts in the peripheral pool may well be a Ly 123 cell. Experiments using chemically modified self cells for the activation of Ts have shown that Ly 123 cells can give rise to Ly 23 Ts cells. This step might be augmented by T_H cells. Furthermore, suppressor factors may act on Ly 123 cells to give rise to Ly Ts cells (Cantor and Boyse 1977).

As mentioned previously, Tc cells share with Ts the same Ly phenotype, namely Ly 23. Both precursor and effector Tc cells recognising allogeneic antigens possess Ly 23 phenotype, but can be differentiated from each other as the effector cells are additionally FcR^+ and Ly 6.2^+ (Woody et al 1977).
In addition, the precursors for Tc cells to altered self might be of Ly 123
phenotype. Data at hand would also suggest that primary Tc cell populations
generated against virus infected self in vivo are partly sensitive to anti-
Ly 1 sera plus complement, arguing for at least two phenotypic populations,
one Ly 123 and one Ly 23 (Pang et al 1976, Mullbacher unpublished data).
The response of Tc cells either in allogeneic or syngeneic systems is deter-
mined by the K and/or D antigens in H-2 (Bach et al 1972b). The require-
ments for induction of primary and secondary Tc cells in vivo and in vitro
in the allogeneic system have been documented extensively (Bach et al 1977,
puzzle which still remains to be solved is the reason for the high precursor
frequency of alloreactive Tc cells in the peripheral T cell population
of Tc precursor cells are specifically activated against a single foreign
H-2 alloantigen. To accommodate the large number of specificities that can
potentially be recognised, populations of cross reactive clones are invoked
(Teh et al 1978, Geib et al 1978) to account for this high number. Hypotheses
concerning reasons for the production of these alloreactive Tc cells have
been advanced but their validity remains unknown (Janeway et al 1977,

4. Tc CELL RESPONSE TO MODIFIED SELF

Tc cells play an important role in defence against most intracellular
parasites, in particular against viral infection (reviewed by Blanden 1974).
It was further shown that lysis of virus-infected target cells by virus-
immune Tc cells occurred only when effector and target cells shared at least
part of the H-2 complex, e.g. LCM virus immune Tc cells from BALB/c mice
(H-2\textsuperscript{d}) lysed only P815 (H-2\textsuperscript{d}) LCM infected targets but not L929 (H-2\textsuperscript{k}) LCM
infected targets (Zinkernagel and Doherty 1974, Doherty et al 1976). Such H-2 restricted Tc cell recognition was found independently by Shearer (1974) for trinitrophenyl (TNP)-immune Tc cells and TNP-modified targets.

Since the original discoveries of H-2 restricted Tc cell lysis with LCM, an ever increasing number of H-2 restricted Tc cell systems became evident. The same restriction phenomena were shown to be operative for the male H-Y antigen (Gordon et al 1975) and other minor transplantation antigens by Bevan (1975). H-2 restriction has been shown for the majority of virus groups as summarised in table I. Some controversy still exists regarding the herpes virus systems, but most of the other systems have been confirmed by at least one other group of workers. H-2 restricted responses to chemical modifications other than TNP have been documented by Rhen et al (1976). In addition, proteins such as foetal calf serum antigens (Peck et al 1977, Forni and Green 1976) or chemically modified bovine serum albumen (BSA) (Schmitt-Verhulst et al 1978) can stimulate Tc cells to develop into H-2 restricted effector cells. Though most of the experimental systems are murine, the phenomenon of MHC restricted Tc cell responses may well be ubiquitous. Evidence for such responses have been reported in rats, humans, chickens and dogs (Zinkernagel and Doherty 1979). The clearest assessment of the relevance of H-2 restricted Tc cell responses in vivo comes from the laboratory of Blanden and co-workers (Kees and Blanden 1976, 1977), experimenting with the response to a natural mouse pathogen, ectromelia virus (mouse pox). Their experimental evidence strongly argues for the idea that virus immune Tc cells, induced very early after an infection, are crucial for viral clearance and survival. This in vivo relevance has been supported by experiments using the LCM system (Mims and Blanden 1972, Doherty and Zinkernagel 1974, Cole et al 1972). Viral clearance from spleen and liver tissue in the ectromelia system coincides with the appearance of immune Tc
cells (Blanden and Gardner 1976) and appears at a time (4-6 days) post-infection, well before antiviral antibody levels have become detectable. Adoptive transfer experiments in mice with characterised and purified immune Tc cells unequivocally established the significance of virus-immune Tc cells in protection and recovery from ectromelia virus infections (Kees and Blanden 1976, 1977). Immune protection was only conferred in those experiments when homologous virus was used e.g. ectromelia immune Tc cells would protect ectromelia infected animals, but would not confer protection to LCM virus infected mice, and vice versa. Besides virus specificity, the Tc cells had also to share either the K or D region of the H-2 complex. Recently Yap and Ada (1978) showed that influenza immune Tc cells can have antiviral effects in the lungs of mice. Purified Ly 23 spleen cells, taken at the peak of the primary anti influenza Tc cell response in vivo dramatically reduced the influenza virus titre in lungs of recipients in adoptive transfer experiments. Homology between the donor Tc cells and the recipient animals was necessary in the K and/or D regions of H-2.

4.1 The induction of H-2 restricted Tc cells

The most common and probably biologically most reliable method of inducing Tc cells is the primary in vivo response. Mice are immunized with virus or injected with syngeneic modified cells. Spleens or lymph node (LN) cells are recovered 4-12 days post immunization and tested on autologous infected or modified targets for lysis. Data from a number of virus systems, such as LCM, Sendai and ectromelia, suggest that peak cytotoxicity occurs 3-4 days post peak antigen load. This occurs with live ectromelia at days 5-6 (with peak virus titre at day 2) for LCM on day 9 (with peak virus titre at day 5). Ectromelia virus inactivated by γ irradiation induces a peak of immune Tc cell response at 4 days post-immunisation (Mullbacher unpublished).
Other immune mechanisms besides antigen dose may well play a role in the kinetics of Tc cell responses. Experiments by Pfizermaier et al (1977), using the herpes virus system, showed that pretreatment of mice with low doses of cyclophosphamide results in selective elimination of hypothetical suppressor cells and allows detection of an otherwise insignificant Tc cell response to herpes virus.

The induction of secondary responses in vivo has been used much less frequently as a tool to investigate H-2 restriction phenomena. In the ectromelia and LCM systems smaller than primary responses are obtained (Gardner and Blanden 1976, Dunlop et al 1977). The cause for this low responsiveness is probably due to the presence of high neutralising antibody titres that eliminate most of the immunogen before infection of putative stimulator cells can occur. Blocking effects by antibody and possible Ts cell mediated suppression cannot be excluded from being partly the reason for low responsiveness after secondary in vivo stimulation (Pang and Blanden 1976a). One exception to this general phenomena occurs in the influenza virus system, where challenge with a serologically different virus elicits a strong secondary response in vivo. The influenza-immune Tc cells so generated lyse targets infected with both the priming and challenging virus (Braciale 1977, Effros et al 1978).

Primary stimulation in vitro has been extensively used for generation of H-2 restricted Tc cells to chemically modified self (Shearer et al 1976). Normal spleen cells are co-cultured with chemically modified autologous cells for 5 days in vitro. Of the virus systems, the two best characterised systems are ectromelia and LCM (Blanden et al 1977), but primary in vitro responses to Sendai, influenza and alphaviruses can be generated using a similar protocol to that described for ectromelia (Mullbacher unpublished).

The secondary in vitro response is probably more extensively used than
any of the other three described above. This is mainly due to the generation of highly potent Tc cells and the somewhat less demanding conditions for stimulation, that might be a reflection of precursor frequency. In short, spleen or IN cells (memory cells) of animals primed 2 to 50 weeks previously with either virus, syngeneic chemically modified-self cells or, in the H-Y system, female animals primed with syngeneic male cells are cocultured with syngeneic stimulator cells (either virus infected, chemically modified or male cells) usually for 5 days at 37°C. Details of kinetics, effector cells and requirements for optimal induction in the virus systems are reported by Gardner and Blanden (1976), Pang and Blanden (1976b) and Dunlop and Blanden (1976). Both primary and secondary stimulation in vitro have the danger of producing artifacts due to a response to foetal calf serum (FCS) (Forni and Green 1976, Peck et al 1977) and this must be checked by appropriate controls. The in vitro secondary method has permitted the analysis of responses to minor H (including H-Y) antigens, tumor viruses and alphaviruses which give a weak or undetectable primary response in vivo. This increased sensitivity of memory cells to stimulation, in contrast to primary precursors, opened the way to a much finer dissection of the specificity of Tc cell recognition. Using the influenza system, both UV inactivated virus and isolated haemagglutinin could be used to generate secondary immune Tc cells in vitro (Zweerink et al 1977, Braciale and Yap 1978). Secondary in vitro immune Tc cells could also be obtained with infected fibroblast cells (Hapel et al 1978) and with glutaraldehyde fixed, infected macrophages (Dunlop and Blanden 1976). Fixed TNP-modified macrophages were also able to induce TNP-immune Tc cells (Forman 1977a). Both primary and secondary in vitro responses were employed to estimate the frequency of cytotoxic precursors for allogeneic (Skinner and Marbrook 1976, Fischer-Lindahl and Wilson 1977a) for TNP-modified self (Fischer-Lindahl and Wilson 1977b) and for viruses such as
influenza, Sendai and ectromelia (Komatsu et al. 1978, Ashman and Mullbacher see chapter 9). These techniques of limiting dilution analysis will be invaluable in analysing antigen recognition patterns of Tc cells, because a clone derived from a single precursor can be employed for specificity analysis.

4.2 The assay system

The usual method used to measure Tc cell cytotoxicity is the $^{51}$Cr release assay reviewed by Cerottini and Brunner (1974). Other systems such as the microcytotoxicity assay (Takasugi and Klein 1970) are rarely employed as the tend to be cumbersome and unreliable. Various cells can be used as targets. Established tumor cell lines such as L929 (H-2$^k$) P815 (H-2$^d$) or EL4 (H-2$^b$) have the advantage of convenience and low spontaneous release of $^{51}$Cr but this is countered by the difficulty of comparing lysis of one target cell with another, and the lack of cell lines syngeneic with H-2 recombinant mice. Primary and secondary fibroblast cell lines on the other hand are easily established but suffer from a rather high spontaneous release and are often very insensitive as targets. Recent publications by the Doherty and Zinkernagel groups used SV40 transformed cell lines, established from H-2 recombinant mice, as targets. These cell lines proved to be quite satisfactory in their hands but caution should be exercised and appropriate controls applied because results obtained with SV40 transformed systems suggest that some major changes in H-2 antigen expression can occur in such cells (Warnatz and Krapf 1976, Maki and Howe 1976). Other groups of workers rely mainly on Con A stimulated lymphohlasts as their targets, but these cells suffer from rather high spontaneous release. Peritoneal macrophages, first used by Zinkernagel and Doherty (1975) have the advantage of being freely available from any recombinant mouse strain and provide very sensitive targets, with reasonably low spontaneous release. Assay times can be shortened con-
siderably and they are susceptible to infection by most viruses (in terms of expressing viral antigens on their surface) as well as being modifiable by TNP. The possibility of a short term assay, as one can use with macrophage targets, reduces the possibility of other phenomena, such as interferon induction and antibody-dependent cell-mediated cytolysis (ADCC) complicating the assays. The drawback with macrophage targets is their refractoriness in antibody blocking experiments (Chapter 7) and their possession of FcR which requires Fab fragments of antiviral antibodies to monitor for infectivity by immunofluorescence.

A very important but much more cumbersome assay system for virus-immune Tc cells is the in vivo method of adoptive transfer of Tc cells and the measurement of the reduced virus titres (Blanden 1974). These in vivo systems have been characterised for both ectromelia and LCM viruses.

4.3 The lytic event

Much of our knowledge about the actual cytolytic event comes from studies using drugs affecting Tc cell metabolism or from studies of physical parameters such as temperature. The process has been divided into three stages (Golstein and Smith 1977). The recognition stage, the lethal hit stage and target cell disintegration stage. Nearly all of the work has been performed with Tc cells in the allogeneic system or in the tumor systems (Henny 1977, Martz 1977) but there appears to be no reason why a similar process should not take place with modified self targets.

(a) The recognition stage

We still know relatively little about the T cell receptor and it has therefore eluded isolation and characterisation. Most of its properties are inferred indirectly by studying antigen specificity or the antigen molecules themselves. Recent anti-idiotype work using antigen-binding regions as
immunogens for antibodies (anti-idiotype antibodies) (Binz and Wigzell 1977a) or Tc cells specific for T cell recognition structures (Binz and Wigzell 1977b) have shed some more light on the Tc cell receptor(s). Some evidence points towards a structure resembling immunoglobulin heavy variable chain region as being at least part of this putative receptor. Regarding the actual event of recognition, contact between Tc cell and target is a prerequisite for the lethal hit stage to occur. Separation of target cells by semipermeable membranes, or coating target cells by antibody prevents recognition and lyses. The recognition phase has mainly been investigated by using specific target cell monolayer to absorb and deplete Tc cell populations (Henny 1977). The following data regarding the recognition event emerged from a number of studies: Divalent cations such as Mg$^{2+}$ and Ca$^{2+}$ are mandatory, but neither DNA or RNA nor protein synthesis is required. The effector cell has to be metabolically active as inhibitors of metabolic functions such as azide, dimethylsulfoxide (DMSO), cytocholasin B, low temperature and others prevent recognition (Golstein and Smith 1977). No requirements except antigen presentation are obligatory for the target cell as glutaraldehyde fixed monolayers sufficed as targets. The inhibitors of recognition only acted before the event of association and not after binding has taken place. The need for specificity in the recognition event was cast in doubt when it was found that "glueing" of targets with Tc cells by plant lectins was sufficient for the triggering of the lytic event, but experiments by Henny's group (see Henny 1977) showed elegantly that close proximity between targets and effectors was not sufficient for triggering lyses. Coculturing of two allogeneic stimulated spleen population A anti B and B anti C reduced only the B anti C population but not A anti B. To reconcile this with the data obtained with lectins requires that lectin binding mimics the specific recognition-signal that triggers the lethal hit.
(b) The lethal hit

Very little data are available which give any definite evidence about the mechanism of the lethal hit event. A hypothesis proposing lymphotoxins as being responsible for lysis has been countered by findings that antilymphotoxin antibodies were unable to prevent target cell lysis (Gately et al 1975). Furthermore, specificity of the lytic event (innocent bystander targets are not killed) and survival of the effector T cell after the event (though they are demonstrably sensitive to their own type of lytic mechanism) make the lymphotoxin hypothesis even less likely. There is abundant evidence that Tc cells survive the lytic event and are able to interact with more than one target cell consecutively (Zagury et al 1975). A model of intercytoplasmic channels between target and effector cells, reminiscent of the killer channel as proposed by Langman (1978) has been proposed by Selin et al (1971). These channels might act in conjunction with lymphotoxins. Time limitation is the main argument against this model, but as long as we do not know how long it would take to form such putative channels, such a model cannot yet be ruled out. Finally, surface enzymes such as phospholipases, that become activated by steric alterations after receptor-antigen binding is another possible mechanism (Frye and Friou 1975).

(c) Target cell disintegration

It is known that target cell destruction does not necessitate contact with the effector cell after the delivery of the lethal hit, and inactivation and/or dissociation of effectors from radioisotope labelled targets by heat shock, EDTA or antibody plus complement, still allows the eventual release of the isotope label from the target cell. Using size markers, a graduation of leakiness of the target cell membrane was noticed, with initial permeability to small ions upsetting the osmotic balance, which lead to further membrane destruction and cell death (Wagner and Rollinghoff 1974).
4.4 The recognition of foreignness

It was shown in early Tc cell transfer experiments and by in vitro cytotoxicity tests that either protection or target cell lysis required homologous virus for induction of Tc cells and infection of recipient or target (Doherty et al 1976). This has been true for all viruses tested so far if unrelated groups were compared. Such discrimination is also observed with regard to Tc cells induced by chemically modified self cells. Forman (1977b) reported little cross reactivity of TNP immune Tc cells on DNP modified target cells and vice versa, indicating good specificity of recognition, considering the similarity of the two haptens. It is not quite clear at present what is seen by Tc cells in the chemically modified self system, or how such antigens are recognised. Some contradictory results in the TNP systems await clarification, especially when carriers such as TNP modified proteins are used to sensitize Tc cells and to modify target cells. Results by Rehn et al (1976) and the ones described in Chapter 2 strongly suggest that TNP immune Tc cells either see part of the protein carrying the hapten and thereby show specificity between different TNP-modified carriers or TNP-modified H-2K and/or H-2D antigens (Forman et al 1977a) are recognised by a different mechanism to the one that recognises TNP bound to other proteins. Maybe a single or a dual recognition structure is involved depending on the method of induction (Schmitt-Verhulst et al 1978). The analysis of the fine specificities of virus immune Tc cells regarding their recognition of the viral-coded or determined antigen moieties has been helped by the accumulated knowledge of serological differences between virus subgroups and the advanced state of molecular virology. The minimum requirements for producing on cell surfaces the antigenic changes recognised by virus-immune Tc cells can be achieved in two ways with most viruses. Firstly, a cell can be infected with a small dose of virus, presumably as low as one
infectious virion, and antigen expression on the cell surface due to translation of viral-coded or viral-induced messenger RNA's renders the cell susceptible to lysis by the appropriate Tc cells (Ada et al 1976, Jackson et al 1976, Hapel et al 1978).

The second method, using high virus input and fusion of viral envelope with the target cell membrane, then allows viral antigen molecules to intercalate in cell membranes. This was first shown with Sendai virus, using UV irradiated virus or isolated fusion proteins to render target cells susceptible to lysis (Schrader and Edelman 1976, Sugamura et al 1977, Chapter 2). This has since been shown for other viruses such as influenza (Braciale and Yap 1978) vaccinia (Hapel et al 1978) and alphaviruses (Mullbacher unpublished). The virus-cell fusion systems only allow virion envelope antigens to become target antigens. However, there may be examples where virus-specified or -induced proteins other than envelope antigens are recognised by Tc cells. For example, Koszinowski and Ertl (1976) obtained blocking of Tc cell-mediated lysis of vaccinia-infected targets only with serum against early vaccinia viral antigens but not late antigens, yet late viral antigens contain the coat proteins for vaccinia virus. Also the matrix protein of influenza is not in the virion envelope, but may be a target for Tc cell recognition on the surface of infected cells. Target cell sensitisation using liposomes containing purified influenza haemagglutinin subunits have been only partially successful (Higgins personal communication) but this approach, if technical problems can be overcome, should prove extremely useful in analysis of the target antigens. Use of ts mutants of VSV by Zinkernagel et al (1978) and Hale et al (1978) confirmed results obtained earlier by Ada et al (1976) using protein synthesis inhibitors. They showed that target cells did not become recognisable by Tc cells at non permissive temperature. Furthermore, employing a number of mutants of known defects
the viral antigen molecule recognised could be identified.

As mentioned above, anti-viral antibody blocking of Tc cell lysis has been obtained with vaccinia (Koszinowski and Ertl 1976), VSV (Hale et al 1978) ectromelia and influenza (Chapter 7) and alphavirus (Chapter 6), but due to the heterogeneity of antibodies in whole serum, little fine analysis of target antigens has yet been achieved. The employment of monoclonal antiviral antibodies could be a more definitive approach.

4.5 The recognition of self

When it was recognised that virus immune Tc cells (Zinkernagel and Doherty 1974) and TNP-immune Tc cells (Shearer 1974) preferentially lysed infected or modified self cells rather than allogeneic infected or modified cells, mapping studies using congenic and H-2 recombinant mice were performed to delineate the required homology. It soon became apparent that homology at either the K or the D region of the H-2 complex was sufficient for lysis to occur, homology in the I region was neither necessary nor sufficient. This was found for all virus-immune Tc cell systems, TNP and minor antigen systems (Blanden et al 1975, Shearer et al 1975, Bevan 1975, Gordon et al 1976). These findings were extended by experiments showing that private rather than public specificities correlated with Tc cell recognition of modified self (Gardner et al 1975, Zinkernagel and Doherty 1977, Blanden and Kees 1978). Wagner et al (1977) reported I region restricted Tc cell responses for TNP and the L antigen may also turn out to be a restriction antigen, at least in the influenza virus system (Biddison et al 1979). Apart from the mapping studies, lack of H-2K and H-2D antigens on targets such as F9 teratoma cells or antibody selected H-2 negative cell lines prevented target cell lysis when modified by TNP (Dennert and Hyman 1977). Furthermore, F9 cells infected with either vaccinia or LCM could not be
lysed by virus immune Tc cells (Zinkernagel and Oldstone 1976). Further evidence for the involvement of H-2K and D antigens comes from blocking studies using anti H-2K or anti H-2D antibodies (Koszinowski and Ertl 1975b). Again a clearer understanding will emerge with the employment of monoclonal antibodies; an example is presented in this thesis (Chapter 8).

The use of mutants further helped to characterize the self recognition element. So far, Tc cell recognition of H-2K and D antigens followed the same pattern as defined by serology, but mutant mouse strains, especially the K^b mutants, K^ba in particular, were serologically silent, but gave exquisite specificity when tested in the ectromelia and vaccinia virus system (Blanden et al. 1976, Zinkernagel 1976a). Wild type K^b ectromelia immune Tc cells lysed wild type infected targets perfectly but little lysis was observed on K^ba infected targets and vice versa. (For a review see McKenzie et al. 1977).

This specificity did not hold true in the TNP system (Forman and Klein 1977). Here strong cross-reactivity was observed between wild type and mutant targets. This is in accordance with earlier findings that TNP immune Tc cells showed a significant level of cross-reactivity with allogeneic TNP modified or unmodified targets (Burakoff et al. 1976), findings which led to speculations about the nature of target antigens and differences between virus immune and TNP immune Tc cell recognition. The role of the K and D antigens in Tc cell lysis is still speculative. They are expressed on all cell types which makes them the perfect marker structure for Tc cell recognition, but whether their role is only a passive one is still uncertain.

Other functions, such as anchoring of "killer channels" as proposed by Langman (1978) is as yet untested. The majority of observations regarding recognition of foreignness and recognition of self have been obtained by measuring lysis resulting from Tc cell recognition of target cells. These findings are generally applicable to Tc cell induction, with a few notable
exceptions which are discussed in Chapter 2.

4.6 The role of thymus in H-2K and H-2D restricted Tc cell responses

The question is H-2 restriction limited to self H-2 antigens only or, in other words, can T cells respond to allogeneic infected or modified cells has been approached in a variety of experimental systems. To overcome the normal high reactivity to alloantigens, a number of techniques have been employed to either tolerize mice to the alloantigen or to selectively deplete alloreactive Tc cell precursors. A successful depletion method has been used by Wilson and co-workers (1977); parental strain mouse T cells are depleted of reactivity to specific alloantigens by "filtration" from blood to lymph in irradiated Fl hybrids. Such depleted T cells were stimulated with TNP modified allogeneic spleen cells. The results obtained showed that a good response to allogeneic modified T cells can be obtained and therefore the T cell pool must contain precursors for allogeneic TNP-modified MHC determinants. Restriction was observed in that stimulator and target cells had to share either H-2K or D antigens. Recently, Bennink and Doherty (1978) obtained different results, using a similar experimental approach with vaccinia and influenza viruses, but later experiments by the same group (Doherty and Bennink unpublished) found that selected H-2^d^ Tc cells could recognise H-2^K^ plus vaccinia, in conformity with earlier findings of Wilson et al (1977) with TNP. Suicide experiments using BUdR and light to deplete alloreactive Tc cells, followed by stimulation of remaining cells with TNP-modified allogeneic cells, gave only very limited lysis on TNP modified allogenic targets, but overall lysis in these experiments was low (Janeway et al 1978). The other major approach is either to induce tolerance to alloantigens neonatally, or by reconstituting adult irradiated animals with T cell depleted bone marrow or foetal liver cells (irradiation chimeras).
Neonatally tolerant mice have been obtained by injecting (A x B) F1 spleen cells into A neonates. These mice have been shown by skin graft rejection tests to be tolerant of B alloantigens. Using such mice, Zinkernagel et al. (1977) and others (Kees personal communication) could not stimulate a response to poxvirus plus B alloantigens. Experiments using the TNP system (Forman et al. 1977b) could obtain H-2 restricted responses to tolerated K or D antigens but response strength varied widely with different haplotypes.

Three different irradiation chimera protocols have been used: (a) parent into F1 combination, (b) F1 into parent and (c) totally allogeneic combinations. Results obtained with parent into F1 (A + A x B) chimeras showed that Tc cells of A phenotype, when allowed to differentiate in the F1 (A x B) hybrid, responded to both A and B modified H-2K and D antigens. This was established for virus, TNP and minor H (including H-Y) antigen systems (Zinkernagel 1976b, von Boehmer et al. 1978, Billings et al. 1978). The significance of those experiments became clearer when the reverse experimental procedure was adopted e.g. F1 (A x B) into F1 (A) or P2 (B) irradiated recipients. Such experiments by Bevan (1977), Fink and Bevan (1978) and Zinkernagel et al. (1978a,b) showed preferential killing of targets homologous with the recipient parent. Thymus graft experiments (Zinkernagel et al. 1978a,b) gave conclusive evidence that the H-2 phenotype of radiation-resistant thymic elements play a major role in determining restriction specificity during Tc cell ontogeny.

Low, but significant lysis of F1 → F1 chimera T cells have been obtained on infected targets of P2 type in the ectromelia virus system (Blanden and Andrew 1978) and for minor H antigens (Bevan 1977). Matzinger and Kirkwood (1978) reported a similar breakdown of exclusive specificity for one parent in total allogeneic chimeras. The significance of these data is still elusive.
### TABLE I

**SYSTEMS IN WHICH H-2 RESTRICTED Tc CELL MEDIATED LYSIS OF VIRUS INFECTED OR VIRUS TRANSFORMED TARGET CELLS HAS BEEN SHOWN**

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>GROUP</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCM</td>
<td>Arena</td>
<td>Zinkernagel and Doherty 1974</td>
</tr>
<tr>
<td>Ectromelia</td>
<td>Pox</td>
<td>Pfizermaier et al 1975</td>
</tr>
<tr>
<td>Vaccinia.</td>
<td>Pox</td>
<td>Blanden et al 1975</td>
</tr>
<tr>
<td>Sendai</td>
<td>Paramyxo</td>
<td>Koszinowski and Ertl 1975(a)</td>
</tr>
<tr>
<td>Influenza</td>
<td>Myxo</td>
<td>Doherty and Zinkernagel 1976</td>
</tr>
<tr>
<td>SV40</td>
<td>Papova</td>
<td>Yap and Ada 1977</td>
</tr>
<tr>
<td>Mouse Adeno</td>
<td>Adeno</td>
<td>Doherty et al 1977</td>
</tr>
<tr>
<td>Sindbis (SIN)</td>
<td>Toga</td>
<td>Inada and Uetake 1978</td>
</tr>
<tr>
<td>Bebaru (BEB)</td>
<td>Toga</td>
<td>Mullbacher and Blanden 1979</td>
</tr>
<tr>
<td>Semliki Forest (SF)</td>
<td>Toga</td>
<td>Mullbacher and Blanden 1979</td>
</tr>
<tr>
<td>Coxsackie</td>
<td>Entero</td>
<td>Mullbacher and Blanden 1979</td>
</tr>
<tr>
<td>Rhabies</td>
<td>Rhabdo</td>
<td>Wong et al 1977</td>
</tr>
<tr>
<td>Vesicular-stomatitis (VSV)</td>
<td>Rhabdo</td>
<td>Wiktor et al 1977</td>
</tr>
<tr>
<td>Herpes simplex (HSV)</td>
<td>Herpes</td>
<td>Hale et al 1978</td>
</tr>
<tr>
<td>Cytomegalo (CMV)</td>
<td>Herpes</td>
<td>Zinkernagel et al 1978(c)</td>
</tr>
<tr>
<td>Rauscher Leukemia (RLV)</td>
<td>Oncorna</td>
<td>Pfizermaier et al 1977</td>
</tr>
<tr>
<td>Friend Leukemia (FLV)</td>
<td>Oncorna</td>
<td>Quinnan et al 1978</td>
</tr>
<tr>
<td>Murine Sarcoma (MSV)</td>
<td>Oncorna</td>
<td>Schrader et al 1975</td>
</tr>
<tr>
<td>Mouse Mammary Tumor (MMT)</td>
<td>Oncorna</td>
<td>Blank et al 1976</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Comard et al 1976</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stutman 1977</td>
</tr>
</tbody>
</table>
FIG. 1 - Genetic Map of H-2 (after Klein 1978)
CHAPTER 2

CROSSREACTIVITY PATTERNS OF MURINE CYTOTOXIC T LYMPHOCYTES

Murine cytotoxic T lymphocytic lymphocytes (Tc cells) generated as a consequence of viral infection in vivo or by in vitro stimulation of nylon-splenic Tc cells with syngeneic virus-infected cells (secondary Tc cells) show dual specificity (Libby et al., 1976a, b). That is, target cells identified with the array of effector cells use K and/or D regions of the I-A
ergene complex (Bleazard et al., 1975). Additionally, they are H-2 restricted. Thus far it seems that specificity for self H-2 determinants is stringent (Parren et al., 1977, Mossman and Reed, 1976); strict viral specificity is also observed with respect to different viral genera (Libby et al., 1976a). A comparable restraint is placed on Tc cells specific for specifically-modified self (Cheever et al., 1977, Forseen, 1977).

A degree of specificity is observed for the foreign antigen molecules as Tc cells distinguish between NP and DP substitutions (Forseen, 1977), but specificity for self H-2 is not apparently so stringent as in the case of virus-infected Tc cells (Forseen and Klein, 1977). Affectorive Tc cells also display marked crossreactivity between certain H-2 antigens (Fischer-Madelin et al., 1977, Forseen and Miller, 1974). This investigation presented crossreactivity between various classes of Tc effector cells (affectorive, virus-infected or virus-bearing) on different target cells (untreated, NA-modified or virus-infected).

The most striking feature of the crossreactivity patterns thus observed was the one-wayness of cross-reactivity. For example, virus-infected Tc cells lysed NA-modified targets, but not vice versa. Thus, this chapter investigates the basis of such phenomena.
INTRODUCTION

Murine cytotoxic thymus-derived lymphocytes (Tc cells) generated as a consequence of viral infection in vivo or by in vitro stimulation of memory splenic Tc cells with syngeneic virus-infected cells (secondary Tc cells) show dual specificity for viral and self H-2 antigens (Doherty et al. 1976a). Thus, target cells are lysed only if they share with the donors of effector cells the K and/or D regions of the H-2 gene complex (Blanden et al. 1975), that is, they are H-2 restricted. Thus far it seems that specificity for self H-2 determinants is stringent (Pang et al. 1977, Blanden and Kees 1978); strict viral specificity is also observed with respect to different viral genera (Doherty et al. 1976a). A comparable restraint is placed on Tc cells specific for chemically-modified self (Shearer et al. 1975, Forman 1975). A degree of specificity is observed for the foreign antigen molecules as Tc cells distinguish between TNP and DNP substitutions (Forman 1977), but specificity for self H-2 is not apparently as stringent as in the case of virus-immune Tc cells (Forman and Klein 1977). Alloreactive Tc cells also display marked crossreactivity between certain H-2 antigens (Fischer-Lindahl et al. 1975, Forman and Moller 1974). This investigation concerned crossreactivity between various classes of Tc effector cells (alloreactive, TNP-immune or virus-immune) on different target cells (untreated, TNP-modified or virus-infected). The most striking feature of the crossreactivity patterns thus revealed was the one-way-ness of cross-lysis. For example, virus-immune Tc cells lysed TNP-modified targets, but not vice versa. This chapter investigates the basis of this phenomenon.

MATERIALS AND METHODS

Animals

All mouse strains were bred at the John Curtin School and used at 7-10
Viruses

Virulent (Moscow) and attenuated (Hampstead egg) strains of ectromelia virus (Gardner et al. 1974), Sendai (Doherty and Zinkernagel 1976), influenza virus strain A/JAP (Yap and Ada 1977), polyoma virus (Ralph and Colter 1977) and adenovirus 5 (Philipson 1961) were grown and titrated as described in detail elsewhere. Bebaru virus was grown in infant mouse brains. Virus was recovered after sonication and differential centrifugation, and titrated on Vero cell monolayers as described previously (Hapel 1975).

Preparation of TNP-Modified Sendai Virus (TNP-Sendai)

$10^{7.5}\text{EID}_{50}$ of Sendai virus in 1 ml phosphate-buffered saline (PBS) was incubated for 10 min at $37^\circ C$ with 1 ml 100mM 2,4,6-trinitrobenzene sulfonic acid (TNBS) and then dialysed against 4 litres of Eagle's Minimal Essential Medium at $4^\circ C$ for 24 h, and for a further 24 h against PBS. The modified virus was concentrated to the original volume and stored at $-70^\circ C$.

Preparation of TNP-Conjugated Stimulator and Target Cells

The method used was basically similar to that reported by other workers (Shearer et al. 1975, Forman 1975). Briefly, normal splenic lymphocytes or peritoneal macrophages, L929 fibroblasts or P815 mastocytoma cells were modified with 10mM TNBS in PBS for 10 min at $37^\circ C$ and thoroughly washed with PBS prior to and post-modification.

Immunization

Immunizations with attenuated ectromelia virus (Gardner et al. 1974), influenza strain A/JAP virus (Yap and Ada 1977) and Sendai virus (Doherty and Zinkernagel 1976) have been described previously. Immunization with TNP-modified Sendai was carried out by a single injection of $10^{6.5}\text{EID}_{50}$ of the TNP-modified virus in gelatine saline intraperitoneally. Immunization with TNP-modified spleen cells was obtained by subcutaneous injection of
Memory Cultures (in vitro Secondary Responses)

The generation of secondary Tc cells with ectromelia (Pang and Blanden 1976) and influenza virus (Yap and Ada 1977) has been reported. Secondary Sendai-immune and TNP-Sendai-immune Tc cells were generated in vitro by culturing responder spleen cells (from mice immunized with Sendai or TNP-Sendai respectively 3-8 wk previously) with normal syngeneic "stimulator" spleen cells infected with 1-2 EID_{50} units of Sendai virus or TNP-Sendai virus per nucleated cell. The stimulator:responder ratio was 1:10. Usually 8 x 10^7 responder cells were cultured with 8 x 10^6 stimulator cells at 37°C in an atmosphere of 10% CO_2 in 40 ml of Eagle's minimal essential medium (GIBCO, Cat. no. 15) supplemented with 10% heat-inactivated foetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), 10^{-4} M 2-mercaptoethanol and antibiotics. Cultures were harvested after 5 d.

The generation in vitro of secondary TNP-immune Tc cells was essentially as for Sendai-immune Tc cells. TNP-modified spleen cells (2 x 10^7 per culture) were used as stimulators with 8 x 10^7 responder cells from mice primed 2-12 wk previously with TNP-modified syngeneic spleen cells.

Mixed Lymphocyte Reactions (MLR)

Effector Tc cells were generated in MLR by the method of Lafferty et al (1974) with responder:stimulator ratios of 4:1.

Cytotoxicity Assay with Macrophage Target Cells

The method has been described in detail elsewhere (Blanden et al 1976). The multiplicity of infection for ectromelia was 50 PFU/cell, influenza A/JAP 1 EID_{50}/cell, Sendai 1 EID_{50}/cell, TNP-Sendai 1 EID_{50}/cell. The duration of infection for all viruses used was 1 h at 37°C followed by 2 washes with complete medium and an incubation time of 1-2 h at 37°C before addition of effector Tc cells. Data given have had spontaneous release
subtracted and are the means of triplicates for assays run at 37°C for 6-16 h. S.E. of the means were usually less than ± 3% and are omitted for clarity. Significance was determined by Student's t test.

Cytotoxicity assay using L929 or P815 target cells

The method has been described previously for ectromelia (Pang and Blanden 1976), influenza (Yap and Ada 1977) and Sendai (Doherty and Zinkernagel 1976).

Infection with Bebaru, polyoma, lymphocytic choriomeningitis (LCM) and adenovirus 5 was essentially the same as for ectromelia virus with the following multiplicities of infection: 50 PFU Bebaru, 50 PFU Polyoma, 35 PFU LCM and 50 PFU adenovirus 5 per cell. Infection of target cells with these viruses was checked by virus-specific immunofluorescence.

Cold Target Competition

L929 cells labeled with Na$_{51}$CrO$_4$ and infected with virus (Sendai or ectromelia respectively) were distributed at 2 x 10$^4$ cells in 50 µl per microtitre well. Unlabeled L929 adapted for 24 h in spinner culture were infected or modified with TNP as described above and added in 50 µl volumes at ratios of 1, 2, 4 and 8 times the number of labeled targets. Effector cells were added in 100 µl medium. The assay was run for 6 h.

RESULTS

Activity of alloreactive Tc cells against Virus-Infected or TNP-Modified Target Cells

C57Bl/6 (H-2$^b$) spleen cells were cultured in MLR with CBA (H-2$^k$) DBA/1 (H-2$^d$) or SJL/J (H-2$^s$) stimulators and effector Tc cells thus generated were tested on P815 (H-2$^d$) targets, either uninfected or infected with ectromelia (poxvirus), influenza (A/JAP) (myxovirus), Sendai (paramyxovirus), Bebaru (alphavirus), LCM (arenavirus), poloma virus (papovavirus) or
adenovirus 5 (adenovirus) (Table I, exp. 1). None of the virus-infected targets was lysed significantly more than uninfected controls. What could be observed was a varying degree of lysis of 3rd-party targets (P815) depending on the stimulator used in the MLR with C57Bl/6 (H-2^b) anti DBA/1 (H-2^a), showing a significant 24% specific lysis. Such a crossreaction has been observed by others (Fischer-Lindahl et al 1975).

In a second experiment, the lysis of targets syngeneic with the responder cells in an MLR was tested (Table I, exp. 2) Thus BALB/c anti-CBA Tc cells did not lyse any virus-infected BALB/c macrophage targets significantly more than uninfected controls, but TNP-modified BALB/c targets were significantly lysed.

TNP-Immune Tc Cells and Alloreactive Tc Cells Crossreact on TNP-Modified and Unmodified Syngeneic and Allogeneic Targets

TNP-Immune Tc cells were generated either in secondary response in vitro (CBA) or in primary response in vitro (BALB/c) and tested on syngeneic or allogeneic TNP-modified and unmodified targets (Table II). Secondary CBA TNP-immune Tc cells (top line) show high lysis of TNP-modified CBA macrophage targets and also significant crossreactive lysis of C57Bl/6 and A.TL TNP-modified macrophages. Furthermore, significant lysis of unmodified BALB/c and A.TL macrophages occurred. Primary BALB/c TNP-immune Tc cells appeared to be more H-2 restricted than the CBA secondary Tc cells since they significantly lysed only BALB/c TNP-modified targets and A.TL TNP-modified targets (which share D^d with BALB/c); no significant lysis of C57Bl/6 targets either modified or unmodified occurred, and there was slight lysis of CBA-TNP targets. This lesser crossreactivity might be a quantitative phenomenon since the lytic power of the BALB/c primary cells was clearly less than CBA secondary cells.

The data in Table II lines 1 and 4 showed that unmodified BALB/c targets were lysed more than BALB/c-TNP targets by CBA TNP-immune T cells..
With C57BL/6 and C57BL/6-TNP targets, the opposite effect occurred with these same killer cells. These results imply that target lysis was due to particular subsets of killer cells, and are not compatible with TNP-modified targets being generally more sticky or more sensitive to cross-reactive lysis than unmodified cells.

Tc cells generated in MLR gave the expected strong lysis of modified or unmodified targets syngeneic with the stimulator cells, but also showed crossreactive lysis of TNP-modified targets syngeneic with the responding Tc cells (as shown also in Table I), and of third-party targets (Table II, lines 3 and 4).

**TNP-Modified Sendai Virus**

To test if TNP-immune Tc cells lyse targets which have TNP inserted into their surfaces other than by direct chemical modification of H-2 antigens, we modified Sendai virus with saturating amounts of TNP (TNP-Sendai). This virus was used for primary and secondary stimulation to obtain Tc cells reactive to TNP-Sendai, which were tested on TNP-modified targets, Sendai-treated targets and targets treated with TNP-Sendai (Table III). TNP-immune Tc cells did not lyse Sendai-infected targets nor TNP-Sendai-treated targets i.e. they did not crossreact on virus-treated targets. On the other hand, Sendai virus-immune Tc cells cross-lysed TNP-modified targets as much as they lysed Sendai-treated targets, but did not lyse TNP-Sendai-treated targets. Effectors generated by TNP-Sendai lysed all three targets with about equal efficiency. This last result reflected first that the TNP-Sendai virus used to prime mice and stimulate secondary secondary responses in vitro was not inactivated, and thus coded for the synthesis of unmodified Sendai proteins in infected cells, which in turn stimulated Tc cells that lysed Sendai-infected targets. (The converse, i.e.
Sendai-immune Tc cells failing to lyse TNP-Sendai targets, is explained by there being insufficient time for significant synthesis of new Sendai proteins in these targets in the 4 h assay time). Second, the lysis of TNP-modified targets is an example of the crossreactivity of virus-immune Tc cells (see below). Third, the lysis of TNP-Sendai targets which was achieved only by TNP-Sendai-immune Tc cells, indicated that TNP treatment of Sendai proteins created unique, TNP-dependent determinants not present on normal Sendai-infected cells, or on TNP-modified cells. Finally, the failure of TNP-immune Tc cells to lyse TNP-Sendai treated targets strongly suggests that TNP-immune Tc cells do not recognize TNP and H-2 determinants as separate 'entities.'

**Virus-Immune Tc Cells Lyse TNP-Modified Targets but not Vice-Versa**

Tc cells were generated in secondary responses in vitro using syngeneic stimulator cells treated with TNP, Sendai virus, influenza (A/JAP) virus or ectromelia virus, and were tested for their cytotoxicity on syngeneic, TNP-modified or appropriately virus-infected macrophage targets (Table IVa) or on H-2 compatible L929 targets (Table IVb). TNP-immune Tc cells showed no appreciable lysis of virus-infected syngeneic targets, but very high lysis of TNP-modified targets. On the other hand, virus-immune Tc cells lysed targets infected with the homologous virus used for induction, and also lysed TNP-modified targets to a very significant extent. This one-way crossreactivity was also consistently observed with other viruses such as Bebaru and LCM (data not shown).

**Cold Target Competition Experiments**

To investigate whether the same Tc cell clones or different clones are responsible for the lysis of virus-infected and TNP-modified target cells, cold TNP-modified targets were used to inhibit lysis of virus-infected 51Cr-labeled targets by virus-immune Tc cells. Figure 1a shows a competition
experiment using Sendai-immune Tc cells on Sendai-infected L929 targets. Figure 1b shows a similar experiment employing ectromelia-immune Tc cells and ectromelia-infected L929 targets. In both experiments, TNP-modified competitors specifically inhibited lysis of virus-infected L929 cells, but less efficiently than virus-infected competitors. As the crossreactive lysis of TNP-modified targets was usually not as great as lysis of virus-infected targets, equal inhibition with TNP-modified and virus-infected competitors would not be expected. It appeared, therefore, that some, but not all, clones of virus-immune Tc cells did recognize both virus-infected and TNP-modified targets. A similar conclusion was reached by Starzinski-Powitz et al (1976) for the case of vaccinia and LCM viruses.

One-Way Crossreactivity of Virus-Infected and TNP-Modified Cells is not Observed at the Level of Precursor Tc Cell Induction

Since the cold target competition experiments indicated that some individual virus-specific Tc cells were responsible for lysis of both virus-infected and TNP-modified targets, the following experiments were designed to investigate this crossreactivity at the induction level.

CBA mice were primed in vivo with either TNP-modified syngeneic cells or with ectromelia virus. Three wk later, the primed spleen cell pools of each category were divided into 2 parts and given a second stimulation in vitro with either TNP-modified or ectromelia virus-infected syngeneic cells. The resulting secondary Tc cells were then tested for lytic activity on either TNP-modified or virus-infected targets. A similar experiment was done with Sendai virus. As can be seen in Table V, primary (1st + 7th line) or secondary (3rd + 9th line) TNP-immune Tc cells show high lytic activity on TNP-modified targets, but none on virus-infected targets.

Primary virus-immune Tc cells generated in vitro did not cause any significant target cell lysis in the case of ectromelia virus (2nd line) and some lysis in the case of Sendai virus (8th line). (It was not expected to
obtain high lysis with primary anti-viral Tc cells at a killer:target ratio of 2:1) (Blanden et al 1977a).

In the case of cross-stimulation of TNP-memory cells with virus-infected stimulator cells (lines 4 and 10), no more lytic activity was observed than with the primary anti-viral responses; and in the converse (lines 5 and 11), TNP-modified stimulator cells did not activate virus-immune memory cells but gave lysis of TNP-modified targets comparable with that given by a primary anti-TNP response. Therefore, as a population, virus-immune Tc cell precursors could not be detectably stimulated by TNP-modified cells, even though some virus-immune effector Tc cells do recognize TNP-modified targets.

Primary virus-immune Tc cells (2nd, 4th, 8th + 10th lines) sometimes lysed TNP-modified targets to a greater extent than virus-infected targets; we assume that this was due to a low-grade multiclonal stimulation of Tc cells and shows up as lysis of TNP-modified targets due to high precursor frequency of TNP-reactive subsets, since the CBA response to TNP-modified self is very strong.

DISCUSSION

In these experiments Tc cells generated in one-way MLR predictably lysed target cells syngeneic with the stimulator cells, but there was also crossreactive lysis of third-party targets sharing certain public H-2 specificities with the stimulator cells as reported elsewhere (Blanden and Kees 1978, Fischer-Lindahl et al 1975, Forman and Moller 1974) and lysis of TNP-modified target cells syngeneic with the responder Tc cells as described previously by several groups of workers (Shearer et al 1976, Lemonnier et al 1977, Janeway et al 1978). Cold target competition experiments by Shearer et al (1976) and Janeway et al (1978) suggest the possible existence
of genuinely crossreactive Tc cells which recognize both a foreign H-2 antigen, and a TNP-modified self H-2 antigen (Forman et al 1977). The converse crossreaction has not always been seen, and therefore has not been so extensively investigated. We have shown here that Tc cell populations generated after stimulation with TNP-modified self cells do indeed lyse certain unmodified allogeneic target cells, but we have not formally tested (e.g. by cold target competition) the existence of crossreactive Tc cells. It should be noted that the observed crossreactions between allogeneic and TNP-modified self cells were one-way e.g. BALB/c anti-CBA Tc cells cross-lysed TNP-modified BALB/c targets, but BALB/c anti-TNP-BALB/c Tc cells did not lyse CBA targets. This might be explained by the fact that the MLR generated a far stronger response than TNP (Table II). We have analysed another example of one way crossreactivity in more detail where response strengths were more similar, however, and will discuss another possible explanation for this phenomenon below.

Another feature of the Tc cell response to TNP-modified self cells was crossreactivity on some TNP-modified allogeneic targets. Cloning experiments by Ching and Marbrook (personal communication) have established that genuinely crossreactive clones of Tc cells exist which recognize TNP-modified cells of both self and allogeneic type.

Forman and Klein (1977) have shown, and we have confirmed (unpublished data) that Tc cells which recognize cells bearing wild-type H-2k\(^b\) plus TNP crossreact on cells with mutant H-2k\(^ba\) plus TNP. This is a further example of H-2 restriction breaking down partially in TNP systems, and contrast strikingly with viral systems in which H-2k\(^b\) and H-2k\(^ba\) do not crossreact (Kees and Blanden 1976, Zinkernagel 1976).

The results discussed thus far suggest that the pools of Tc cells which respond to alloantigens and TNP-modified self overlap to some extent
i.e. same position of the alloreactive spectrum may crossreact with TNP-modified self. A similar situation has been suggested by Bevan (1977) for crossreactions between alloreactive Tc cells and those which recognize self H-2 plus minor histocompatibility antigens. However, thus far we have been unable to demonstrate any significant lysis of virus-infected targets (over and above uninfected controls) by Tc cells stimulated by alloantigens (Table I) or TNP-modified cells (Table IV), despite the use of many different viral groups.

In general it seems that crossreactions, particularly those involving alloreactive and TNP-reactive Tc cells have not been reproducible from one laboratory to another (Shearer et al 1975, Burakoff et al 1976). Our impression is that using more sensitive target cells such as macrophages or certain cell lines, rather than unstimulated lymphoid cells or blast cells, may reveal otherwise undetectable crossreactions. Cloning experiments should give a more definitive answer to the nature of crossreactive subsets in TNP-immune and alloreactive Tc cells (Skinner and Marbrook 1976, Fischer-Lindahl and Wilson 1977).

TNP-immune Tc cells were investigated further to determine whether some subsets could recognize target cells in which TNP was inserted into the cell membrane via a Sendai viral protein. No cross-lysis of TNP-immune Tc cells on TNP-Sendai-treated targets was observed. TNP-modified Sendai antigens were displayed on these target cells, however, since TNP-Sendai-immune Tc cells were able to lyse them.

These results are in agreement with those obtained by Rhen et al (1976) using TNP-β-alanylglycylglycyl (TNP-AGG) to induce immune Tc cells and to modify targets, but are in contrast to results of Schmitt-Verhulst et al (1978) using TNP-modified BSA and EGG. We have no explanation for this discrepancy.
The data from the TNP-AGG approach (Schmitt-Verhulst et al 1978) and the TNP-Sendai experiments reported here (Table III) suggest that the majority of Tc cells induced by self stimulator cells modified directly by TNP do not recognize self H-2 determinant(s) and TNP as separate antigenic determinants via a dual receptor system (Doherty et al 1976b, Langman 1978, Janeway et al 1976, Blanden and Ada 1978). Dual receptor models must invoke recognition of TNP plus carrier amino acids by one receptor, and H-2 by another receptor. Alternatively, most Tc cells stimulated by TNP-modified self cells may recognize TNP-altered self H-2 determinants via a single receptor. We favour this latter alternative because of the observed low specificity of TNP-reactive Tc cells for self H-2 determinants (Forman and Klein 1977, Burakoff et al 1976). In contrast, virus-immune Tc cell subsets which lyse virus-infected self cells are exquisitely specific for self H-2 determinants (Pang et al 1977, Blanden and Kees 1978, Kees and Blanden 1976, Blanden et al 1977, Zinkernagel 1976); these observations are more compatible with dual recognition (Langman 1978, Blanden and Ada 1978).

A major objective in this work was to analyse cross-reactions between virus-infected and TNP-modified cells. First, we could demonstrate a subset of virus-immune Tc cells which was able to lyse both virus-infected and TNP-modified syngeneic targets. Second, we and other workers (Starzinski-Powitz 1976) have established that this Tc cell subset is truly crossreactive by employing cold target competition. In such experiments TNP-modified unlabeled targets inhibited the lysis of labeled, virus-infected syngeneic targets in both the ectromelia and Sendai virus systems.

We observed this type of crossreactivity between infected and TNP-modified cells with every virus system we tested, which ranged from pox virus (ectromelia), paramyxovirus (Sendai) arenavirus (LCM), myxovirus (influenza) to alphavirus (Bebaru). These results suggest to us that the
phenomenon is ubiquitous, but we have no explanation for the failure of Biddison et al. (1977) to observe it with influenza or vaccinia virus-immune Tc cells. Starzinski-Powitz et al. (1976) on the other hand, did observe crossreactivity of vaccinia and LCM-immune Tc cells on syngeneic TNP-modified targets but did not see it with Sendai-immune Tc cells. None of the available data, however, gives any indication of the nature of the TNP-modified target structures which are responsible for the crossreactivity of the virus-immune Tc cells.

The results with crucial significance for the basis of the one-way crossreactivity of virus-immune Tc cells on TNP-modified targets were that crossreactivity which was so apparent in the expression of Tc cell effector function was not seen at the level of induction of secondary responses (Table V). Thus, when virus-primed memory spleen cells were cultured with TNP-modified stimulator cells, no effector Tc cell activity against virus-infected targets was generated; the only observable activity was directed against TNP-modified targets and its magnitude was similar to a primary in vitro anti-TNP response (line 5, Table V). However, memory cells capable of giving rise to crossreactive effector Tc cells which recognize both virus-infected and TNP-modified targets must have been present in the virus-primed populations, since after culturing with virus-infected stimulators, Tc cells which lysed TNP-modified targets were generated, and the magnitude of this crossreactive response was greater than that seen in primary antiviral responses. We conclude that the cause of the one-way crossreactivity phenomenon resides in a difference between the requirements for inducing a response in precursors of effector Tc cells (including memory cells) and the expression of effector function, with induction being the more demanding of the two, as reported elsewhere in a different context (Blanden et al. 1977b). Virus-infected stimulator cells would seem to fulfil the requirements for
inducing crossreactive Tc cell precursors, whilst TNP-modified cells do not.

The differences between precursor and effector Tc cells which form the basis of the one-way phenomenon described here are potentially complex. However, the findings that qualitative and/or quantitative differences in cell surface antigens correspond with differences in ability to stimulate a particular subset of precursor Tc cells (Blanden et al 1977b) has led us to consider various hypotheses involving quantitative and/or qualitative differences in the antigen-receptors or precursor versus effector Tc cells which are currently under investigation. If these differences can be more clearly defined, they may in turn shed light on the current controversy surrounding the "dual recognition" and "altered self" or "single receptor" models for Tc cell responses to modified cells.

SUMMARY

Crossreactivity of TNP-immune, virus-immune and alloreactive Tc cells was investigated at the level of target cell lysis. Alloreactive Tc cells crossreacted on TNP-modified and unmodified third-party targets and on syngeneic TNP-modified targets, but did not crossreact on syngeneic virus-infected targets. TNP-immune Tc cells showed marked crossreactivity on certain allogeneic targets modified by TNP (loss of H-2 restriction) and also on certain unmodified allogeneic targets, but did not cross-lyse virus-infected syngeneic targets. Targets treated with TNP-Sendai virus were not lysed by TNP-immune Tc cells, but Tc cells stimulated by cells treated with TNP-Sendai virus lysed such targets readily. These results are consistent with the view that Tc cell recognition of foreign H-2 antigens and TNP-modified self H-2 antigens are mechanistically similar (possibly via one receptor), whereas recognition of viral plus H-2 antigens is different
Virus-immune Tc cells ubiquitously exhibited strong crossreactivity on syngeneic TNP-modified targets using pox-, arena-, alpha-, myxo- and paramyxoviruses for Tc cell induction. The lysis of virus-infected targets by virus-immune Tc cells could be inhibited by cold TNP-modified competitors, thus establishing that some individual virus-immune Tc cells could recognize both types of target cells. This genuine crossreactivity at the effector level was not observed at the level of induction of secondary responses, since the crossreactive subset of virus-immune memory Tc cells could not be activated by TNP-modified stimulator cells, but could be activated by virus-infected stimulators. These results implied that requirements for stimulation of precursor Tc cells are sometimes different from antigenic requirements for recognition and lysis of effector Tc cells.
REFERENCES


### Table I

Cytolytic Activity of Alloreactive Tc Cells on Virus-infected and TNP-Modified Targets

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator</th>
<th>Uninfect.</th>
<th>TNP</th>
<th>Ectrom.</th>
<th>Influ.</th>
<th>Sendai</th>
<th>Bebaru</th>
<th>LCM</th>
<th>Polyoma</th>
<th>Adeno</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
<td>CBA (K(^k), D(^k))</td>
<td>8.0</td>
<td>NT</td>
<td>7.0</td>
<td>12.0</td>
<td>12.5</td>
<td>9.5</td>
<td>10.2</td>
<td>12.9</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>SJL (K(^s), D(^s))</td>
<td>14.7</td>
<td>NT</td>
<td>16.3</td>
<td>20.1</td>
<td>20.8</td>
<td>19.5</td>
<td>19.1</td>
<td>20.7</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td>DBA/1 (K(^q), D(^q))</td>
<td>24.0</td>
<td>NT</td>
<td>25.1</td>
<td>30.7</td>
<td>26.9</td>
<td>26.9</td>
<td>23.3</td>
<td>28.0</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>C57Bl/6</td>
<td>0</td>
<td>NT</td>
<td>1.2</td>
<td>0</td>
<td>2.1</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>% Specific Lysis of BALB/c Macrophage Targets(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
</tr>
<tr>
<td>CBA (K(^k), D(^k))</td>
</tr>
<tr>
<td>7.4</td>
</tr>
<tr>
<td>25.5</td>
</tr>
<tr>
<td>8.6</td>
</tr>
<tr>
<td>2.9</td>
</tr>
<tr>
<td>4.8</td>
</tr>
<tr>
<td>3.1</td>
</tr>
<tr>
<td>4.3</td>
</tr>
<tr>
<td>NT</td>
</tr>
<tr>
<td>NT</td>
</tr>
</tbody>
</table>

---

\(^a\) Percent \(^{51}\text{Cr}\) release from virus-infected, TNP-modified and uninfected targets over a 6 h period with spontaneous release subtracted. Spontaneous release for the P815 targets tested above was 6.7, 7.0, 6.2, 7.9, 6.8, 7.4, 6.3 and 7.1 respectively and for macrophage targets 23.0, 26.1, 27.4, 29.6, 20.8 respectively. Killer:target ratio was 2:1. Means of triplicates are given with S.E. of the mean never greater than 3.1%.

\(^b\) The infection of targets was tested by cytotoxic assays using secondary virus-immune Tc cells and the specific lysis was 48.2% for ectromelia, 62.8% for influenza JAP, and 58.3% for Sendai virus at a killer:target ratio of 2:1.

\(^c\) Infection was confirmed by virus-specific immunofluorescence.
Table II
Cytolytic Activity of TNP-Immune and Alloreactive Tc Cells on TNP-Modified and Unmodified Targets

<table>
<thead>
<tr>
<th>Responder Stimulator</th>
<th>CBA</th>
<th>CBA-TNP</th>
<th>BALB/c</th>
<th>BALB/c-TNP</th>
<th>C57BL/6</th>
<th>C57BL/6-TNP</th>
<th>A.TL</th>
<th>A.TL-TNP</th>
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</thead>
<tbody>
<tr>
<td>CBA (TNP-primed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA-TNP</td>
<td>11.1</td>
<td>95.0</td>
<td>17.3</td>
<td>8.5</td>
<td>5.8</td>
<td>27.1</td>
<td>31.7</td>
<td>45.8</td>
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<tr>
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<td>13.6</td>
<td>4.4</td>
<td>40.6</td>
<td>3.0</td>
<td>0.1</td>
<td>6.4</td>
<td>29.8</td>
</tr>
<tr>
<td>BALB/c-TNP</td>
<td>2.9</td>
<td>11.1</td>
<td>100.0</td>
<td>97.6</td>
<td>17.7</td>
<td>17.0</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>CBA</td>
<td>100.0</td>
<td>92.2</td>
<td>8.2</td>
<td>28.0</td>
<td>13.7</td>
<td>8.2</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

a Percent $^{51}$Cr release from TNP-modified or unmodified targets over a 6 h period with spontaneous release subtracted. Killer:target ratio was 2:1. Means of triplicates are given with S.E. of the mean never greater than 2.4%. Spontaneous release for the targets tested above, was 20.7, 24.4, 18.4, 21.9, 20.8, 20.1, 19.7 and 21.3 respectively.
Table III
Lysis of Target Cells Treated with TNP, Sendai Virus, or TNP-Modified Sendai Virus

<table>
<thead>
<tr>
<th>Secondary in vitro effectors (CBA, H-2k)</th>
<th>% Specific Lysis of L929 (H-2k) targets^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
</tr>
<tr>
<td>TNP</td>
<td>0.8</td>
</tr>
<tr>
<td>Sendai</td>
<td>4.8</td>
</tr>
<tr>
<td>TNP-Sendai</td>
<td>2.9</td>
</tr>
</tbody>
</table>

^a Percent 51Cr release over a 4 h assay period with spontaneous release subtracted.

Killer:target ratio was 3:1. Means of triplicates are given with S.E. of the mean never greater than 2.7%. Spontaneous release was 12.5, 11.8, 9.8 and 10.9 respectively.
<table>
<thead>
<tr>
<th>Secondary in vitro generated effector cells (CBA, H-2^k)</th>
<th>% Specific Lysis of CBA Macrophage Target Cells^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNP</td>
</tr>
<tr>
<td>I.</td>
<td></td>
</tr>
<tr>
<td>TNP</td>
<td>80.6</td>
</tr>
<tr>
<td>Sendai</td>
<td>41.5</td>
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<tr>
<td>Ectromelia</td>
<td>28.5</td>
</tr>
<tr>
<td>Influenza (A/JAP)</td>
<td>39.3</td>
</tr>
<tr>
<td>II.</td>
<td></td>
</tr>
<tr>
<td>TNP</td>
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<tr>
<td>Sendai</td>
<td>46.7</td>
</tr>
<tr>
<td>Ectromelia</td>
<td>33.3</td>
</tr>
<tr>
<td>Influenza (A/JAP)</td>
<td>49.2</td>
</tr>
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</table>

^a Percent ^51Cr release from virus-infected, TNP-modified or unmodified targets over a 6 h period with spontaneous release subtracted. Killer:target ratio was 2:1. Means of triplicates are given with S.E. of mean never larger than 1.8% (I) or 2.7% (II). Spontaneous release for macrophage targets was 10.6, 13.1, 22.5, 11.2, 14.3 respectively; for L929 targets, 9.8, 11.9, 9.9, 13.7 and 10.8 respectively.
### Table V

Cross-Stimulation of Viral and TNP-Immune Memory Cells

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Primary Stimulation</th>
<th>Secondary Stimulation</th>
<th>% Specific Lysis of CBA Macrophage Targets</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>TNP</td>
</tr>
<tr>
<td>1.</td>
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<td>55.9</td>
</tr>
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<td></td>
<td>None</td>
<td>Ectromelia</td>
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</tr>
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<td></td>
<td>TNP</td>
<td>TNP</td>
<td>79.5</td>
</tr>
<tr>
<td></td>
<td>TNP</td>
<td>Ectromelia</td>
<td>7.5</td>
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<td></td>
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<td>TNP</td>
<td>58.3</td>
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<tr>
<td></td>
<td>Ectromelia</td>
<td>Ectromelia</td>
<td>21.7</td>
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<tr>
<td>2.</td>
<td>None</td>
<td>None</td>
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<td></td>
<td>None</td>
<td>TNP</td>
<td>74.4</td>
</tr>
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<td></td>
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<td>Sendai</td>
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<td></td>
<td>TNP</td>
<td>TNP</td>
<td>89.7</td>
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<td></td>
<td>TNP</td>
<td>Sendai</td>
<td>18.7</td>
</tr>
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<td>Sendai</td>
<td>TNP</td>
<td>82.5</td>
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<tr>
<td></td>
<td>Sendai</td>
<td>Sendai</td>
<td>29.4</td>
</tr>
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</table>

*a* Primary stimulation was 3 wk prior to experiment.

*b* Percent $^{51}$Cr release over a 6 h period with spontaneous release and minor lysis of uninfected controls subtracted. Killer:target ratio was 2:1. Means of triplicate are given with S.E. of mean never larger than 2.4%. Spontaneous release for Exp. 1 was 12.8 and 16.6, for Exp. 2, 20.3 and 19.8 respectively.
FIG. 1 - Inhibition of cell-mediated cytotoxicity by unlabelled "cold" competitors. Secondary virus-immune Tc cells were tested for cytotoxicity on 2x10⁴ virus-infected ⁵¹Cr-labelled L929 targets in the presence of increasing numbers of unlabelled competitors either unmodified, TNP-modified or virus-infected. The ratio of effectors to targets is 2:1. Values for specific lysis are the means of triplicates with S.E. of the mean always less than 2.1%.
CHAPTER 3

SPECIFIC ADSORPTION OF H-2-RESTRICTED CYTOTOXIC T CELLS TO MACROPHAGE MONOLAYERS
INTRODUCTION

Cytotoxic T cells (Tc cells) generated in response to virus-infected or hapten-modified self cells exhibit H-2 restriction, i.e. they apparently recognize antigenic patterns dependent on self H-2K or H-2D antigents plus the foreign antigen (Doherty et al 1976a, Shearer et al 1976). Models to explain this phenomenon fall into two general categories. The first (dual recognition) states that an individual Tc cell expresses on its surface membrane two different types of antigen binding sites, one specific for a self H-2K or H-2D antigenic determinant, and the other specific for a foreign antigenic determinant (X). There are many points of detail which distinguish different versions of this model (Doherty et al 1976b, Janeway et al 1976, Blanden et al 1977, Langman 1977, Blanden and Ada 1978), but they all demand a paired set of binding sites (anti-H-2 plus anti-X) to be essential in triggering T cell function, whereas antigen binding to two anti-H-2 sites, or two anti-X sites is not operationally important. The second model (altered self) states that an individual Tc cell expresses only one type of antigen binding site which is specific for an antigenic pattern that is dependent upon an interaction or complex between a self H-2K or H-2D molecule and a foreign antigen. This antigenic pattern can be viewed as a "new antigenic determinant" caused by allosteric change in one or the other, or both, of the interacting antigenic components, but nonetheless dependent for its specificity on both components. Alternatively, it can be viewed as a "junction zone" antigenic determinant formed by a complex of the self H-2 and foreign molecules and containing a component from both of them. In this latter case however, the Tc cell binding site must have affinity only for the complex and not for each of the separate components or it becomes, by definition, a class of dual recognition.
Thus far, there is no conclusive evidence which excludes either model. However, the exquisite specificity of self H-2 recognition, as illustrated particularly by H-2K mutants (Blanden et al 1976, Kees and Blanden 1976, Zinkernagel 1976, McKenzie et al 1977), the wide variety of viral and other antigens which cause H-2 restricted responses (Doherty et al 1976a, Shearer et al 1976, Blanden et al 1977) and considerations of Tc cell ontogeny (Langman 1977, Zinkernagel et al 1978, Blanden and Ada 1978) impose severe strain on altered self models. On the other hand, cold target competition experiments are readily explained by altered self, but require qualification for dual recognition. The salient observations are as follows: Tc cell-mediated lysis of radio-labelled virus-infected target cells is specifically inhibited only by addition of virus-infected unlabelled competitors which have the same H-2 antigens as the labelled targets; H-2 compatible uninfected competitors, or infected allogeneic competitors are not specifically inhibitory. The precise mechanism of competition is unknown, but several possibilities may be considered. First, it may be simply a case of competition for Tc cell antigen-binding sites. If this be the case, the problem for dual recognition is that multipoint binding of Tc cells to either H-2 or X antigents, not necessarily both, should cause competition. Dual recognition can be salvaged, however, by postulating that cold competition operates through the inability of Tc cells to lyse more than one target cell simultaneously (Bevan et al 1976, Langman 1978). Thus, it could be that while many targets bind simultaneously to an individual Tc cell, only one is being lysed within a certain finite time period.

The present experiments address this problem by testing whether Tc cells specific for viral antigen plus self H-2 bind to macrophage monolayers displaying either viral antigens with allogeneic H-2, self H-2
but no viral antigen, or both viral and self H-2 antigens together. Specific binding could be demonstrated only in the last case, a result compatible with altered self and requiring further operational assumptions in dual recognition models.

MATERIALS AND METHODS

Mice

Mice were bred at the John Curtin School and were used at 6-8 weeks of age. In any one experiment, mice were of the same sex.

Viruses

Ectromelia virus stocks of both virulent (Moscow) and attenuated (Hampstead egg) strains were used. The methods of growing and titrating these strains have been described by Gardner et al (1974). Sendai and influenza virus strain A/JAP were grown and titrated as described by Doherty and Zinkernagel (1976) and Yap and Ada (1977) respectively.

Immunization

Mice were infected i.v. with attenuated ectromelia virus (Gardner et al 1974), influenza virus strain A/JAP (Yap and Ada 1977) and Sendai virus (Doherty and Zinkernagel 1976) as described previously. Spleen cells were used as a source of memory responder cells 3-5 weeks later.

Preparation of TNP (trinitrophenyl)-conjugated stimulator and target cells

The method used was basically similar to that reported by other workers (Shearer et al 1975, Forman 1975). Briefly, normal splenic lymphocytes or L929 fibroblasts were modified with 10mM TNBS (trinitrobenzenesulphonic acid) in phosphate-buffered saline (PBS) for 10 min at 37°C and thoroughly washed with PBS prior to and post-modification.

Generation of effector Tc cells in vitro

The method used was described for ectromelia virus by Gardner and
Blanden 1976). Briefly, spleen cells from mice previously immunized with virus (ectromelia, influenza or Sendai) were cultured with syngeneic splenic stimulator cells infected with the same virus as used for immunization. 2 PFU virulent ectromelia virus/cell was used for the infection of stimulator cells. The other stimulator cells were infected with 1-2 EID$_{50}$ units of Sendai virus or 2 EID$_{50}$ units of influenza virus strain A/JAP. A stimulator:responder ratio of 1:10 was used for all viral systems. Cultures were set up in tissue culture flasks (Falcon Plastics, 75 cm$^2$ growth area) at a final concentration of 2 x 10$^6$ responder cells/ml in 40 ml/flask. Usually 8 x 10$^7$ responder cells were cultured with 8 x 10$^6$ stimulator cells at 37°C in an atmosphere of 10% CO$_2$ for 5 days. (Cultures using ectromelia virus were incubated at 39°C, a non-permissive temperature for ectromelia replication, so preventing the virus from exerting cytopathic effects against responder cells). The complete culture medium was Eagle's minimal essential medium (GIBCO cat. no. F-15) with non-essential amino acids, 10% foetal calf serum, 10$^{-4}$M 2-mercaptoethanol and antibiotics.

The generation in vitro of secondary TNP-immune Tc cells was essentially as for virus-immune Tc cells. TNP-modified spleen cells (2 x 10$^7$ per culture) were used as stimulators with 8 x 10$^7$ responder cells from mice primed 2-12 weeks previously with TNP-modified syngeneic spleen cells. Immunization with TNP-modified spleen cells was obtained by subcutaneous injection of 4 x 10$^6$ TNP-modified spleen cells in 40 µl PBS into the hind footpad.

**Removal of dead cells**

After each harvest of responder cells from cultures, dead cells were removed by centrifuging through Ficoll-Isopaque as described by Parish et al (1974).
Adsorption of immune lymphocytes on macrophage monolayers

The method described by Brondz et al (1975) was modified as follows: Peritoneal macrophages were harvested 3 days after intraperitoneal injection of an irritant (4 ml Thioglycollate medium, DIFCO, Detroit, Michigan, USA). Almost confluent monolayers were prepared by seeding 7 x 10^6 peritoneal macrophages in 10 ml F-15 containing 10% FCS in a tissue culture flask (25 cm^2 growth area, Falcon Plastics) and incubating overnight at 37°C. Monolayers were rigorously washed with warm medium to remove all non-adherent cells; The cells remaining in the flask were then uniformly large, strongly adherent macrophages. They were then either left untreated, infected with virus, or modified with TNBS as described for target cells used in the cytotoxicity assay. Following washing, all medium was drained from the flasks and 2 ml of complete medium containing 10^7 lymphoid cells (including effector Tc cells) from the cultures described above were added to each flask. Adsorption was achieved by incubating at 30°C for 2.5 hrs in a horizontal position. The non-adherent cells were collected by removing the supernatant with a pipette without prior agitation. This procedure inevitably resulted in some loss of non-adherent cells in the small volume of medium remaining in the flask. There could also be a degree of nonspecific adherence and trapping as reported in several laboratories (Brondz et al 1975, Neefe and Sachs 1976, Geib et al 1978). However, this method avoided the problems of detachment of cells from the adsorbing monolayer which would then contaminate the effector cell suspension and act as cold competitors (Neefe and Sachs 1977). Because of their large size and morphology, Thioglycollate-induced macrophages which became detached could be easily identified during counting of the non-adherent cells harvested after the adsorption procedure. They were never more than 1.5% of the total non-adherent cell population, which
was then tested for effector activity on $^{51}$Cr labelled target cells. Since the maximum effector:target ratio used was 10:1, this level of contamination was never high enough for the macrophages to act as cold competitors.

**Cytotoxicity assay**

The methods used for L929 and macrophage targets have been described in detail by Gardner et al (1974). P815 cells were treated as L929 cells and were grown in Eagle's minimal essential medium (GIBCO, cat. no. H-16) containing 10% FCS. Target cells were infected with 2 EID$_{50}$ of Sendai or influenza virus strain A/JAP. Data given are the means of triplicates and have had spontaneous release subtracted for assay times of 6 hrs at 37°C. Standard errors of the means were always < 3% and are omitted for clarity. Significance was determined by Student's t test.

**RESULTS**

Specific depletion of Tc cells sensitized to TNP-modified or virus-infected cells

CBA/H effector cells generated against TNP-modified or influenza virus-infected syngeneic spleen cells were placed on a panel of monolayers of CBA/H or BALB/c macrophages, either TNP-modified, infected with influenza virus (JAP) or untreated (Table I). Specific adsorption occurred only when monolayers were homologous with the stimulator cells used in culture, i.e. CBA/H TNP-immune Tc cells were specifically adsorbed only on TNP-modified CBA/H monolayers. Further, CBA/H influenza-immune Tc cells adsorbed specifically on influenza virus-infected CBA/H monolayers while no reduction of cytotoxicity was observed after contact with TNP-modified or virus-infected BALB/c monolayers. To ensure that the BALB/c macrophages were capable of specific adsorption, alloreactive Tc cells generated in a MLR were tested on the same panel of monolayers. Adsorption of anti-
BALB/c Tc cells occurred on all BALB/c monolayers irrespective of infection by influenza virus or TNP modification.

These observations were extended to include BALB/c Tc cells. Influenza-immune Tc cells from BALB/c mice can be specifically adsorbed on the appropriate virus-infected monolayers (Table II).

**Virus-specificity of adsorption**

To test the specificity of adsorption of virus-immune Tc cells, Sendai and influenza viruses were used. Table III illustrates that adsorption is highly virus-specific. Both types of virus-immune Tc cells showed significantly reduced cytotoxicity only after contact with syngeneic monolayers infected with the same virus as used for immunization.

**DISCUSSION**

The present experiments showed that Tc cell subsets which lyse allogeneic or various virus-infected or TNP-modified self or H-2 compatible targets can be specifically depleted from cell suspensions by adsorption onto appropriate Thioglycollate-stimulated macrophage monolayers. Adsorption of alloreactive Tc cells has been reported previously by several groups (Kedar et al 1974, Brondz et al 1975, Neefe and Sachs 1976) using various types of adsorbing cell layers. In a single adsorption cycle, the method used here, it consistently gave 3-fold specific depletion of effector activity below the small amount of nonspecific depletion inherent in the procedure. Three-fold specific depletion was ample for the main purpose of this report, though a second cycle of adsorption can be used to reduce effector activity by 10-fold or more (unpublished data).

Our prime objective was to determine whether Tc cells which recognize antigenic patterns dependent on both self H-2 and a given foreign
antigen (X) could bind specifically to macrophage displaying (a) self H-2 alone: (b) X alone, or (c) self H-2 plus X together. The results clearly indicated that significant specific binding occurred only in case (c) i.e. where the adsorbing macrophages displayed both self H-2 and the X antigen used for immunization. This adsorption was specific for both H-2 and X antigens in the examples tested i.e. there was a clear discrimination between H-2^k and H-2^d (acting as "self markers") and between TNP, influenza (JAP) and Sendai viral antigens (acting as X). Since no specific adsorption of H-2 restricted Tc cells (specific for self H-2 plus X) could be detected on either H-2-bearing or on X-bearing macrophages, these results are consistent with "altered self" as defined in the Introduction. At face value they are not compatible with dual recognition models in which multiple copies of anti-H-2 and anti-X binding sites, each with measurable affinity for antigen, are displayed on Tc cell surface membranes and are thus available to participate in multipoint (high avidity) interaction with either self H-2 or with X antigenic determinants on adsorbing cell surface membranes.

However, the biophysical and genetic problems inherent in altered self models in the light of available information (see refs. Langman (197), Blanden and Ada (1978) for review of salient points) may justify formulation and testing of modifications of dual recognition models which can accommodate the present data. Such modifications necessarily invoke new assumptions concerning the expression and control of Tc cell receptors and/or the mechanisms of antigen-dependent triggering of lytic function as a consequence of receptor-antigen interaction.

SUMMARY

Adsorption on macrophage monolayers was used to determine the binding
specificity of cytotoxic T cells generated in response to virus-infected or hapten-modified self-cells. The results clearly indicate that significant specific binding occurred only in cases where the adsorbing macrophages displayed both viral (or hapten) and self H-2 antigens together. There was clear discrimination between H-2\(^k\) and H-2\(^d\) (acting as "self markers"), and between TNF, influenza (JAP) and Sendai viral antigens (acting as foreign antigen). These results are consistent with an "altered self" model as an explanation for H-2 restriction.
REFERENCES


Table I
Specific Adsorption of TNP-immune, virus-immune and 
Alloreactive Tc cells on macrophage monolayers\textsuperscript{a}

<table>
<thead>
<tr>
<th>Adsorption monolayers</th>
<th>CBA/H anti-CBA/H-TNP Tc cells\textsuperscript{b} on TNF-L929(H\textsuperscript{-2}k) targets</th>
<th>CBA/H anti-CBA/H-JAP Tc cells\textsuperscript{b} on JAP-L929(H\textsuperscript{-2}k) targets</th>
<th>CBA/H anti-BALB/c Tc cells\textsuperscript{b} on P815(H\textsuperscript{-2}d) targets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3:1</td>
<td>10:1</td>
<td>3:1</td>
</tr>
<tr>
<td>CBA/H (H\textsuperscript{-2}k)</td>
<td>46.1</td>
<td>70.6</td>
<td>26.1</td>
</tr>
<tr>
<td>CBA/H-TNP</td>
<td>12.0\textsuperscript{c}</td>
<td>30.1\textsuperscript{c}</td>
<td>34.8</td>
</tr>
<tr>
<td>CBA/H-JAP</td>
<td>49.8</td>
<td>88.0</td>
<td>12.8\textsuperscript{c}</td>
</tr>
<tr>
<td>BALB/c(H\textsuperscript{-2}d)</td>
<td>53.0</td>
<td>83.9</td>
<td>35.0</td>
</tr>
<tr>
<td>BALB/c-TNP</td>
<td>42.2</td>
<td>71.3</td>
<td>28.5</td>
</tr>
<tr>
<td>BALB/c-JAP</td>
<td>44.7</td>
<td>85.4</td>
<td>26.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data given are means of triplicates of \% specific \textsuperscript{51}Cr release at effector:target ratios of 3:1 and 10:1.

\textsuperscript{b} Effector Tc cells were generated \textit{in vitro} in secondary responses in the case of TNP and influenza virus (JAP) or in primary MLR.

\textsuperscript{c} Significantly less than appropriate controls at the same effector:target ratio (\(P < 0.001\)).
### Table II
Specific adsorption of BALB/c Influenza-JAP-Immune Tc Cells\textsuperscript{a}

<table>
<thead>
<tr>
<th>Adsorption monolayers</th>
<th>Effector:target ratio</th>
<th>% Specific (^{51})Cr release from P815 (H(^{-2d})) targets\textsuperscript{b}</th>
<th>JAP-infected</th>
<th>uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/H (H(^{-2k}))</td>
<td>3:1</td>
<td>49.9</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>77.1</td>
<td>0.1</td>
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<tr>
<td>BALB/c (H(^{-2d}))</td>
<td>3:1</td>
<td>50.5</td>
<td>0.5</td>
<td></td>
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<tr>
<td></td>
<td>10:1</td>
<td>83.4</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>BALB/c-JAP</td>
<td>3:1</td>
<td>11.3(\textsuperscript{c})</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>52.7(\textsuperscript{c})</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Effector cells were generated in secondary responses in vitro.

\textsuperscript{b} Data given are means of triplicates.

\textsuperscript{c} Significantly lower (P < 0.001) than all other groups on infected target cells tested at the same effector:target ratio.
### Table III

Virus-specific adsorption of CBA/H Tc cells stimulated by Sendai or Influenza (JAP) Viruses

<table>
<thead>
<tr>
<th>Adsorption monolayer</th>
<th>Sendai-immune Tc cells&lt;sup&gt;b&lt;/sup&gt; on Sendai-L929(H-2&lt;sup&gt;k&lt;/sup&gt;) targets</th>
<th>JAP-immune Tc cells&lt;sup&gt;b&lt;/sup&gt; on JAP-L929(H-2&lt;sup&gt;k&lt;/sup&gt;) targets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3:1</td>
<td>10:1</td>
</tr>
<tr>
<td>CBA/H (H-2&lt;sup&gt;k&lt;/sup&gt;)</td>
<td>15.9</td>
<td>38.2</td>
</tr>
<tr>
<td>CBA/H-JAP</td>
<td>17.3</td>
<td>36.6</td>
</tr>
<tr>
<td>CBA/H-Sendai</td>
<td>7.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BALB/c (H-2&lt;sup&gt;d&lt;/sup&gt;)</td>
<td>18.3</td>
<td>38.4</td>
</tr>
<tr>
<td>BALB/c-Sendai</td>
<td>14.7</td>
<td>31.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data given are means of triplicates of % specific <sup>51</sup>Cr release at effector:target ratios of 3:1 and 10:1.

<sup>b</sup> Effector Tc cells were generated in vitro in secondary responses.

<sup>c</sup> Significantly less lysis than all other groups on the same target at the same effector:target ratio (P < 0.001).
CHAPTER 4

Murine cytotoxic T-cell response to alphavirus is associated mainly with H-2D<sup>k</sup>
INTRODUCTION

Murine cytotoxic thymus-derived lymphocytes (Tc cells) reactive to virus-infected self cells, or to H-2 compatible cells bearing foreign minor histocompatibility (H) antigens, show dual specificity i.e., they apparently recognize antigenic patterns that are dependent upon both the foreign antigen(s) and self H-2 antigens (Doherty et al 1976, Bevan 1975, Gordon et al 1975). The H-2 antigens recognized by Tc cells are coded in the K and/or D regions of the H-2 complex. I region genes defined thus far are not directly relevant to Tc cell recognition, though they seem to be necessary for stimulation and function of a separate class of co-operating or helper T cells that facilitates the Tc cell response (Cantor and Boyse 1975, Erb and Feldman 1975, Pang et al 1976, Pang and Blanden 1977, Zinkernagel et al 1978a).

In the case of Tc cell responses of inbred female mice against the Y antigen of otherwise identical males, some mouse strains are responders and others are non-responders. Further, some F_1 hybrid females produced by mating two non-responder strains are responders to Y antigen. One possible explanation of this phenomenon was implicit in recent results of von Boehmer et al (1978). Their data suggested that one type of non-responder possessed Tc cell precursors specific for Y antigen plus H-2K or or H-2D antigens, but not T_H cell precursors specific for Y antigen plus H-2I antigens, and that another type of non-responder possessed T_H but not Tc cell precursors. An F_1 hybrid would thus inherit the potential to develop both classes of T cell precursors which are necessary for a detectable Tc cell response.

With most viral systems studied thus far, a Tc cell response is associated with the majority of H-2K and H-2D alleles carried by the available repertoire of inbred strains. Occasional alleles give a weak or
undetectable response with a particular virus, e.g. H-2K\textsuperscript{b} with Friend virus (Bubbers et al 1977) and H-2D\textsuperscript{k} with ectromelia (Blanden et al 1975) and vaccinia virus (Doherty et al 1978, Zinkernagel et al 1978b). In contrast to this general pattern, we report here on the Tc cell response to alphaviruses Bebaru, Sindbis and Semliki Forest, which are detectable almost exclusively in association with the H-2D\textsuperscript{k} allele. Unlike the case of Y antigen F\textsubscript{1} hybrids of non-responders (lacking H-2D\textsuperscript{k}) are also non-responders.

MATERIALS AND METHODS

Animals

All mouse strains were bred at the John Curtin School and used at 7-10 wk of age.

Viruses

Bebaru (BEB), Sindbis (SIN) and Semliki Forest Virus (SFV) were grown in infant mouse brains. Virus was recovered after sonication and differential centrifugation, and titrated on Vero cell monolayers as described previously (Hapel 1975). Ectromelia, influenza (JAP) and Sendai virus were grown and titrated as described elsewhere (Gardner et al 1974, Yap and Ada 1977, Doherty and Zinkernagel 1976).

Immunization

Immunizations with attenuated ectromelia virus (Gardner et al 1974), influenza strain A/JAP virus (Yap and Ada 1977) and Sendai virus (Doherty and Zinkernagel 1976) have been described previously. Immunization with alphavirus was usually done 2-3 wk prior to use in secondary responses in vitro with either 5x10\textsuperscript{5} PFU BEB or SIN intravenously (i.v.). SFV was inactivated by irradiation with 1x10\textsuperscript{6} rad from a 60Co source prior to use, and the equivalent of 5x10\textsuperscript{5} PFU SFV was injected i.v.
Memory cultures (in vitro secondary responses)

The generation of secondary Tc cells with ectromelia (Pang and Blanden 1976) influenza virus (Yap and Ada 1977) and Sendai virus (Mullbacher and Blanden, 1979) has been reported. Secondary immune Tc cells to alpha-virus were generated in vitro by culturing responder spleen cells (from mice immunized with BEB, SIN or SFV respectively 2-3 wk previously) with normal syngeneic "stimulator" spleen cells infected with 1-2 PFU of BEB, SIN or SFV per nucleated cell (2x10^7 viable cells/ml). The stimulator:responder ratio was 1:10. Usually 8x10^7 responder cells were co-cultured with 8x10^6 stimulator cells at 37°C in an atmosphere of 10% CO_2 in 40 ml of Eagle's minimal essential medium (GIBCO, Cat. no. 15) supplemented with 10% heat-inactivated foetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia), 10^{-4} M 2-mercaptoethanol and antibiotics. Cultures were harvested after 5 days.

Separation of T and B lymphocytes and removal of adherent cells

The procedure for separating T and B lymphocytes has been published in full (Parish et al 1974). The removal of adherent cells has been described in detail (Pang and Blanden 1976).

Serum treatment of secondary effectors

The methods used for treatment with anti-0 serum plus complement (Pang and Blanden 1976) and anti-Ly 1.1, anti-Ly 2.1 treatment (Pang et al 1976) have been fully described. The anti-Ly sera were a gift of Dr. I. F. C. McKenzie.

Cytotoxicity assay with macrophage target cells

The method has been described in detail elsewhere (Blanden et al 1976). The multiplicity of infection for ectromelia was 50 PFU/cell; influenza A/JAP, 1 EID_{50}/cell; Sendai, 1 EID_{50}/cell; BEB, SIN and SFV, 50 PFU/cell (2x10^6) macrophage/ml). The duration of infection for all viruses used was
1 h at 37°C followed by 2 washes with complete medium and an incubation time of 1-2 h at 37°C before addition of effector Tc cells. Data given have had spontaneous release subtracted and are the means of triplicates for assays run at 37°C for 6-16 h. S.E. of the means were usually less than ±3% and are omitted for clarity. Significance was determined by Student's t test.

RESULTS

Primary and secondary responses to alphaviruses

McFarland (1974) and Rodda and White (1976) have previously reported primary Tc cell responses to SIN and SFV respectively in BALB/c mice which reached a peak of 6 days post infection. In preliminary experiments with BALB/c, C57BL/6 and CBA/H mice of both sexes and BEB, SIN and SFV viruses, we could not obtain a reproducible primary response either in the spleen 6 days after i.v. infection or in lymph nodes or spleen 6 days after subcutaneous infection. At best we saw no more than 10% greater lysis of infected than uninfected control targets at killer:target ratios up to 45:1 for spleen and 100:1 for lymph node (data not shown).

We decided, therefore, to employ in vitro stimulation of memory cells from virus-primed mice to investigate further parameters of the response to alphaviruses, since this approach has proven to be an effective tool with several viruses (Gardner and Blanden 1976, Dunlop and Blanden 1976, Yap and Ada 1977, Mullbacher and Blanden 1979) and minor histocompatibility antigens (Bevan 1975), including detailed investigation of the basis for responder or non-responder status in the HY system (Gordon et al 1975). In characterisation of the system, we used BEB virus and CBA/H mice. A dose of 5x10^5 PFU i.v. was found to prime satisfactorily for a subsequent secondary response in vitro. Memory cells were present in the spleen within 2 wk of
virus inoculation and persisted without waning for at least 55 wk.

Fig. 1 shows the kinetics of a typical secondary response in vitro obtained by using as responders, spleen cells from CBA/H (H-2^k) mice immunized 3 wk previously with BEB virus and cultured with infected syngeneic spleen cells as the stimulators. Lytic activity against infected CBA/H macrophage target cells was then assayed at various times after culture. Lytic activity was first detected on day 3, reached a peak at day 5-6 and declined from then onwards.

**Nature of cytotoxic cells from secondary cultures**

Secondary in vitro generated BEB-immune cytotoxic cells from CBA/H mice were characterised in the following way: Cells were treated with anti-θ, anti-Ly 1.1 and anti-Ly 2.1 plus complement and cell separation into Ig^+ and Ig^- populations was carried out by a rosetting method (Parish et al 1974). The results (Table I) indicate that secondary BEB-immune cytotoxic cells show similar characteristics to other secondary virus-immune Tc cells. They are mainly θ^+ , Ly 2.1^+ , Ly 1.1^- and Ig^-.

**Virus specificity of secondary BEB-immune Tc cells**

CBA/H secondary immune Tc cells generated against BEB, ectromelia, influenza or Sendai virus were tested against CBA/H macrophage targets infected with the above listed viruses. The results (Table II) clearly show virus specificity in all cases.

**H-2 restriction of BEB-immune Tc cells**

Mice bearing five different haplotypes (H-2^k, H-2^d, H-2^b, H-2^q, H-2^s) were primed in vivo with BEB virus 3 wk prior to removal of their spleens. Cell suspensions were stimulated in vitro with syngeneic BEB-infected cells. The Tc cells thus produced were tested on syngeneic and allogeneic macrophage targets, either BEB-infected or uninfected (Table III). CBA/H immune Tc cells exerted high lytic activity on syngeneic infected targets with no
lysis on allogeneic (BALB/c) infected targets, the typical H-2 restriction phenomenon. On the other hand, BALB/c, C57BL/6, DBA/1 and SJL/J immune Tc cells did not lyse infected syngeneic targets significantly more than uninfected syngeneic targets, but there was significant lysis of syngeneic uninfected targets compared with allogeneic uninfected targets. Essentially the same pattern of response was obtained with SFV and SIN (data not shown), except in the case of SJL/J SIN-immune Tc cells, where we observed significantly more lysis of infected syngeneic targets than uninfected (42.4% versus 19.8% respectively). These data suggest that with respect to secondary Tc cell responses to the 3 alphaviruses tested, CBA/H is a high-responder strain and the other mouse strains are low-responders or non-responders.

Tests for virus-neutralizing antibody against BEB, SIN and SFV in mice of these five haplotypes indicated no significant strain differences (data not shown) and virus-specific immunofluorescence testing on infected macrophages of all strains showed viral antigen expression on all cell surfaces.

Region on the H-2\(^k\) gene complex associated with the Tc cell response to alphavirus

In vitro secondary alphavirus-immune Tc cells from CBA/H, B10.BR, A.TL and C3H.OH were tested on a panel of virus-infected macrophage targets (Table IV). Significant lysis occurred only in those combinations where both donors of effector Tc cells and infected targets possessed the \(\beta^k\) allele, e.g. CBA/H, B10.BR and C3H.OH effectors on CBA/H, B10.BR or C3H.OH targets. Neither I region homology nor K region homology was sufficient for lysis to occur.

The lysis of uninfected self targets consistently observed when low responder strains were used (Table III) was further investigated. Two interesting points emerged (Table V). First, though lysis of uninfected self targets was seen, its generation required infected self cells (not uninfected) as the stimulus for memory spleen cells.
Second, we could tentatively map a requirement for this phenomenon to the D end of the H-2 complex in the BALB/c and DBA/1 strains tested. Thus, BALB/c (K^d,D^d) effectors lysed A.QR (K^q,D^q) targets as much as BALB/c targets, but lysed C3H.OH (K^d,D^d) targets significantly less; DBA/1 (K^q,D^q) effectors lysed DBA/1 targets significantly more than A.QR (K^q,D^q) targets.

The Tc cell secondary response to BEB virus in F_1 hybrids

A number of hybrid (F_1) mice were tested for their ability to mount a secondary response to BEB virus. F_1 mice had either one low- and one high-responder parent (lines 1-3) or two low-non-responder parents (lines 4-8). All the effectors were tested on macrophage targets from F_1 mice homologous to the F_1 source of effector cells and also on an appropriate selection from a panel of targets which covered the repertoire of H-2 haplotypes under test (Table VI). Only F_1 hybrids with a CBA/H (H-2^k) parent gave high specific lysis of infected H-2-compatible targets. The failure of F_1 hybrids containing the A/J and A.TL haplotypes to respond confirmed that the D^k region was essential for a high response and that the K-end and I region of the H-2^k haplotype were insufficient. All F_1 hybrids of two low-responder parents gave 20-30% lysis of uninfected self targets in this experiment (as might be expected from Tables III and V).

DISCUSSION

Results in this report clearly establish that strong secondary Tc cell responses to alphaviruses can be produced in vitro, even though in our hands primary Tc responses in vivo are weak and hard to detect. The characterisation of the cells involved (8^+, Ly 1.1^-, Ly 2.1^+, Ig^-) clearly indicated similarity to other virus-immune Tc cells, or Tc cells generated in response to virus infection, as might be expected from Tables III and V).
to minor H antigens, including HY, or chemically-modified self cells.

The most striking result with this virus system was the clear division of mouse strains into high-responder or low-responder categories. Mice with the H-2\(^k\) haplotype were high-responders, but genetic background seemed unimportant since CBA/H and B10. BR (both H-2\(^k\)) responded similarly. Other mouse strains tested bearing the H-2\(^b\), H-2\(^d\), H-2\(^q\) and H-2\(^s\) haplotypes were low-responders. The critical part of the H-2\(^k\) haplotype was the H-2D region, since C3H.OH (K\(^d\), I\(^d\), D\(^k\)) were high responders, while the I\(^k\) region seemed unimportant as further indicated by A.TL mice (K\(^s\), I\(^k\), D\(^d\)) being low responders. The results are against the idea that T helper cells, requiring certain I region genes for their induction or function, are lacking in low-responders. This conclusion is reinforced by the findings that in F\(_1\) hybrids between high-responder CBA/H (H-2\(^k\)) mice and mice with low-responder haplotypes (H-2\(^b\) and H-2\(^d\)) were high responders, but only virus-specific Tc cells which lysed infected H-2\(^k\) targets, not infected H-2\(^d\) or H-2\(^b\) targets were generated. F\(_1\) hybrids between two low responder strains were always low responders with respect to Tc cells. Low responder cells could be infected as well as high responder cells and displayed alphavirus antigens on their surfaces as detected in immunofluorescence tests. Finally, low responder strains produced similar virus-neutralizing antibody responses to high responders; these responses are likely to be T cell dependent (Burns 1975), though we did not formally investigate this point.

In sum, these results suggest that only the H-2D\(^k\) gene product plus alphavirus coded antigen(s) produce an immunogenic association on infected cell surface membranes, i.e., that the defect in low-responders is simply a lack of appropriate H-2K or H-2D gene product on cell surfaces. If this is true, then Tc cells of a low-responder genotype should be converted to high-responder phenotype to alphavirus if allowed to develop in a thymic environ-
ment of high-responder type (Zinkernagel et al. 1978c, von Boehmer et al. 1978), and if stimulated by alphavirus-infected cells of high-responder type. We are currently testing this prediction using irradiation chimeras. It is also of interest that preliminary experiments (not presented here) show that BEB virus grown in C3H.OH (K^d, L^k) brain can be weakly neutralized by anti-H-2^k serum, but not other anti-H-2 sera. These data point to the tentative conclusion that H-2D^k molecules may be present in the virion envelope and indicate that it may be worth investigating if low-responder H-2K or H-2D molecules are selectively excluded from the envelope, or vice-versa, if H-2D^k is selectively included.

The present results are generally similar to findings with murine oncorna viruses (Gomard et al. 1977, Bubbers et al. 1977) in which it also seems likely that only certain H-2K or H-2D molecules can associate with viral proteins in a manner immunogenic for Tc cells. These viral systems seem to be less complicated examples of a low-responder defect than the HY system in which I region genes and T helper cells have been implicated (Simpson and Gordon 1977, von Boehmer et al. 1977, Hurme et al. 1977).

One other intriguing observation was the lysis of uninfected self target cells caused by spleen cells of primed low responder mice after secondary stimulation in vitro with infected but not uninfected self cells. This failure of uninfected stimulators to provoke the response, plus indications of the same phenomena during primary responses to infection in vivo (Rodda and White 1976) make it unlikely to be due to a response to FCS antigens (Schmitt-Verhulst et al. 1978, Forni and Green 1976). Though we have not characterised the effector cell responsible, it seems unlikely to be a natural killer cell or macrophage because of its preoccupation with antigens dependent on the H-2D region, at least in the H-2^d and H-2^q haplotypes investigated here. Further work on this phenomenon seems
warranted, since it could be an example of an autoimmune reaction provoked by viral infection, and under H-2 linked control.

SUMMARY

A secondary in vitro response to alphaviruses Bebaru, Sindbis and Semliki Forest virus is described. The optimum of the response appears at day 5-6 of culture. The cells responsible for lytic activity are non-adherent, ε-positive, Ig- and mainly Ly-2.1 positive. Out of five haplotypes tested (H-2d, H-2b, H-2s, H-2q, H-2k) only H-2k was a responder. Genetic mapping of the response located it solely in the D region of the H-2 complex. The other four haplotypes responded with a high anti-self activity after a second stimulation with viruses. This anti-self response also maps in the D region of the H-2 complex. No complementation was observed in F1 hybrids between responder and non-responder strains.
REFERENCES


### Table I
Characterization of CBA/H secondary in vitro BEB-immune cytotoxic cells

<table>
<thead>
<tr>
<th>Treatment of Effectors</th>
<th>% specific lysis&lt;sup&gt;a&lt;/sup&gt; ±S.E.</th>
<th>% reduction in specific lysis</th>
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</thead>
<tbody>
<tr>
<td><strong>Exp. I</strong></td>
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<tr>
<td>None</td>
<td>63.8 ± 1.7</td>
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</tr>
<tr>
<td>Complement (C')</td>
<td>60.5 ± 2.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Anti-Ø serum</td>
<td>61.5 ± 2.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Anti-Ø + C'</td>
<td>7.2 ± 0.9</td>
<td>88.7</td>
</tr>
<tr>
<td>Anti-Ly 1.1 + C'</td>
<td>49.9 ± 0.3</td>
<td>21.8</td>
</tr>
<tr>
<td>Anti-Ly 2.1 + C'</td>
<td>9.5 ± 1.2</td>
<td>85.1</td>
</tr>
<tr>
<td><strong>Exp. II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>49.7 ± 2.4</td>
<td>0</td>
</tr>
<tr>
<td>Ig&lt;sup&gt;+&lt;/sup&gt; fraction</td>
<td>3.6 ± 1.1</td>
<td>92.8</td>
</tr>
<tr>
<td>Ig&lt;sup&gt;−&lt;/sup&gt; fraction</td>
<td>44.9 ± 0.7</td>
<td>9.6</td>
</tr>
<tr>
<td>Ig&lt;sup&gt;−&lt;/sup&gt; + Ig&lt;sup&gt;+&lt;/sup&gt; recombined</td>
<td>46.0 ± 0.5</td>
<td>7.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent <sup>51</sup>Cr release from infected macrophage targets over a 6 h period with spontaneous release subtracted. Killer to target cell ratio was 3:1. Release from uninfected control targets was not significant (p < 0.001).
## Table II

**Virus specificity of secondary virus-immune Tc cells**

<table>
<thead>
<tr>
<th>CBA/H secondary virus-immune effectors</th>
<th>% specific lysis of CBA/H macrophage targets&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
</tr>
<tr>
<td>BEB</td>
<td>0.4</td>
</tr>
<tr>
<td>Ectromelia</td>
<td>0</td>
</tr>
<tr>
<td>Influenza (JAP)</td>
<td>0.3</td>
</tr>
<tr>
<td>Sendai</td>
<td>5.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent $^{51}$Cr release from uninfected and infected targets over a 6 h period with spontaneous release subtracted. Killer to target cell ratio was 2:1. Means of triplicates given with S.E. of mean never greater than 1.4%.
### Table III

**H-2 restriction of BEB-immune Tc cells**

<table>
<thead>
<tr>
<th>Secondary in vitro effectors</th>
<th>Macrophage targets</th>
<th>% specific $^{51}$Cr release&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BEB-infected</td>
</tr>
<tr>
<td>CBA/H ($H^{-2k}$)</td>
<td>CBA/H</td>
<td>99.7</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>5.5</td>
</tr>
<tr>
<td>BALB/c ($H^{-2d}$)</td>
<td>BALB/c</td>
<td>45.7</td>
</tr>
<tr>
<td></td>
<td>CBA/H</td>
<td>4.3</td>
</tr>
<tr>
<td>C57Bl/6 ($H^{-2b}$)</td>
<td>C57Bl/6</td>
<td>43.6</td>
</tr>
<tr>
<td></td>
<td>CBA/H</td>
<td>7.7</td>
</tr>
<tr>
<td>DBA/1 ($H^{-2q}$)</td>
<td>DBA/1</td>
<td>32.5</td>
</tr>
<tr>
<td></td>
<td>CBA/H</td>
<td>6.4</td>
</tr>
<tr>
<td>SJL/J ($H^{-2s}$)</td>
<td>SJL/J</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>CBA/H</td>
<td>NT</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent $^{51}$Cr release of targets over a 6 h period with spontaneous release subtracted. Killer to target cell ratio was 3:1. Means of triplicates given with S.E. of mean never greater than 2.2%.
Table IV
Genetic mapping of alphavirus specific Tc cell response

<table>
<thead>
<tr>
<th>Effectors</th>
<th>CBA/H</th>
<th>BIO.BR</th>
<th>C3H.OH</th>
<th>A.TL</th>
<th>BIO.A(4R)</th>
<th>BALB/c</th>
<th>C57/BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kkkka</td>
<td>kkkkk</td>
<td>ddddk</td>
<td>sddkd</td>
<td>kkbbb</td>
<td>ddddd</td>
<td>bbbbb</td>
</tr>
<tr>
<td>Exp. I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA/H-BEB</td>
<td>59.6</td>
<td>44.9</td>
<td>54.2</td>
<td>0.2</td>
<td>8.4</td>
<td>8.7</td>
<td>NT</td>
</tr>
<tr>
<td>BIO.BR-BEB</td>
<td>40.5</td>
<td>49.5</td>
<td>55.0</td>
<td>4.8</td>
<td>7.0</td>
<td>NT</td>
<td>4.4</td>
</tr>
<tr>
<td>A.TL-BEB</td>
<td>3.8</td>
<td>NT</td>
<td>5.5</td>
<td>15.7</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>C3H.OH-BEB</td>
<td>62.8</td>
<td>NT</td>
<td>58.6</td>
<td>NT</td>
<td>4.7</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Exp. II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA/H-SIN</td>
<td>70.4</td>
<td>NT</td>
<td>58.9</td>
<td>1.6</td>
<td>5.5</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Exp. III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBA/H-SFV</td>
<td>48.8</td>
<td>NT</td>
<td>38.1</td>
<td>7.4</td>
<td>5.9</td>
<td>1.1</td>
<td>NT</td>
</tr>
</tbody>
</table>

a  H-2 map corresponding to K, IA, IR, IC and D region.
b  Secondary in vitro generated virus-immune Tc cells.
c  Percent $^{51}$Cr release of targets over a 6 h period with spontaneous release subtracted.
Killer to target cell ratio was 2:1. Means of triplicates given with S.E. of mean never greater than 2.2%. Lysis of uninfected targets was not significant, except in the case of A.TL-BEB effectors on A.TL targets which gave specific lysis of 14.8%. 
Table V
Requirements for lysis of uninfected self targets
induced by secondary stimulation with BEB virus

<table>
<thead>
<tr>
<th>Memory responder</th>
<th>Secondary stimulation</th>
<th>% specific lysis of macrophage targets&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BALB/c&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>BALB/c-BEB</td>
<td>BALB/c-BEB</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td>BALB/c-BEB</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>BALB/c-BEB</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>DBA/1-BEB</td>
<td>9.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent 51 Cr release of targets over a 6 h period with spontaneous release subtracted. Killer to target ratio was 9:1. Means of triplicates given with S.E. of mean never greater than 1.8%.

<sup>b</sup> Memory spleen primed 2 wk prior to experiment.

<sup>c</sup> Secondary stimulation with infected or uninfected syngeneic spleen cells in vitro.

<sup>d</sup> H-2 map corresponding to K, IA, IB, IC, and D region.

<sup>e</sup> Significantly less (p < 0.01) specific lysis than on target cells in which D region shared.
Table VI
Secondary T<sub>c</sub> cell response of F<sub>1</sub> mice to BEB virus

<table>
<thead>
<tr>
<th>Secondary BEB-effectors</th>
<th>Responder haplotype&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% specific lysis of BEB-infected macrophage targets&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>CBA (kkkkk)</td>
</tr>
<tr>
<td>(CBA/H x BALB/c)&lt;sub&gt;F1&lt;/sub&gt;</td>
<td>(kkkkk/ddddd)</td>
<td>41.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(CBA/H x C57Bl/6)&lt;sub&gt;F1&lt;/sub&gt;</td>
<td>(kkkkk/bbbbb)</td>
<td>59.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(CBA/H x BIO.D2)&lt;sub&gt;F1&lt;/sub&gt;</td>
<td>(kkkkk/ddddd)</td>
<td>59.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(BALB/c x C57Bl/6)&lt;sub&gt;F1&lt;/sub&gt;</td>
<td>(ddddd/bbbbb)</td>
<td>7.2</td>
</tr>
<tr>
<td>(BIO.A(5R) x ATL)&lt;sub&gt;F1&lt;/sub&gt;</td>
<td>(bbbbbb/kkkkd)</td>
<td>7.4</td>
</tr>
<tr>
<td>(BIO.A(3R) x DBA/2)&lt;sub&gt;F1&lt;/sub&gt;</td>
<td>(bbbbdd/ddddd)</td>
<td>3.1</td>
</tr>
<tr>
<td>(A/J x C57Bl/6)&lt;sub&gt;F1&lt;/sub&gt;</td>
<td>(kkkdd/bbbbb)</td>
<td>2.8</td>
</tr>
<tr>
<td>(C57Bl/6 x BIO.D2)&lt;sub&gt;F1&lt;/sub&gt;</td>
<td>(bbbbbb/ddddd)</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent <sup>51</sup>Cr release over a 6 h period with spontaneous release and lysis of uninfected targets subtracted for clarity. Killer to target cell ratio was 9:1. Means of triplicates are given with S.E. of mean never larger than 2.1%.

<sup>b</sup> Refers to derivation of K, IA, IB, IC and D respectively.

<sup>c</sup> F<sub>1</sub> targets were always homologous to the secondary effectors used.

<sup>d</sup> Significantly more (<i>p < 0.001</i>) specific lysis than control uninfected targets.

<sup>e</sup> NT = not tested.
FIG. 1 - Kinetics of generation of cytotoxic cells from BEE-primed CBA/H spleen precursors cultured with infected syngeneic stimulator spleen cells and assayed for cytotoxic activity on BEE-infected syngeneic macrophages at various days after culture. Values are mean of 3 wells, with standard error being less than ± 2.1%. Killer to target ratio was 3:1.
CHAPTER 5

H-2-LINKED CONTROL OF CYTOTOXIC T CELL
RESPONSIVENESS TO ALPHAVIRUS INFECTION.

PRESENCE OF H-2D<sup>k</sup> DURING DIFFERENTIATION AND
STIMULATION CONVERTS STEM CELLS OF LOW RESPONDER
GENOTYPE TO T CELLS OF RESPONDER PHENOTYPE.
INTRODUCTION

The alphaviruses are a globally distributed viral genus; they are transmitted by insect vectors (Andrewes and Pereira 1972) and in appropriate animal hosts they cause a variety of disease syndromes, frequently involving the central nervous system (Fenner and White 1970, Baker 1974). In recent studies of the murine cytotoxic T cell (Tc cell) response to three different alphaviruses, Sindbis (SIN), Semliki Forest (SFV) and Bebaru (BEB), we found that only mice carrying the D^k region of the H-2 gene complex were high responders (k and o haplotypes) whereas other haplotypes (d, s, q, b) were low responders (Chapter 4).

Two general classes of low or non-responder with respect to H-2 restricted Tc cell responses have been reported thus far. One class is characterised simply by an apparent inability of certain self H-2K or H-2D molecules to associate with a particular foreign antigen (X) in a manner immunogenic for self (Chapter 4, Blanden et al 1977, Gomard et al 1977, Hurme et al 1977). The other class is more complicated in that genes elsewhere in the H-2 complex influence in some way the immunogenicity of a particular combination of self H-2K or H-2D plus X (Simpson and Gordon 1977, von Boehrmer et al 1977, Doherty et al 1978, Zinkernagel et al 1978a,b). We report here evidence that low responders to alphavirus infection may be examples of the former, simple class of low responder outlined above.

MATERIALS AND METHODS

Animals

Mice were bred at the Curtin School and used at 5 to 11 wk of age.

Viruses and immunization

Sindbis (SIN) and Bebaru (BEB) viruses were grown and titrated as described before (Mullbacher and Blanden 1979). Mice were immunized i.p.
with either $5 \times 10^5$ plaque-forming units (PFU) of SIN or BEB.

**In vitro secondary cultures (memory culture)**

The method has been described in detail elsewhere (Mullbacher and Blanden 1979). In brief, $8 \times 10^7$ spleen cells of mice primed with SIN or BEB 2 to 10 wk previously were cultured with $8 \times 10^6$ syngeneic SIN or BEB-infected stimulator spleen cells (1-2PFU cell at $2 \times 10^7$ cells/ml) for 5 days at $37^\circ C$.

**Cytotoxicity assay with macrophage target cells**

The method has been described in detail elsewhere (Blanden et al 1976). The multiplicity of infection was 50 PFU/cell SIN or BEB ($2 \times 10^6$ macrophages/ml). Data given have had spontaneous release subtracted and are the means of triplicates for assays run at $37^\circ C$ for 6 hr. S.E. of the means were less than ±5% and are omitted for clarity. Significance was determined by Student's t test.

**Preparation of chimeras**

C3H.OH mice were irradiated with 950 rads from a $^{90}$Co source 24 h prior to reconstitution i.v. with $4 \times 10^7$ BALB/c foetal liver cells (14-15 day embryos). Chimeric mice were primed with either BEB or SIN 5-9 wk post-reconstitution as described above.

**RESULTS**

**Tc cell response to SIN is restricted to the $D^k$ region of H-2**

In our initial studies (Mullbacher and Blanden 1979), out of 5 mouse strains tested (CBA/H, BALB/c, SJL/J, C57Bl/6 and DBA/1) only CBA/H mice gave a significant secondary Tc cell response to SIN. On mapping of the response, an absolute requirement for $D^k$ in effector and target cells became apparent. Results showing a $D^k$ requirement at the target cell level for lysis to occur are presented in Table I. CBA/H, C3H.OH and (CBA/H x BALB/c) $F_1$ hybrids gave high specific lysis of CBA/H, C3H.OH or $F_1$
SIN-infected targets. No lysis of infected targets was associated with $K^k$ as shown by B10.A(4R). BALB/c, a low responder strain to SIN and other alphaviruses, did give lysis of BALB/c and $F_1$ targets, but uninfected targets were lysed as much as infected targets, a result discussed in detail elsewhere (Mullbacher and Blanden 1979).

There was a lack of complementation in $F_1$ hybrids between high and low responder, even though responsiveness was dominant. Thus (CBA/H x BALB/c) $F_1$ Tc cells only lysed targets which carried the $D^k$ region, and did not lyse BALB/c targets.

Stem cells of low responder genotype can give rise to Tc cells which recognize SIN, if allowed to differentiate in high responder, irradiated recipients. BALB/c foetal liver cells were allowed to differentiate in the thymus of irradiated C3H.OH mice. Splenic Tc cells taken 9 wk after reconstitution and 2 wk after priming with SIN were boosted in vitro by coculturing with SIN-infected C3H.OH spleen stimulator cells inactivated with 2000 rad. The responder cells were tested by complement-mediated lysis with the appropriate anti-H-2 sera and found to be > 95% of $D^d$ (i.e. BALB/c) phenotype. Table II shows the results of such an experiment in which Tc cells of BALB/c origin lysed SIN-infected targets bearing $D^k$ antigen, provided $D^k$ was present during differentiation and stimulation of the Tc cells. A similar result was obtained using BEB virus instead of SIN in the same experimental protocol (data not shown).

DISCUSSION

In these experiments, we converted Tc cells of BALB/c origin (low responder genotype) to responder phenotype, by allowing them to differentiate in a responder thymus, and then stimulating them with alphavirus infected responder-type cells. This is a similar observation in principle to that
reported for the vaccinia system by Zinkernagel et al (1978a). Paradoxically, however, H-2Dk, which is the only K or D region out of the 10 tested thus far which gives a high response to alphaviruses (Mullbacher and Blanden 1979), is associated with low responsiveness to ectromelia (Blanden et al 1975), vaccinia and Sendai viruses (Doherty et al 1978, Zinkernagel 1978b). Our results, and those of others (von Boehmer et al 1978, Doherty et al 1978, Zinkernagel et al 1978a, Matsunaga and Simpson 1978), exclude the possibility that low- or non-responder genotypes are absolutely unable to produce Tc cells which can recognize or respond to particular viral antigens. They emphasize the point that the phenotype (low or high responder) is dependent upon the effects of a particular H-2K or H-2D region gene product either at the level of T cell ontogeny (von Boehmer et al 1978, Zinkernagel et al 1978, Matsunaga and Simpson 1978) and/or at the level of stimulation of the response during viral infection (Doherty et al 1978).

The latter of these two possibilities is the simplest. It implies that in low responders there may be a failure of self H-2K or H-2D molecules to associate (or form a complex) with an alphavirus antigen molecule in the same infected cell membrane; thus, there would be no antigenic moiety available to stimulate alphavirus-specific H-2-restricted Tc cells. Recent evidence shows that H-2 antigens bind to alphavirus particles (Fries et al 1978), i.e. H-2 and viral molecules can form complexes when present in different membranes, but this may not be relevant to their relationship when adjacent to each other in the same membrane, since the orientations of the molecules in these two cases would be different.

The other possibility is that even if H-2K or H-2D and alphavirus antigens can form an appropriate complex the Tc cell repertoire lacks antigen-receptor(s) which recognize this complex. von Boehmer et al (1978)
have recently suggested an explicit dual recognition hypothesis to account for this: their hypothesis states that a Tc cell uses two receptors to recognize H-2K (or H-2D) plus foreign antigen (X), that the gene coding for the anti-X variable (V) region is derived by mutation from the germ line anti-H-2 V region gene expressed by the same Tc cell, and that a particular anti-H-2 V gene gives rise to a limited repertoire of mutants, and thus may not generate a mutant V gene appropriate for a certain X.

Our results are compatible with both of the possibilities outlined above i.e. either H-2Dk is the only H-2 antigen of those tested able to form a complex with alphavirus-coded protein(s), or an anti-H-2Dk V gene is the only V gene which mutates to give an anti-alphavirus V gene. At present we cannot resolve between them.
REFERENCES


<table>
<thead>
<tr>
<th>Secondary Representative T&lt;sub&gt;c&lt;/sub&gt; Cells</th>
<th>Killer: Target Ratio</th>
<th>% Specific Lysis of Macrophage Targets&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/H</td>
<td>3:1</td>
<td>SIN Normal SIN Normal SIN Normal SIN Normal SIN Normal</td>
</tr>
<tr>
<td></td>
<td>42.2 0</td>
<td>7.9 5.2 39.2 7.2 9.1 6.2 40.4 11.1</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>20.6 0</td>
</tr>
<tr>
<td>BALB/c</td>
<td>3:1</td>
<td>8.6 9.2 28.3 25.3 8.3 8.1 N.T. N.T. 26.4 25.6</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>5.3 2.1 10.8 14.3 4.2 3.7 N.T. N.T. 12.7 14.4</td>
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<tr>
<td>C3H.OH</td>
<td>3:1</td>
<td>46.6 7.9 10.2 8.1 47.0 12.2 N.T. N.T. 40.6 13.7</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>19.5 4.9 5.3 5.1 18.0 0.1 N.T. N.T. 28.7 0</td>
</tr>
<tr>
<td>(CBA/HxBALB/c)</td>
<td>3:1</td>
<td>53.5 6.3 22.5 17.7 49.2 7.9 N.T. N.T. 49.9 11.3</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>25.9 3.4 10.2 9.1 29.8 3.7 N.T. N.T. 27.8 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent <sup>51</sup>Cr release of targets over a 6 h period with spontaneous release subtracted (<20%). Means of triplicate given with S.E. of mean never greater than 3.1%.

<sup>b</sup> Secondary responses generated in vitro 2 wk after priming as described in Materials and Methods.

<sup>c</sup> H-2 map corresponding to K, IA, IB, IC, and D region.

<sup>d</sup> Not tested.
### Table II
Development of responder capability in Tc cells of BALB/c low responder origin in irradiated C3H.OH high responder mice

<table>
<thead>
<tr>
<th>Secondary Tc cells</th>
<th>Killer: target ratio</th>
<th>% Specific Lysis of Macrophage Targets&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CBA/H SIN</td>
</tr>
<tr>
<td>C3H.OH (ddddk)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9:1</td>
<td>75.8</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
<td>68.3</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>50.4</td>
</tr>
<tr>
<td>BALB/c (dddddd)</td>
<td>9:1</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>7.4</td>
</tr>
<tr>
<td>(BALB/c + C3H.OH CHIMERA)&lt;sup&gt;d&lt;/sup&gt; (dddd)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9:1</td>
<td>45.1</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>25.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percent 51Cr release of targets over a 6 h period with spontaneous release subtracted (< 20%). Means of triplicates given with S.E. of mean never greater than 4.7%.

<sup>b</sup> Secondary responses generated <i>in vitro</i> 2 wk after priming as described in Materials and Methods.

<sup>c</sup> H-2 map corresponding to K, IA, IB, IC, and D region.

<sup>d</sup> 7 wk post-reconstitution chimera; phenotype of spleen cells tested by complement mediated lysis with anti-H-2D<sup>d</sup> serum to be > 95% of BALB/c phenotype.
CHAPTER 6

CROSSREACTIVE CYTOTOXIC T CELLS TO ALPHAVIRUS INFECTION

The alphaviruses are a widespread viral genus, transmitted by arthropods that cause several encephalitides in various host animals, including man (Arrighi et al. 1974). In the past, study of the immune response of infected animals has focused mainly on natural reactions, and classification of alphaviruses into complements, types and subtypes relied substantially on serological tests such as virus neutralization (NDV) and hemagglutination inhibition (HI) tests (Casals 1957).

Protection from alphavirus infection has been demonstrated after immunization with a distinctly related virus, as defined by NDV and HI tests (Parkes and Price 1957, Frye et al. 1974, Ferguson et al. 1975), and such crossprotection has been tentatively ascribed to T cells in experiments using infected mice (Peck et al. 1975). Protection against alphavirus-induced meningoencephalitis by T cells has also been documented (Gürgel 1975).

The concept of cross-protection by T cells between serologically distinct viruses is currently attracting much attention in the influenza virus system (Yao and Ma 1977, Flach and Werner 1972). Crossreactivity in vivo and in vitro by subpopulations of influenza-virus cytotoxic T cells (Tc cells) is thought to reside in an antigen molecule (matrix protein) which is shared by otherwise serologically distinct subtypes, and is expressed on membranes of infected cells but not on the virus envelope (Ogata 1977, Frye et al. 1977, Riddler et al. 1977, Brian 1977c). Tc cell responses to influenza viruses also modulate a specific Tc cell population probably directed at the viral hemagglutinin molecule (Rennie et al. 1977).

We report here that Tc cells innate to alphaviruses, in contrast to the influenza virus system, comprise only a crossreactive population as tested by target lysis and by induction of secondary responses with homologous or
INTRODUCTION

The alphaviruses are a widespread viral genus, transmitted by arthropods that cause serious encephalitis in various host animals, including man (Andrewes and Pereira 1972, Baker 1974). In the past, study of the immune response to alphaviruses has focussed mainly on humoral reactions, and classification of alphaviruses into complexes, types and subtypes relied extensively on serological methods such as virus neutralisation (NEUT) and haemagglutination inhibition (HI) tests (Casals 1957).

Protection from alphavirus infection has been demonstrated after immunization with a distantly related virus, as defined by NEUT and HI tests (Parks and Price 1958, Fine et al 1974, Ferguson et al 1978), and such crossprotection has been tentatively ascribed to T cells in experiments using outbred mice (Peck et al 1975). Protection against alphavirus-induced meningoencephalitis by T cells has also been documented (Hapel 1975).

The concept of cross protection by T cells between serologically distinct viruses is currently attracting much attention in the influenza virus system (Yap and Ada 1978, Floch and Werner 1978). Crossreactivity in vivo and in vitro by subpopulations of influenza-immune cytotoxic T cells (Tc cells) is thought to reside in an antigen molecule (matrix protein) which is shared by otherwise serologically distinct subgroups, and is expressed on membranes of infected cells but not on the virion envelope (Braciale 1977, Effros et al 1977, Biddison et al 1977, Braciale 1977b). Tc cell responses to influenza viruses also contain a specific Tc cell population probably directed at the viral haemagglutinin molecule (Zweerink et al 1977).

We report here that Tc cells immune to alphaviruses, in contrast to the influenza virus system, comprise only a crossreactive population as tested by target lysis and by induction of secondary responses with homologous or
heterologous viruses.

MATERIALS AND METHODS

Animals

CBA/H mice were bred at the John Curtin School and females were used at 6-12 wk of age.

Viruses

Bebaru (BEB), Sindbis (SIN) and Semliki Forest Virus (SFV) were grown in infant mouse brains. Virus was recovered after sonication and differential centrifugation, and titrated on Vero cell monolayers as described previously (Hapel 1975).

Immunization

CBA/H mice were immunized with virus 2-3 wk prior to use in secondary responses in vitro. For BEB and SIN, 5x10^5 plaque-forming units (PFU) of active virus was injected i.v. For SFV, virus was inactivated by 1.5x10^6 rads of γ-irradiation and the equivalent of 5x10^5 PFU was given i.v.

Serum

CBA/H mice were immunized i.v. twice 2 wk apart with 1x10^7 PFU BEB or 1x10^7 PFU SIN and were bled 2 wk after the second injection. The serum was heat inactivated at 56°C for 30 min and stored at -20°C.

Serum titrations

Plaque reduction neutralisation (NEUT) tests were carried out on Vero cells as described in full elsewhere (Clarke et al 1974).

Complement mediated lysis

This was performed as described in detail elsewhere (Braciale 1977b). In short, ^51^Cr labelled P815 mastocytoma cells were infected with either 50 PFU/cell BEB or SIN for 1 h at 37°C at 2x10^6 cells/ml and distributed at
2x10^4 cells/well in 0.05 ml aliquots. Dilutions of either virus-immune or normal sera were added in 0.05 ml aliquots, and after 30 min incubation at 37°C, 0.1 ml guinea pig complement at 1/10 dilution was also added. After 3 h incubation at 37°C 0.1 ml aliquots of the supernatant was removed and the radioactivity was estimated. Percent specific ⁵¹Cr release was obtained from the formula:

\[
\text{Percent specific ⁵¹Cr release} = \frac{\text{Test counts} - \text{spontaneous release}}{\text{Water lysis counts} - \text{spontaneous release}} \times 100
\]

All values given were the mean of 3 replicate wells with S.E. of the mean never greater than 1.7%.

**Memory cultures (in vitro secondary responses)**

Secondary immune Tc cells to alphavirus were generated in vitro by culturing responder spleen cells (from CBA/H mice immunized with BEB, SIN or SFV respectively 2-3 wk previously) with normal syngeneic "stimulator" spleen cells infected with 1-2 PFU of BEB, SIN or SFV per nucleated cell (2x10⁷ viable cells/ml). The stimulator:responder ratio was 1:10. Usually 8x10⁷ responder cells were cultured with 8x10⁶ stimulator cells at 37°C in an atmosphere of 10% CO₂ in 40 ml of Eagle's minimal essential medium (GIBCO, Cat. no.F15) supplemented with 10% foetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Australia) 10⁻⁴ M 2-mercaptoethanol and antibiotics. Cultures were harvested after 5 days.

**Cytotoxicity assay with macrophage target cells**

The method has been described in detail elsewhere (Blanden et al 1976) The multiplicity of infection for BEB, SIN and SFV was 50 PFU/cell (2x10⁶ macrophages/ml). The duration of infection for all viruses used was 1 h at 37°C followed by 2 washes with complete medium and an incubation time of 1-2 h at 37°C before addition of effector Tc cells. Data given have had
spontaneous release subtracted and are the means of triplicates for assays run at 37°C for 6h. S.E. of the means were usually less than ± 3% and are omitted from the figures for clarity. Significance was determined by Student's t test.

Cold target competition

Macrophage target cells were labelled with $^{51}$Cr, and infected with alphaviruses as described above, except that they were distributed at $5 \times 10^4$ cells/well. Unlabelled thioglycolate-activated macrophages were either left uninfected or were infected with 50 PFU/cell BEB, SIN or SFV ($2 \times 10^6$ cells/ml) for 1 h. They were washed twice with complete medium and added in 0.1 ml aliquots/well at ratios of 1, 2, 4 and 8 times the number of labelled targets. Secondary Tc cells ($1 \times 10^5$) were added in 0.1 ml/well. The assay was run for 6 h.

Serum inhibition of Tc cell lysis

L929 (H-2k) cells were adapted for 24 h in spinner cultures (Eagle's Minimum Essential Medium GIBCO Cat. no. F13 supplemented with 10% heat inactivated FCS) at $10^5$ cells/ml.

Cells were labelled with $^{51}$Cr and infected with 50 PFU/cell SIN or BEB ($5 \times 10^6$ cells/ml) for 1 h at 37°C, followed by two washes in complete medium. Cells were distributed at $2 \times 10^4$ cells in 0.05 ml/well and incubated for 1 h at 37°C. Dilutions of either normal or virus-immune sera were added in 0.05 ml aliquots/well and further incubated for 1 h before addition of secondary Tc cells in 0.1 aliquots/well, at a killer to target cell ratio of 5:1, for a 6 h assay time.

RESULTS

Serological characterisation of viruses used in this study

BEB, SIN and SFV virus were NEUT-tested with homologous and heterologous
sera. The results shown in Table I clearly indicate that BEB and SFV, two viruses of the same type, crossreact slightly, whereas SIN virus, belonging to a separate complex of alphaviruses, does not significantly crossreact by this test. Similar results using either NEUT or HI tests have been extensively documented (Casals 1957).

**Induction of secondary alphavirus-immune Tc cells, and target cell recognition across serologically distinct subgroups**

Spleen cells from CBA/H mice, primed 3-12 wk earlier with either BEB, SIN or SFV, were restimulated *in vitro* with stimulator spleen cells infected with either the homologous or a heterologous virus and tested on 4 types of CBA/H macrophage targets, uninfected, or infected with the 3 viruses used for infection of stimulator cells. As can be seen from Table II, secondary stimulation of alphavirus memory Tc cells can be achieved with homologous or heterologous viruses. Similarly, no specificity was seen at the level of target cell lysis; all infected targets were lysed to a similar extent, regardless of the relationship between viruses used for stimulation and target cell infection. The lower lysis obtained with SFV memory spleen cells can be explained by lower priming efficiency, because inactivated virus was used for inoculation. Low lysis of uninfected target cells (Table II), and other controls (using normal spleen cells as stimulators *in vitro*) that did not give significant lysis of any target cells (data not shown) excluded the possibility of anti-FCS responses being implicated in this crossreactivity (Forni and Green 1976).

**Cold target competition experiments**

The results in Table II gave no hint of the existence of Tc cell subsets specific for a single virus out of the 3 tested. In contrast, influenza-immune Tc cell populations can be divided into specific and cross-reactive subsets (Braciale 1977a, Effross et al 1977). Cold target competition can reveal the presence of specific Tc cells. Thus, if competition
is more efficient using cold targets infected with the same virus used for both stimulation and hot target infection than with cold targets infected with a heterologous virus, then this would indicate the presence of virus-specific Tc cells.

Therefore, BEB-, SIN- or SFV-immune Tc cells were assayed on $^{51}$Cr labelled macrophage targets infected with the homologous virus and unlabelled macrophage competitors were added, infected with either the homologous or heterologous viruses, at various ratios to labelled targets. The results in Fig. 1 show that no difference in the ability to compete was observed between macrophages infected with either homologous or heterologous viruses, results that argue against the existence of virus-specific Tc cells in these alphavirus systems.

Specific inhibition of Tc cell-mediated lysis by antiviral antibody

Secondary BEB- or SIN-immune Tc cells were tested on homologous virus-infected targets in the presence of normal, anti-BEB or anti-SIN sera. The results obtained from such a blocking experiment are shown in Fig. 2.

Lysis of BEB-infected L929 targets by BEB-immune Tc cells could be specifically inhibited by BEB-antiserum but not by SIN-antiserum. A nonspecific reduction in lysis was observed at $1/20$ serum dilution with normal as well as antiviral sera. The reverse was true in the SIN-assay system, using SIN-immune Tc cells on SIN-infected targets. Only SIN antiserum inhibited lysis of SIN-infected L929 targets; BEB antiserum did not inhibit lysis to a greater extent than the normal serum control. Because alphavirus-immune Tc cells do not distinguish between targets infected with homologous or heterologous viruses (Table II, Fig. 1) it appears either that alphavirus-immune Tc cells are less specific for the virus-specified antigenic determinant(s) than antibody, or that Tc cells see different viral antigenic structures than B cells and the blocking is due to steric
Detection of serologically crossreactive antigens on infected cell membranes

The same sera used for the Tc cell blocking and virus neutralization studies were tested for the presence of crossreactive antibodies between BEB and SIN, recognizing a virus-specified determinant antigen on the surface of infected cells.

P-815 (H-2\textsuperscript{d}) mastocytoma cells, \textsuperscript{51}Cr labelled and infected with either SIN or BEB were treated with a series of dilutions of either the homologous or heterologous serum raised in CBA/H (H-2\textsuperscript{k}) mice against these viruses, and after guinea pig complement treatment \textsuperscript{51}Cr release was estimated. The data presented in Fig. 3 illustrate one such experiment.

Both BEB and SIN antiviral sera could lyse either BEB- or SIN-infected P-815 cells in the presence of complement, though the heterologous serum had less lytic activity than the homologous serum on both BEB- and SIN-infected cells. These data indicate that the sera contain both specific and crossreactive antibodies that recognize antigen(s) on the surfaces of BEB- and SIN-infected P-815 cells. These antigens are probably virus-specified. They cannot be modified H-2 antigens because the sources of the sera, CBA/H mice were H-2\textsuperscript{k}, and the target cells used were H-2\textsuperscript{d}.

DISCUSSION

Secondary stimulation in vitro of alphavirus-primed CBA/H spleen cells resulted in the generation of Tc cells which showed apparently complete crossreactivity between BEB, SIN and SFV. The crossreactivity was observed at the level of target lysis and also occurred at the level of induction of the secondary response in vitro, a more demanding test for specificity in other systems (Chapter 2). Cold target competition experiments could not detect any evidence for virus-specific Tc cells, thus suggesting that
only crossreactive Tc cell populations were generated. In contrast, the Tc cell response to influenza virus appears to comprise two distinct populations, one specific for the viral haemagglutinin molecule (Zweerink et al 1977) and a crossreactive subset possibly recognizing the viral matrix protein (Biddison et al 1977, Braciale 1977b).

The lack of specificity of Tc cell recognition of BEB-, SIN-, and SFV-infected cells also contrasts with the specificity of antibody recognition of viral envelope antigens as revealed in NEUT and HI tests (Casals 1957).

Two broad propositions could account for such data.

(a) Alphavirus-immune Tc cells recognize the same viral antigen molecule as the antibodies detected in NEUT and/or HI tests. This can be subdivided. Either Tc cells and antibody recognize the same area of the molecule, or different areas.

(b) Tc cells recognize virus-induced cell surface antigen(s) common to BEB-, SIN-, and SFV-infected cells that are different from the viral envelope antigens detected serologically.

In testing these two propositions we used two antiviral sera raised against BEB and SIN that distinguished clearly between these viruses in NEUT tests, but exhibited some crossreactivity in complement-dependent lysis of virus-infected cells. However, the activity of these sera in blocking Tc cell-mediated lysis of infected target cells was completely virus-specific. This result is consistent with proposition (a) but does not distinguish between the subdivisions. For proposition (b) to remain viable, one must assume that blocking occurred via steric hindrance, which in turn requires that the putative common and virus-specific antigen molecules are physically close together in infected cell membranes and that antigens seen by Tc cells were not detectable in any of the serological
tests employed. Testing these assumptions will depend upon identification of the alphavirus-specified protein(s) relevant to Tc cell recognition of infected cells.

If proposition (a) does indeed turn out to be correct, i.e. if Tc cells recognize the same viral antigen molecule as antibodies, but with less specificity, then this contrasts with Tc cell recognition of H-2 molecules. Studies thus far with H-2 mutants suggest that Tc cells distinguish between mutant and wild-type H-2 molecules more specifically than antibodies (Blanden et al 1976).

**SUMMARY**

Secondary Tc cells immune to alphaviruses (BEB, SIN and SFV) crossreact between serologically defined sub-groups at the level of target lysis and at the level of induction of response.

Despite this, apparently complete Tc cell crossreactivity between BEB, SIN and SFV, antisera raised against BEB and SIN showed virus-specificity in their ability to block Tc cell-mediated lysis of alphavirus-infected targets. This result suggests that Tc cells recognize the same viral antigen molecule as antibodies, but with less specificity. Other possible interpretations are discussed.
REFERENCES


### Table I

Sero logical characterisation of viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Neutralization of virus by antisera (log NI)(^a)</th>
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<tr>
<td></td>
<td>SIN</td>
</tr>
<tr>
<td>SIN</td>
<td>4.4</td>
</tr>
<tr>
<td>BEB</td>
<td>1.5</td>
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<tr>
<td>SFV</td>
<td>0.9</td>
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</tbody>
</table>

\(^a\) Log neutralization index = \(\log_{10}\) infectivity titre (virus plus normal serum) minus \(\log_{10}\) infectivity titre (virus plus immune serum)
<table>
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<tr>
<th>CBA/H Tc cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Specific Lysis of CBA/H Macrophage Targets&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>BEB</td>
</tr>
<tr>
<td>PRIMARY STIMULATION</td>
<td>SECONDARY STIMULATION</td>
<td></td>
</tr>
<tr>
<td>NONE</td>
<td>BEB</td>
<td>12.7 ± 2.3</td>
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<tr>
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<td>SIN</td>
<td>9.8 ± 0.6</td>
</tr>
<tr>
<td>NONE</td>
<td>SFV</td>
<td>13.6 ± 1.2</td>
</tr>
<tr>
<td>BEB</td>
<td>BEB</td>
<td>7.4 ± 0.2</td>
</tr>
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<td>BEB</td>
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<td>7.7 ± 2.8</td>
</tr>
<tr>
<td>BEB</td>
<td>SFV</td>
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</tr>
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<td>SIN</td>
<td>BEB</td>
<td>7.1 ± 1.2</td>
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<td>SIN</td>
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<td>SIN</td>
<td>SFV</td>
<td>3.2 ± 0.9</td>
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<tr>
<td>SFV</td>
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</tr>
<tr>
<td>SFV</td>
<td>SIN</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>SFV</td>
<td>SFV</td>
<td>7.4 ± 1.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Priming was by i.v. route 3 wk prior to secondary stimulation in vitro as described in Materials and Methods.

<sup>b</sup> Percent 51Cr release over a 6 h period with spontaneous release subtracted. Means of triplicates ± S.E. of mean are given. Killer to target cell ratio was 3:1. Killer to target cell ratio of 10:1 was performed in the same experiment, but data are only given for the lower ratio because this was below the plateau of lysis.
FIG. 1 - Similar inhibition of Tc cell-mediated cytotoxicity by "cold" competitors infected with different viruses. Secondary BEB-, SIN- or SFV-immune Tc cells were tested for cytotoxicity on 51Cr-labelled macrophage targets infected with the homologous virus in the presence of various numbers of "cold" competitors either uninfected (■), BEB-infected (▲), SIN-infected (●) or SFV-infected (◇). The ratio of Tc cells to 51Cr labelled targets was 2:1. Values for specific lysis are the means of triplicates with spontaneous release subtracted. S.E. of the mean was always less than 2.7%. * denotes specific lysis of control targets without competitors. For detail see Materials and Methods.
FIG. 2 – Inhibition of Tc cell-mediated infected target lysis by antiviral antibody. Dilutions of mouse sera immune to BEB (▲) to SIN (■), or normal mouse serum (●) were added to 51Cr-labelled L929 (H-2k) targets infected with either BEB or SIN. CBA/H (H-2k) BEB- or SIN-immune Tc cells were then added to targets infected with homologous virus at a killer:target ratio of 5:1 and the assay was run over a period of 6 h in the presence of the antiviral serum as described in Materials and Methods. *denotes control without serum. All values are the means of triplicates with spontaneous release subtracted. S.E. of the means was less than 3.5%.
FIG. 3 - Cytotoxicity of CBA/H mouse (H-2^k) anti-BEB or anti-SIN antisera for \textsuperscript{51}Cr-labelled alphavirus-infected P815 (H-2^d) cells. P815 cells were infected with either BEB or SIN and tested along with uninfected P815 cells for complement-mediated lysis in the presence of anti-serum to BEB (▲) or to SIN (■) or control normal mouse serum (●). * represent spontaneous \textsuperscript{51}Cr release. Uninfected P815 cells were not lysed in the presence of either virus-immune or normal serum.
THE EFFECT OF VIRUS–IMMUNE SERUM ON ANTIVIRAL CYTOTOXIC T CELLS IN VIVO AND IN VITRO
Virus infection of murine cells leads to the expression of virus-coded and/or virus-induced antigens on the cell surface. Virus-immune cytotoxic T cells (Tc cells) recognize and lyse infected cells, but display dual specificity for the virus and for the H-2K and/or H-2D antigens of the murine host (Doherty et al. 1976). Two different mechanisms can produce the antigenic patterns recognized by Tc cells. Virion envelope proteins can be transferred into the cell surface membrane by fusion of the viral envelope with the cell membrane. This mechanism occurs most efficiently with paramyxoviruses (Schrader and Edelman 1977), but with a high multiplicity of infection it can also occur with other virus groups (Hapel et al. 1978). With lower multiplicity of infection, new protein synthesis early after penetration and uncoating is required for antigenic changes detectable by Tc cells (Ada et al. 1976, Jackson et al. 1976). Proteins specified either by the viral genome, or by host cell genes derepressed after infection could produce the relevant antigenic changes.

Models to account for Tc cell recognition fall into two general categories, dual receptor or single receptor (Langman 1978, Blanden and Ada 1978). Dual receptor models invoke two separate Tc recognition sites for H-2 and virus-induced antigens, though available evidence suggests that to trigger Tc cell function these sites must act as a paired set to recognize two antigen molecules close together in the same cell membrane. Single receptor models envisage one class of recognition site that binds to a modified self H-2 molecule, or to a new antigenic determinant resulting from a complex of self H-2 and virus-induced molecules. It is apparent that subclasses of each model can be proposed, depending upon whether the new virus-induced protein(s) are coded by the viral genome or the host cell genome. Theoretically these sub-models can be tested if Tc cell recognition and
ysis of virus-infected target cells can be blocked by applying antibodies to the relevant target cell antigens. Thus far, significant blocking has been achieved with anti-H-2 sera (Burakoff et al 1976, Schmitt-Verhulst et al 1976, Koszinowski and Ertl 1976), but little success has been met with anti-viral sera (Zinkernagel and Doherty 1979). We report here on investigations that define the experimental conditions required for specific blocking with antiviral sera using ectromelia virus infection as the basic system.

An additional objective was to determine if antiviral antibodies contribute to the regulation of primary Tc cell responses to infection in vivo. Though Tc cells seem to be crucial in recovery from ectromelia and other infections, little is known about the regulation of such responses.

MATERIALS AND METHODS

Animals

All mouse strains were bred at the John Curtin School and used at 7-10 wk of age.

Viruses and Immunization

Ectromelia and influenza A/JAP viruses were grown, titrated and used for immunisation of mice as described elsewhere (Gardner et al 1974, Yap and Ada 1977).

Antisera

Convalescent sera was prepared from mice 2 wk after immunization with either 5x10^4 FFU attenuated ectromelia virus i.v., 8x10^6 influenza A/JAP i.v. or 250 FFU WE 3 strain of LCM i.v. Hyperimmune serum was obtained after 2 further immunizations at one wk intervals using the same dose of virus. Mice were bled one wk after the last injection. Rabbit and anti-rabbitpox and rabbit anti-vaccinia hyperimmune sera were obtained after 3 inoculations
s.c. of either $5 \times 10^4$ pock-forming units of rabbit pox or vaccinia at weekly intervals. Rabbits were bled 1 month after the last immunization. All sera were heat-inactivated at $56^\circ C$ for 30 min and stored at $-20^\circ C$. The LCM serum was a gift of Dr. M. Dunlop and the rabbit sera a gift of Dr. K. Lafferty.

**In vivo primary responses**

In vivo primary responses to ectromelia infection have been described before (Gardner et al. 1974).

**Memory cultures (in vitro secondary responses)**

The generation of secondary Tc cells with ectromelia (Gardner and Blanden 1976) and influenza virus (Yap and Ada 1977) has been reported in detail.

**Cytotoxic assay**

The basic method has been fully reported elsewhere (Gardner et al. 1974). In brief, L929 cells, P815 cells or 3T3 cells were labelled with $^{51}$Cr for a period of 1 h in suspension, and infected for 1 h in suspension ($2 \times 10^6$ cells/ml) with either 50 PFU/cell of virulent ectromelia virus or 1 EID$_{50}$/cell A/JAP influenza virus. $2 \times 10^4$ cells per well were distributed in 50 µl aliquots. After a 1 h incubation 50 µl of a $1/10$ dilution of immune or control sera (if not otherwise specified) was added and incubated for 30 min at $37^\circ C$, before the addition of effector Tc cells in 100 µl aliquots. Final volume was 200 µl/well.

The method for macrophage target cells has been described in detail elsewhere (Blanden et al. 1976). The multiplicity of infection and serum addition was as described above.

Data given have had spontaneous release subtracted and are the means of triplicates for assays run at $37^\circ C$ for 6-16 h. S.E. of the means were usually less than ± 3% and are omitted for clarity. Significance was
Specific $^{51}$Cr release was calculated according to the formula:

$$\% \text{ specific } ^{51}\text{Cr} = \frac{\text{test well release - spontaneous release}}{\text{total release - spontaneous release}} \times 100$$

% Blocking was calculated as follows:

$$\% \text{ Blocking} = 100 - \frac{\% \text{ specific } ^{51}\text{Cr release with immune serum}}{\% \text{ specific } ^{51}\text{Cr release with normal serum}} \times 100$$

Fractionation of serum on sucrose gradients

The method of Baxter (1972) was used for the preparation of linear gradients of 10-30% sucrose in isotonic saline. 1.0ml serum diluted 1:1 with isotonic saline was overlaid on a 12 ml gradient and centrifuged at 30,000rpm for 20 h at 15°C in an SW41 rotor. 1 ml fractions were collected from the top and dialyzed against phosphate-buffered saline (PBS) overnight at 4°C.

Isolation of IgG

Isolation of IgG on Sepharose A protein columns was performed using the method of Goding (1976).

RESULTS

Kinetics of appearance of serum blocking activity in relation to primary and secondary Tc cell responses to ectromelia virus infection

Serum and spleens of ectromelia-infected CBA/H mice were taken from 2-21 days after infection. The spleen cells were tested in vitro for primary Tc cell-mediated cytotoxicity against ectromelia-infected H-2 compatible L929 target cells and an aliquot of those spleen cells was cultured in vitro for 5 days at 39°C with ectromelia-infected syngeneic spleen stimulator cells for the detection of secondary Tc cell response capability (memory). The sera of these primed mice was tested for
blocking activity in a standard secondary ectromelia-immune Tc cell assay as described in Materials and Methods.

The result of such an experiment is shown in Fig. 1. Primary Tc cell-mediated lysis generated in vivo was highest at day 6 and disappeared by day 10. A primary in vitro-response was evident at day 0 i.e. with spleen cells taken from the mice before infection. This capability disappeared by 2 days post-infection, but cells capable of a in vitro response reappeared by day 4 and increased in potential until the end of the experiment. The primary response level (day 0) was exceeded by 14-16 days post-infection and presumably is a reflection of memory cell production during the course of the primary response to infection.

The blocking activity of the serum reached near plateau levels at day 6 with only slight increases up to day 21. The decline of the primary in vivo Tc cell response after day 6 coincided with the plateau of blocking activity, a finding consistent with, but not proving, a causal relationship.

Effect of serum on the primary ectromelia-immune Tc cell response in vivo

The effect of hyper-immune serum on a primary Tc cell response in vivo was investigated by inoculation of mice with serum prior and post-infection with virus, and measuring the primary Tc cell response in the spleen 6 days post-infection. Injection of serum from 3 days prior till the day of infection significantly reduced the capability of the mouse to mount a primary Tc cell response. Serum administration at a later time had less effect (Table I). This confirmed earlier results (Pang and Blanden 1976) and suggested that virus neutralization or blocking of virus-induced antigens on infected cells in vivo could be a factor in regulating the primary Tc cell response to ectromelia infection.

Virus specificity of blocking

A number of convalescent and hyperimmune sera raised against different
viruses were tested for their ability to inhibit lysis by secondary influenza-A/JAP and ectromelia-immune Tc cells of influenza-A/JAP- and ectromelia-infected L929 targets respectively (Fig. 2). Virus specificity of blocking was observed throughout when unrelated viruses were used e.g. LCM, and influenza virus-immune sera did not inhibit lysis in the ectromelia system. The ability of sera raised against related viruses of the poxvirus group (Downie and MacDonald 1950) such as anti-rabbitpox and anti-vaccinia sera raised in rabbits were equally able to inhibit lysis by ectromelia-immune CBA/H Tc cells (Table II).

Using the ectromelia system we were also able to inhibit lysis of ectromelia-infected targets (59.3% inhibition) with hyperimmune serum from mice inoculated with syngeneic, ectromelia-infected, glutaraldehyde-fixed spleen cells (data not shown). Again, inhibition of lysis was virus-specific but independent of the mouse strain used for raising the serum.

Characterisation of blocking factor in serum

Ectromelia hyperimmune serum was fractionated on a 10-30% linear sucrose gradient, and dialysed fractions were tested for their ability to inhibit lysis of ectromelia-infected L929 targets by secondary ectromelia-immune Tc cells. The profile of the blocking activity of fractions from the gradient is shown in Fig. 3. Marker mouse IgG and IgM were run in separate tubes. Blocking activity coincided with the IgG peak in serum and to a lesser extent with IgM. Furthermore, purification of IgG from serum by Na$_2$SO$_4$ precipitation, or isolation of IgG on Sepharose A-protein columns clearly established the blocking factor in hyperimmune serum to be immunoglobulin, mainly IgG.

Target cell type required for efficient blocking

We observed in our initial studies using CBA/H macrophages and L929 cells as targets that only the L929 cell line could be protected from Tc
cell-mediated lysis by ectromelia-immune serum. We investigated a number of target cell lines for differences in their susceptibility to blocking. Table III summarises the data obtained using 3 different strains of mice (CBA/H, BALB/c, C57Bl/6) as responders and different H-2 compatible or syngeneic target cell lines. Macrophage targets, irrespective of mouse strain, were not susceptible to blocking, nor were P815 cells. On the other hand, all 3T3 fibroblast lines, L929 and an SV40 transformed BALB/c 3T3 line gave good blocking results. In all cases, a CBA/H hyperimmune serum at 1/20 final dilution was used, indicating no mouse strain specificity in the blocking activity of the serum.

**DISCUSSION**

The ability of serum from mice recovering from virus infection, or of hyperimmune serum, to specifically inhibit Tc cell-mediated lysis of virus-infected target cells in vitro has been demonstrated. This blocking activity was found in the immunoglobulin fractions of hyperimmune serum after purification on Sepharose-A protein columns or by sucrose density gradient centrifugation. The main peak of activity was present in the IgG fraction, and minor blocking activity in the IgM fraction, which probably reflects a quantitative rather than qualitative difference. Blocking activity was prominent in the serum of infected mice by the sixth day post-inoculation. This early activity could well be mediated mainly by IgM antibodies, but this remains to be determined.

The ability of immune sera to inhibit Tc cell lysis was virus-specific as far as unrelated virus groups were concerned, but extensive crossreactivity was observed by virus-immune sera raised to related members of the poxvirus group. Serological crossreactivity of this group has been well documented (Downie and MacDonald 1950). Serum raised against infected glutaraldehyde-
fixed cells was also able to inhibit lysis. These findings indicate that the same virus-induced antigen(s) on infected cell membranes seen by B cells are physically close to, or a part of, the antigenic pattern seen by Tc cells. The lack of requirement for homology of strain and species between target cells and donors of virus-immune sera suggests that the blocking antibodies recognize virus-specified protein(s). We could find no evidence for blocking attributable to antibodies against host-specified antigens. Thus models for Tc cell recognition involving modified H-2 molecules or derepressed host cell genes seem unlikely.

One interesting aspect of these experiments was the behaviour of different cell types. Fibroblast cell lines (L929 and 3T3) were found to be the only targets which could be protected from Tc cell lysis by virus-immune serum. Macrophage and P815 mastocytoma target cells were refractory to blocking. The nature of this difference between cells remains to be elucidated but one could envisage greater fluidity of the membrane and/or a faster rate of capping and endocytosis of the antigen-antibody complexes on macrophages and mast cells being a possible factor.

This differential behaviour of target cells used for blocking is important for two reasons. Firstly, it is a crucial practical factor that should allow precise identification of the virus-specified proteins involved in Tc cell-recognizable changes in infected cell membranes, particularly when appropriate monoclonal antibodies are applied. Secondly, infected macrophages and other lymphomyeloid cells are probably the main stimulators of the Tc cell response in vivo, at least with poxviruses (Pang and Blanden 1976b, Zinkernagel et al 1978). If such cells are refractory to the blocking effect of antiviral antibodies on Tc cell recognition, then any regulatory effect of antibodies would have to operate through neutralization of viral infectivity or other mechanisms of viral clearance (Blanden 1971) rather than
SUMMARY

Virus-immune sera applied to infected target cells inhibited Tc cell-mediated lysis in vitro. Blocking activity was clearly present in serum by 6 days after ectromelia virus-infection. Activity was found in the IgG and IgM fractions of hyperimmune sera and was specific for the immunizing virus when ectromelia and influenza viruses were used, but did not distinguish between the serologically related poxviruses ectromelia, vaccinia and rabbitpox. There was no requirement for the donors of immune serum to be the same mouse strain as the target cells, or the same species, since rabbit sera blocked similarly to mouse sera.

These findings imply that virus-specified antigens recognized by B cells are physically close to, or identical to, the viral antigens involved in Tc cell-recognizable antigenic changes in infected cell surfaces. There was no evidence for modified H-2 molecules alone, or other proteins coded by derepressed host cell genes, being recognized by virus-immune Tc cells. Significant inhibition of lysis by antiviral antibody was only observed on fibroblast type target cells. Macrophage and P815 targets were refractory to blocking. These findings are discussed in practical terms and in relation to possible regulation of Tc cell responses in vivo by antiviral antibody.
REFERENCES


Suppressive effect of hyperimmune serum on primary Tc cell response to ectromelia virus in vivo

<table>
<thead>
<tr>
<th>Day of serum injection b</th>
<th>% specific lysis of a ectromelia infected L929 cells</th>
<th>% Suppression</th>
</tr>
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<tbody>
<tr>
<td>No serum</td>
<td>23.9</td>
<td>0</td>
</tr>
<tr>
<td>-3</td>
<td>10.5</td>
<td>56.1</td>
</tr>
<tr>
<td>-1</td>
<td>9.8</td>
<td>59.0</td>
</tr>
<tr>
<td>0</td>
<td>10.2</td>
<td>57.3</td>
</tr>
<tr>
<td>+1</td>
<td>18.1</td>
<td>24.3</td>
</tr>
<tr>
<td>+3</td>
<td>19.8</td>
<td>17.1</td>
</tr>
</tbody>
</table>

a Percent $^{51}$Cr release over a 6 h period with spontaneous release subtracted. Killer to target ratio was 15:1. Means of triplicates are given with SE of mean never greater than 1.8%. Spontaneous release was 14.6%. Lysis of uninfected L929 was insignificant ($P < 0.001$).

b 0.2ml Ectromelia hyperimmune serum diluted in Hanks saline $^1/10$ was injected i.v.
Table II
Virus specificity of blocking

<table>
<thead>
<tr>
<th>Serum Additiona</th>
<th>Ectromelia assayb % Spec. lysisd</th>
<th>% Blocking</th>
<th>Influenza-A/JAP assayc % Spec. lysisd</th>
<th>% Blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>64.3e</td>
<td>0</td>
<td>58.9e</td>
<td>0</td>
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<tr>
<td>Normal CBA/H</td>
<td>60.3</td>
<td>6.1</td>
<td>57.6</td>
<td>2.2</td>
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<tr>
<td>CBA/H anti-ectromelia (convalescent)</td>
<td>32.9</td>
<td>48.8</td>
<td>55.6</td>
<td>5.6</td>
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<tr>
<td>CBA/H anti-ectromelia (hyperimmune)</td>
<td>28.6</td>
<td>55.5</td>
<td>54.8</td>
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<tr>
<td>CBA/H anti-LCM (convalescent)</td>
<td>66.9</td>
<td>-4.0</td>
<td>60.2</td>
<td>-2.2</td>
</tr>
<tr>
<td>CBA/H anti-influenza-A/JAP (hyperimmune)</td>
<td>68.6</td>
<td>-6.7</td>
<td>19.4</td>
<td>67.1</td>
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<tr>
<td>CBA/H anti-influenza-A/WSN (convalescent)</td>
<td>69.9</td>
<td>-8.9</td>
<td>37.0</td>
<td>37.1</td>
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<tr>
<td>Rabbit anti-Rabbitpox (hyperimmune)</td>
<td>42.4</td>
<td>34.0</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Rabbit anti-Vaccinia (hyperimmune)</td>
<td>19.0</td>
<td>70.4</td>
<td>50.1</td>
<td>14.9</td>
</tr>
</tbody>
</table>

a Serum was added to a final concentration of 1/20 in 0.2ml.
b Ectromelia infected L929 target cells and CBA/H secondary ectromelia-immune Tc cells.
c Influenza-A/JAP infected L929 target cells and CBA/H secondary influenza-A/JAP-immune Tc cells.
d Percent $^{51}$Cr release from virus-infected targets over a 10 h period with spontaneous release subtracted, at a killer to target cell ratio of 3:1 Means of triplicates given with S.E. of the mean never greater than 3.4% for ectromelia assay and 2.4% for influenza assay.
e Significantly greater than from uninfected L929 cells (P<0.001).
## Table III

Target cell differences to antiviral antibody blocking

<table>
<thead>
<tr>
<th>Secondary Ectromelia immune T cells</th>
<th>Target (^a)</th>
<th>% Specific lysis (^b)</th>
<th>% Blocking</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CBA/H(^c) normal serum</td>
<td>CBA/H(^c) Ectromelia hyperimmune</td>
</tr>
<tr>
<td>CBA/H</td>
<td>CBA/H-MØ</td>
<td>74.3</td>
<td>72.1</td>
</tr>
<tr>
<td></td>
<td>L929</td>
<td>58.3</td>
<td>27.1</td>
</tr>
<tr>
<td></td>
<td>CBA/H-3T3</td>
<td>48.5</td>
<td>15.7</td>
</tr>
<tr>
<td>BALB/c</td>
<td>BALB/c-MØ</td>
<td>82.4</td>
<td>75.4</td>
</tr>
<tr>
<td></td>
<td>P815</td>
<td>69.9</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>BALB/c 3T3</td>
<td>72.4</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>BALB/c 3T3 SV 40</td>
<td>42.5</td>
<td>16.1</td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>C57Bl/6-MØ</td>
<td>52.8</td>
<td>53.9</td>
</tr>
<tr>
<td></td>
<td>C57Bl/6 3T3</td>
<td>39.6</td>
<td>18.2</td>
</tr>
</tbody>
</table>

\(^a\) Targets were used at 2x10\(^4\)/per well

\(^b\) Percent \(^{51}\)Cr release over a 8 h period with spontaneous release subtracted. Killer to target ratio was 3:1. Means of triplicates are given with S.E. of mean never greater than 2.7%.

\(^c\) Serum was added to give a final concentration of 1/20 in 0.2ml.
FIG. 1 - Primary cytotoxicity (▲), cytotoxicity after 5 day in vitro stimulation (●) and blocking activity in serum, as tested in a standard secondary ectromelia-immune Tc cell assay, (○) of mice primed for 2–21 days with 2x10⁴ PFU ectromelia virus i.v. Primary cytotoxicity was assayed at 30:1 killer to target ratios, and secondary cytotoxicity and blocking at 3:1 killer to target cell ratio on ectromelia-infected L929 cells (means of 3 wells with % S.E. of mean never greater than 2.1). Lysis of uninfected controls was negligible.
FIG. 2 - CBA/H convalescent serum to ectromelia (circles) or influenza (squares) was tested for blocking activity using secondary ectromelia-immune Tc cells on ectromelia-infected L929 targets (open symbols) or secondary influenza A/JAP Tc cells on influenza A/JAP-infected L929 targets (closed symbols). Serum was added to 2x10^4 51Cr-labelled targets at 1/16 to 1/512 dilution 30 min prior to addition of Tc cells. Killer to target cell ratio was 3:1. Assay time was 10 h. Specific lysis in the ectromelia and influenza assays without serum was 64.7% and 48.3% respectively, and lysis of uninfected controls was 1.3% and 0.7% respectively. All assays were done in triplicate with S.E. of mean never greater than 2.7%.
FIG. 3 - Sedimentation analysis on a neutral sucrose density gradient of convalescent ectromelia serum. 0.5 ml of serum diluted with 0.5 ml normal saline was overlaid on a 12 ml 10-30% linear sucrose gradient and spun in an SW41 rotor at 30,000rpm for 20 h at 15°C. 1 ml fractions collected from the top of the gradient were dialysed overnight against PBS and tested for blocking activity using secondary ectromelia-immune Tc cells against L929 ectromelia-infected targets. Killer to target ratio was 3:1. Specific lysis of the control test without serum was 48.3%. Arrows indicate marker positions of purified mouse IgG and mouse IgM run on separate gradients.
DIFFERENT D-END-DEPENDENT ANTIGENIC DETERMINANTS
ARE RECOGNIZED BY H-2 RESTRICTED CYTOTOXIC T CELLS
SPECIFIC FOR INFLUENZA AND BEBARU VIRUSES
INTRODUCTION

Murine cytotoxic lymphocytes (Tc cells) that recognize modified self cells are specific for both the foreign antigen (X) and a self component dependent on gene(s) in either the K or the D region of the H-2 complex (Doherty et al. 1976) i.e. they are "H-2 restricted". Studies with several mutants of the \( H-2K^b \) region have shown that, with a variety of viruses and minor histocompatibility (H) antigens (Blanden et al. 1976, Zinkernagel 1976, Chiang and Klein 1978, Brown and Nathenson 1977), the genetic unit coding for the polypeptide chain of the classical, serologically-defined H-2K glycoprotein is also responsible for a restriction antigen, though most of the mutants are largely, if not completely, undetectable by serological means (McKenzie et al. 1977, Klein 1978). The specificity of H-2 restricted Tc cell discrimination between wild-type and mutant H-2K molecules is exquisite (Blanden et al. 1976, Zinkernagel 1976, McKenzie et al. 1977, Kees and Blanden 1976) and seems similar, regardless of the X antigen involved, thus suggesting that the same antigenic determinant or area of the same H-2K molecule is recognized in each case. This idea is reinforced by the finding that, of the first 10 mutants in the same genetic unit in the \( H-2K^b \) region, 3 are phenotypically identical (McKenzie 1977, Melvold and Kohn 1976). Provided these 3 mutants arose independently, this implies that only small section(s) of the H-2K polypeptide sequence are relevant to Tc cell recognition.

Thus far, there are insufficient mutants of other K or D regions to know how far the conclusions drawn from the \( H-2K^b \) mutants can be extrapolated. However, in contrast to \( H-2K^b \) mutants, the BALB/c-\( H-2d^{m2} \) mutation (formerly \( H-2d^b \)) has generated data suggesting that the D end contains two genetic units concerned with H-2 restriction (Blanden et al. 1977, Blanden and Kees 1978, Biddison et al. 1978). This mutation has revealed
a new locus (H-2L) which is apparently responsible for a molecule similar in many respects to the serologically-defined H-2K and H-2D molecules (Hansen et al. 1977, Demant et al. 1978). All three are approximately 45000 in molecular weight, are associated in the cell membrane with β-2 microglobulin, are recognized by allogeneic Tc cells and share certain serological public specificities, though major private specificities are carried by H-2K and H-2D, not H-2L. Evidence to date suggests that, for ectromelia virus and some minor H antigens, H-2L does not act as a restriction antigen (Blanden and Kees 1978) i.e. it is not recognized by H-2 restricted self Tc cells, whereas it may be so recognized with influenza virus antigens (Biddison et al. 1978).

An H-2L locus may be present in other H-2 haplotypes as illustrated by recent information on the D-end of H-2k (Neauport-Sautes et al. 1978). We report here that monoclonal antibody that binds to molecule(s) dependent on gene(s) in the D-end of H-2k can block lysis by Tc cells specific for these H-2 antigen(s) and influenza virus, but not Bebaru virus. This indicates that two different D-end-dependent antigenic determinants, possibly carried on two different H-2 molecules, are recognized by Tc cells specific for the two different viruses.

MATERIALS AND METHODS

Animals

CBA/H, BALB/c and C3H.OH and BIO.A mice were bred at the John Curtin School and were used in experiments at 8 wk of age.

Viruses

Ectromelia virus, Sendai virus, Bebaru virus (BEB) and influenza A strains JAP and WSN were grown, titrated and used as described in detail previously (Chapter 2).
Generation of Tc cells

Alloreactive Tc cells were generated in primary mixed lymphocyte reactions (MLR) as described in chapter 2. Secondary anti-viral Tc cells were produced by culturing responder spleen cells from mice primed i.p., with virus 3 wk previously with virus-infected syngeneic spleen cells; the complete method is given in chapter 2.

Target cells

L929 cells were grown overnight in spinner culture after routine harvest of monolayers. They were labelled with $^{51}$Cr and used in cytotoxicity assays, either uninfected or infected with viruses, as described in chapter 2.

Blocking with monoclonal anti-H-2-antibodies

The monoclonal antibodies 27R9 and 30R3 used were produced and described in detail by Lemke et al (1978). Briefly, these antibodies were stimulated by H-2$^k$ antigens (BALB/c anti-CBA) and they bind to molecules coded in both the K and D ends (Lemke et al 1978).

Triplicate samples of 50µl containing 2x10$^4$ $^{51}$Cr-labelled, spinner L929 target cells of different categories (uninfected or 1.5 hr post virus-infection) were incubated (30 min at 37°C) with 50µl of serial 2-fold dilutions of the monoclonal antibodies (from 1/50 to 1/1600) in wells of 96-hole Linbro trays. Tc cells of appropriate specificity were then added (10$^5$ cells in 100µl/well) and the cytotoxicity assay was run for 6 hr at 37°C.

RESULTS

Blocking with monoclonal anti-H-2 antibodies of target cell lysis mediated by various Tc cells specific for H-2$^Kk$ or H-2$^Dk$

Four different Tc cell populations specific for K$^k$ antigens were generated in vitro and assayed on appropriate L929 (k$^k$, D$^K$) target
cells

(a) Anti-\(K^k\): C3H.OH (\(k^d, D^k\)) anti-CBA/H (\(k^k, D^k\)) MLR.

(b) Anti-\(K^k\)-ectromelia: secondary BIO.A (\(k^k, D^d\)) anti-ectromelia-infected BIO.A.

(c) Anti-\(K^k\)-Sendai: secondary BIO.A (\(k^k, D^d\)) anti-Sendai-infected BIO.A.

(d) Anti-\(K^k\)-WSN: secondary BIO.A (\(k^k, D^d\)) anti-WSN-infected BIO.A.

The L929 targets were left uninfected for the alloreactive Tc cells (a), were infected with ectromelia virus (b), with Sendai virus (c) or with WSN (d) for assay of the virus-immune Tc cells. Additional targets were infected with JAP and used with WSN-immune Tc cells to reveal the subset that crossreacts with different influenza A strains (Fig. 1, e).

Lysis of uninfected control targets by all of the antiviral Tc cell populations was negligible at the killer:target ratio chosen, and the data given for these Tc cells concern only infected targets. Serial dilutions of monoclonal antibodies 30R3 and 27R9, that bind to H-2\(^k\) molecules, showed different blocking activities (Fig. 1, a,b,c,d,e). 30R3 consistently blocked lysis by all of the \(K^k\)-specific Tc cells, though its activity was more pronounced with alloreactive (Fig. 1 a) than with virus-immune cells (Fig. 1 b-e). 27R9 blocked alloreactive Tc cells significantly, though less than 30R3 (Fig. 1, a), and showed little if any activity with virus-immune cells (Fig. 1, b-e).

The same monoclonal antibodies were tested for blocking of lysis mediated by various \(D^k\)-specific Tc cells. Anti-\(k^k\) alloreactive Tc cells were included for comparison with anti-\(D^k\) alloreactive cells, but responses to ectromelia and Sendai viruses in association with \(D^k\) are weak (Blanden et al 1975, Doherty et al 1978, Zinkernagel et al 1978) and were omitted. Instead, Tc cells were generated against Bebaru virus (BEB), an alphavirus
with which $D^k$ antigens give good responses (Chapter 4). Thus, the four
different Tc cell populations used in this second experiment were as
follows:

(a) Anti-$K^k$, C3H.OH ($K^d, D^k$) anti-CBA/H ($K^k, D^k$) MLR.
(b) Anti-$D^k$, BALB/c ($K^d, D^d$) anti-C3H.OH ($K^d, D^k$) MLR.
(c) Anti-$D^k$-BEB; secondary C3H.OH ($K^d, D^k$) anti-BEB-infected C3H.OH.
(d) Anti-$D^k$-JAP; secondary C3H.OH ($K^d, D^k$) anti-JAP-infected C3H.OH.

As in the previous experiment, the Tc cells were assayed against L929
targets which were left uninfected for the alloreactive Tc cells (a and b),
infected with BEB (c), or infected with JAP (d), and additional WSN-infected
L929 targets were used with anti-JAP Tc cells to reveal the crossreactive
(Fig. 2, e) subset. Lysis of uninfected control targets by the antiviral
Tc cells was again negligible at the killer:target ratio chosen, and the
data given (Fig. 2) for these Tc cells concern infected targets only. 30R3
blocked anti-$K^k$ Tc cell-mediated lysis very efficiently (Fig. 2, a) as
expected, but showed little activity with anti-$D^k$ alloreactive Tc cells
(Fig. 2, b), and did not block any lysis by Tc cells recognizing $D^k$ plus any
of the viral antigens (Fig. 2, c,d,e). In striking contrast, 27R9 blocked
anti-$K^k$ Tc cell lysis less than 30R3 (Figs. 1 and 2, a), blocked anti-$D^k$
lysis more than 30R3 (Fig. 2, b), blocked anti-$K^k$-JAP Tc cell lysis assayed
on both JAP- and WSN-infected targets (Fig. 2, d,e), and did not block
anti-$D^k$-BEB lysis (Fig. 1, c).

DISCUSSION

In the case of interactions between Tc cells and a variety of target
cells studied previously (Blanden et al 1977, Lindahl and Lemke 1979,
could apparently block killer-target interaction by binding to the target,
not the killer cell. This seemed to be true in the present experiments, since lysis mediated by Tc cells from C3H.OH mice was blocked in some cases and not others, depending upon the nature of the target cell antigen being recognized (Fig. 2). The degree of blocking obtained with a particular anti-H-2 antibody in a killer-target system should therefore depend upon the concentration of antibody, its affinity for a certain H-2 determinant on the target cell, and the spatial relationship between that determinant and the determinant recognized by the Tc cell receptor. Steric hindrance must be a possible cause of blocking, but the data in Fig. 2 (a,b) strongly suggest that for blocking to occur the determinant(s) recognized by antibody and Tc cell receptor must be on the same H-2 molecule, since the same target cell-antibody combination showed different degrees of blocking when the Tc cells used were of different specificity. Thus, 30R3 antibody blocked anti-K\textsuperscript{k} Tc cells strongly, but showed little activity with anti-D\textsuperscript{k} Tc cells. Conversely, 27R9 antibody blocked anti-D\textsuperscript{k} Tc cells strongly and had weaker effects with anti-K\textsuperscript{k} Tc cells.

All K end-specific virus-immune Tc cells were blocked in a similar manner by 30R3. Thus, there was no indication that antigenically different K-end-dependent molecules were recognized by Tc cells in the different viral systems. However, there was a suggestion that blocking by 30R3 and 27R9 of alloreactive Tc cell-mediated lysis of uninfected targets was slightly more efficient than blocking of lysis of infected targets by the various virus-immune cells (Fig. 1). This may be a reflection either of hindrance of antibody access to H-2 determinants by adjacent viral antigens, or of allosteric change in H-2 determinants on molecules complexed with virus-specified molecules.

The findings with D-end-specific virus-immune Tc cells contrasted strikingly with K-end-specific cells. 27R9 antibody strongly blocked Tc
cell-mediated lysis specific for antigens dependent on influenza plus the D-end of \( H-2^k \) thus indicating that 27R9 binds to an H-2 determinant that is identical to, or is located on the same molecule as, a determinant recognized by influenza-specific Tc cells. However, lysis mediated by Tc cells specific for antigens dependent on BEB plus the D-end of \( H-2^k \) was not blocked by 27R9.

Two quite separate explanations may be considered. First, it could be that different D-end-dependent determinants are recognized by BEB-immune and influenza-immune Tc cells. The former would need to be sufficiently distant from the site of 27R9 binding so that the IgG2a molecule does not sterically inhibit access of the Tc cell receptor. One form of this model would be that different D-end coded molecules serve as restriction antigens with influenza and BEB. The BALB/c-\( H-2^{d\text{cm2}} \) mutation has shown that H-2L as H-2D molecules are coded in the D-end of \( H-2^d \) (Hansen et al 1977, Demant et al 1978) and evidence exists that \( H-2^k \) may similarly code for two different D-end molecules (Neauport-Sautes et al 1978). Also, blocking studies with anti-\( H-2^L^d \) antibodies suggest that \( H-2^L^d \) may act as a restriction antigen with influenza virus (Biddison et al 1978). Antibodies specific for \( H-2^D^k \) and \( H-2^L^k \) should be able to test this idea.

A second explanation allows both BEB-immune and influenza-immune Tc cells to recognize the same D-end-coded molecule, even the same area of that molecule, but postulates that when complexed with BEB antigen (not influenza antigen) there is sufficient allosteric change in the determinant bound by 27R9 to render it unrecognizable. This idea appears to be difficult to test experimentally and seems less likely than the first explanation.

Our results complement those of Lindahl and Lemke (1978) who have shown that 27R9 blocks Tc cell-mediated lysis specific for H-Y plus D\(^k\)-dependent antigens, but not other minor H. antigens.
We showed that 27R9 antibodies strongly blocked JAP-immune Tc cell lysis of both JAP-infected and WSN-infected target cells. Thus, in a crossreactive Tc cell subset which recognizes an identical or related determinant common to JAP and WSN, possibly on the viral matrix protein (Braciale 1977, Biddison et al 1977), was definitely blocked. From the data presented (Fig. 1, d,e) it is not possible to say whether a JAP-specific Tc cell subset, probably recognizing a determinant on the haemagglutinin molecule, was also blocked. We are attempting to clarify this point using specific anti-JAP or anti-WSN Tc cells and antibodies to various virus-specified antigens. Together with the answer to the question concerning which of the H-2 antigens (H-2D^k or H-2L^k) are recognized together with the various viral antigens, this information may allow better evaluation of current models of H-2-linked "Ir gene" control of H-2 restricted Tc cell responses (von Boehmer et al 1978, Langman 1978).

SUMMARY

Two different BALB/c anti-CBA(H-2^k) monoclonal antibodies that bind to K^k and D^k antigens blocked Tc cell-mediated lysis of L929 (K^k, D^k) target cells, but with quite different specificity. One antibody (30R3) powerfully blocked K^k-specific lysis mediated by alloreactive or by K^k-restricted Tc cells immune to ectromelia, Sendai or influenza viruses. The other antibody (27R9) blocked these anti-K^k Tc cells much less than 30R3, but in contrast, 27R9 blocked anti-D^k lysis much more than 30R3. Most importantly, 27R9 strongly blocked D^k-restricted anti-influenza Tc cells, but did not significantly block D^k-restricted anti-Bebaru lysis. This result indicated that different H-2 determinants coded in the D-end of H-2^k were recognized by influenza- and Bebaru-immune Tc cells. These determinants may be carried on two different molecules coded by the H-2D and H-2L loci, but other possibilities are not yet excluded.
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FIG. 1 - Inhibition by monoclonal antibodies 30R3 (■) and 27R9 (●) mediated by K<sup>k</sup>-specific Tc cells. (a) anti-K<sup>k</sup> alloreactive Tc cells on uninfected targets (b) K<sup>k</sup>-ectromelia-immune Tc cells on ectromelia-infected targets (c) K<sup>k</sup>-Sendai-immune Tc cells on Sendai-infected targets (d) K<sup>k</sup>-WSN immune Tc cells on WSN-infected targets or (e) on JAP-infected targets. Details of Tc cell generation are given in the text under Materials and Methods. The horizontal lines show the level of specific lysis in the absence of antibodies. A killer:target ratio of 5:1 was used throughout.
FIG. 2 - Inhibition by monoclonal antibodies 30R3 (■) and 27R9 (●) of specific lysis of L929 target cells mediated by Kk- and Dk- specific Tc cells. (a) anti-Kk alloreactive Tc cells on uninfected targets (b) anti-Dk alloreactive Tc cells on uninfected targets (c) Dk-BEB-immune Tc cells on BEB-infected targets (d) Dk-JAP-immune Tc cells on JAP-infected targets or (e) on WSN-infected targets. Details of Tc cell generation are given in the text under Materials and Methods. The horizontal lines show the level of specific lysis in the absence of antibodies. A killer:target ratio of 5:1 was used throughout.
CHAPTER 9

FREQUENCY OF PRECURSORS OF CYTOTOXIC LYMPHOCYTES

IN PRIMARY AND SECONDARY H-2 RESTRICTED

RESPONSES TO MODIFIED SELF ANTIGENS
INTRODUCTION

In mice, cytotoxic T cells (Tc cells) have been implicated in the recovery from a primary infection with a number of different viruses (Blanden 1974, Doherty et al 1976). It is also well known that previous exposure to a virus induces long-lasting immunity, or memory. However, the importance of Tc cells in recovery or protection from a second infection with the same virus is less clear-cut, as, in most systems, (Dunlop et al 1976, Gardner and Blanden 1976), it has been found to be difficult to elicit secondary Tc cell responses in vivo.

The development of techniques for the induction of virus-immune Tc cells in vitro (Gardner and Blanden 1976, Dunlop et al 1976, Blanden et al 1977) has made possible a comparison of the relative efficiency of killing by Tc cells generated in primary and secondary responses. By comparing the killer to target ratios that give equivalent specific lysis of infected target cells, it can be estimated that secondary Tc cells are from two to ten times as potent as primary Tc cells (Blanden et al 1977, Gardner and Blanden 1976). The increased potency of secondary, compared to primary, Tc cells can be explained by three possible mechanisms. First, the frequency of Tc cell precursors may be much greater in primed mice; second, the 'burst' size of clones derived from memory Tc cell precursors might be larger and, third, there may be a qualitative difference in the efficiency of killing between Tc cells generated in primary and secondary infections. The most direct way to discriminate between these alternatives is to estimate the precursor frequency.

We have recently developed a limiting dilution assay that detects precursors of virus-specific Tc cells and we report here the application of this assay to the estimation of the frequency of precursors of Tc cells in primary and secondary responses to modified self.
MATERIALS AND METHODS

Animals

All mouse strains were bred at the John Curtin School and only females 5 to 11 wk of age were used in experiments.

Viruses and Immunization

Virulent (Moscow) and attenuated (Hampstead egg) strains of ectromelia virus (Gardner et al. 1974), Sendai and influenza virus strain A/JAP (Doherty and Zinkernagel 1976, Yap and Ada 1977) were grown and titrated as described in detail elsewhere.

BALB/c mice were immunized with either $2 \times 10^4$ PFU attenuated ectromelia virus i.v., $10^6$ EID$_{50}$ of Sendai i.p., or $10^7$ EID$_{50}$ on influenza A/JAP i.p. and used for primed spleen populations 3 to 8 wk later.

TNP modification of target and stimulator cells has been described previously (Chapter 2).

Allogeneic stimulation

Stimulator cells were either BIO.D2 or DBA2 spleen cells and were irradiated with 2000 rad from a $^{60}$Co source prior to use. Responders were BIO.BR spleen cells and limiting dilution assay was performed as described below.

Limiting Dilution Assay

Spleens were removed aseptically from primed or unprimed mice, dispersed by forcing through a wire grid, and washed once with Eagle's minimum essential medium (GIBCO, Cat no. F 15) supplemented with 10% FCS and $10^{-4}$M 2ME. Normal spleen cells to be used as stimulators were infected with virus at a multiplicity of infection of either 10 PFU ectromelia virus 1 EID$_{50}$ Sendai or 1.5 EID$_{50}$ influenza A/JAP at a concentration of $1 \times 10^2$ cells/ml for 1 h at 37°C and washed three times. $1 \times 10^5$ stimulator cells mixed with varying numbers of primary or secondary spleen responder cells
were dispensed in 0.1 ml aliquots into round bottom Linbro tissue culture trays, and incubated at 39°C in 10% CO₂. After five or six days of culture, 5x10³ virus-infected P815 mastocytoma target cells, prepared as described previously (Gardner and Blanden 1976), were added to each well in 0.1 ml of medium, and the trays incubated for a further 6h at 37°C.

The assays were harvested by removing 0.1 ml of the supernatant from each well and ⁵¹Cr release from the target cells determined. The total releasable ⁵¹Cr was determined by lysing triplicate aliquots of target cells in water. The lysis of targets in each well was calculated as follows:

\[
\text{Percent lysis} = \frac{\text{⁵¹Cr released from targets}}{\text{Total releasable ⁵¹Cr}}
\]

Wells showing lysis greater than 3 standard deviations above the mean of the spontaneous release in the presence of medium alone were considered to contain a clone of cytotoxic lymphocytes.

Calculation of Precursor Frequencies

The proportion of negative wells in limiting dilution assays can be described by the zero order term of the Poisson probability distribution

\[
P(o) = e^{-\nu N}
\]

where \(\nu\) = frequency of precursors and \(N\) is the number of spleen cell responders/well. Thus, a semilogarithmic plot of data should yield a straight line, the slope of which gives the precursor frequency.

RESULTS

Semilogarithmic plots of the proportion of negative wells versus the number of responders in primary and secondary ectromelia infection are shown in Fig. 1. The intercepts on the Y-axis, calculated by simple linear regression, ranged from -0.1 to 1.49 with a mean of 0.38 ± 0.08 SE.

1. Natural logarithm of the proportion of negative wells.
However, in individual experiments, the intercepts were not significantly different from zero. In accordance with Poisson probability theory, this indicated that only one cytotoxic precursor was necessary to produce a detectable response in a well.

The estimated frequency of cytotoxic precursors in secondary infections was from two to tenfold greater than in primary infections. Similar estimates of precursor frequencies were obtained using three different viruses: ectromelia, Sendai, and influenza-A/JAP (Fig. 2). Precursor frequencies in primary and secondary responses to TNP-modified syngeneic cells were also within the observed range of the precursor frequencies in primary and secondary anti-viral responses. Some secondary responses gave estimates of precursor frequencies that were very close to those obtained for allogeneic interactions (Fig. 2).

It thus seemed that at least part of the difference between primary and secondary responses could be attributed to an increase in the precursor frequency. However, when the levels of specific lysis, in individual wells were tabulated and compared by $\chi^2$ analysis, clones derived from secondary cytotoxic precursors were found to give significantly higher levels of specific lysis than clones derived from primary precursors (Table 1).

DISCUSSION

The precursor frequencies obtained for allogeneic interactions, and for H-2 restricted responses to TNP-modified syngeneic cells are very close to those reported by other workers (Skinner and Marbrook 1976, Lindahl and Wilson 1977). In relation to the total nucleated spleen cell population, minimal estimates for the frequency of cytotoxic precursors in primary viral infections range from $1 \times 10^{-6}$ to $1.2 \times 10^{-5}$. Marbrook and his colleagues
(Komatsu et al 1978) have recently reported that the precursor frequency in a primary response against the A/JAP strain of influenza virus is $1.9 \times 10^{-5}$; our estimates are entirely consistent with this figure. After priming, the number of cytotoxic precursors increases by up to a factor of ten. This may represent a clonal expansion, during a primary infection, of virus-immune Tc cells, some of which later differentiate into long-lived memory cells (Teh et al 1977). This would suggest that at least part of the differences between primary and secondary responses can be attributed to an increase in precursor frequency. However, the observation that clones of secondary killer cells give higher lysis of infected target cells than do clones of primary killers suggests that there may be a qualitative, as well as a quantitative difference between primary and secondary responses. It may be that Tc cells generated during a primary infection are predominantly of low affinity, whereas stimulation of precursors from immune animals generates Tc cells having a higher affinity.

It should be noted that, by using a limiting dilution assay, we are not only diluting out precursors of cytotoxic lymphocytes, but also other populations, such as accessory or 'helper' cells. Several studies have implicated cells other than cytotoxic precursors in the development of killer cells. These accessory cells include non-specific cells such as macrophages (Wagner et al 1972), non-T-cells from the spleens of nude mice (Schilling et al 1976), amplifier cells (Cantor and Boyse 1975, Wagner et al 1973) and proliferating helper cells (Bach et al 1975). Antigen specific, radioresistant helper T-cells have also been shown to be essential for the induction of cytotoxic T-cells in allogeneic interactions (Pilarski 1977). One or more of these accessory cell types may also undergo clonal expansion after primary stimulation: if so, the limiting unit in our assays may not necessarily be the cytotoxic precursor. If accessory cells, functionally
different in primary and secondary responses, are involved in the generation of virus-specific killer cells, then factors affecting the sensitivity of our assay, such as the 'burst' size of the clones, may not be constant. Thus, if positive clones in primary responses were below the threshold of detection, this would lead to erroneously low estimates of the precursor frequency.

Burnet's theory of clonal selection (1959) requires that each precursor cell be committed to a particular antigenic determinant; however, because the number of antigens to which an animal can respond has been shown to be very large, it follows that only a limited number of precursors can be committed to any one determinant. If we take the average precursor frequency in primary responses to be $1 \times 10^{-5}$, and the average number of Ly $1^{-2+3^+}$ Tc cell precursors in the spleen to be $4 \times 10^6$, then we can calculate that there would be about 40 precursors per spleen committed to determinants specified by a single virus. If all of the Tc cell precursors were available for H-2 restricted responses, then the total repertoire of responses possible under the clonal selection theory would encompass about $10^5$ different viruses. However, as the majority of Tc cell precursors in the spleen are reactive to MHC alloantigens (Wilson et al 1976), the real figure is likely to be at least an order of magnitude lower. Such a restricted range of specificities seems improbable, so in order to resolve this paradox it may be necessary to consider the possibility that there may be multiple receptors on the Tc cell precursor (Wilson et al 1976).

**SUMMARY**

Using a limiting dilution assay, estimates have been made of the frequency of precursors of cytotoxic T lymphocytes in H-2-restricted responses to modified self antigens. Precursor frequencies in primary responses ranged from $1 \times 10^{-6}$ to $3 \times 10^{-5}$, and in secondary responses from
Clones derived from memory precursors showed significantly higher specific lysis than clones derived from primary responders. These results are discussed in relation to the repertoire of H-2-restricted Tc cell responses, and possible models for T cell recognition.
REFERENCES


Comparison of levels of specific lysis of ectromelia-infected target cells by clones of cytotoxic lymphocytes generated in primary and secondary responses.\(^a\)

<table>
<thead>
<tr>
<th>Percent Negative</th>
<th>Primary Response</th>
<th>Secondary Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>wells</td>
<td>56.3</td>
<td>57.8</td>
</tr>
<tr>
<td>Estimated no. of wells containing more than one precursor</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Percent specific lysis per well</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>20-30</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^a\) A representative comparison is shown. In order to make a statistical analysis, we assumed that the wells containing more than one precursor would show specific lysis that lay in the higher frequency classes, and so these were decreased by the appropriate number. A \(\chi^2\) analysis on the resultant 2x2 contingency table gave a value of 4.89: this indicated that the possibility that the observed differences were due to chance was less than 5%. 
FIG. 1 - Semilogarithmic plot of percent negative wells against the number of responders per well in primary and secondary responses to ectromelia-infected syngeneic cells. The lines were fitted by simple linear regression. (●) primary response; (■) secondary response.
FIG. 2 - Summary of estimates of precursor frequency in responses to allogeneic stimuli, and H-2 restricted responses to virus infected and TNP modified syngeneic cells.
CONCLUSION

The phenomena of self-recognition have been conceptualized mainly by two hypotheses: the altered self and the dual-recognition models of T-cell recognition.

The altered self model was developed by Kisker and Brossart (1978) to explain the finding of preferential lysis of H-2 compatible L3T4 infected targets by L3T4-activated T cells, over allogeneic L3T4 infected targets. This model exists in two versions: firstly, the modified altered self model and, secondly, the complement altered self model (Largman 1978). The first version can further be subdivided according to which molecules act as the antigen. In the one version it is envisaged that altered antigens (X)—especially altered histocompatibility antigens (K or D) either by biochemical modification or by other alteration and to which T cells recognize only the modified K or D antigen exclusive of X. The other version proposes a modification of X by K or D molecules. In the complement altered self model, antigens (viral or otherwise) create new antigenic determinants by close association with K and D molecules, and these new antigenic determinants are recognized by T cells. The determinant so created would be the reaction pair between K and X or D antigen. All altered self models propose recognition with a receptor carrying a single antigen binding region (V region).

The somatic generation of such a receptor necessitates that a variable "memory" of self be retained during generation of diversity by gene-controlled mutation of a gene like anti-self receptor. "Altered self models" accommodate data which imply that restriction is imposed during antigen presentation.

The dual receptor model, on the other hand, suggests the presence, one for self H-2 antigens, the other for X antigen. Receptors in this context refer to V region. This model does not generally differentiate...
The phenomena of H-2 restricted responses have been conceptualised mainly by two hypotheses, the altered self and the dual recognition models of Tc cell recognition.

The altered self model was rediscovered by Zinkernagel and Doherty (1974b) to explain their findings of preferential lysis of H-2 compatible LCM infected targets, by LCM-immune Tc cells, over allogeneic LCM infected targets. This model exists essentially in two versions, firstly, the modified altered self model and, secondly, the complexed altered self model (Langman 1978). The first version can further be subdivided according to which molecules act as the modifier. In the one version it is envisaged that foreign antigens (X) (especially viruses) modify self antigens (K or D) either by biochemical modification or by steric alteration and Tc cells recognize only the modified K or D antigens exclusive of X. The other version proposes a modification of X by K or D molecules. In the complexed altered self model, antigens (viral or otherwise) create new antigenic determinants by close association with K and D molecules, and those new antigenic determinants are recognized by Tc cells. The determinants so created would be the junction zone between X and K or D antigens. All altered self models propose recognition with a receptor carrying a single antigen binding region (V region). The somatic generation of such a receptor necessitates that a rudimentary "memory" of self is retained during generation of diversity e.g. controlled mutation of a germ line anti-self receptor. Altered self models accommodate data which imply that restriction is imposed during antigen presentation.

The dual receptor model, on the other hand, envisages two receptors, one for self H-2 antigens, the other for X antigen. Receptors in this context refer to V region. The model does not generally differentiate
between molecular arrangements of these two V regions. They may be presented on one molecule or on two, either linked or unlinked, but operationally they act as a unit. Furthermore, the two V regions are assumed to be independently clonally expressed on T cells (Janeway et al 1976, Langman 1978, Blanden and Ada 1978). Some versions of this model do assume that restriction is imposed independently of antigen presentation (Langman 1978). Although the models are postulated as alternatives, they will be discussed separately, as evidence in favour of one is not always contradictory to the other.

A number of experimental observations are hard to reconcile with some of the versions of altered self models, and modified self versions in particular. The general idea of X modifying self has been refuted conclusively by the following experiments: UV-inactivated Sendai virus can be used to induce Sendai-immune Tc cells and can sensitize targets (Schrader and Edelman 1977). This has also been shown for poxvirus (Hapel et al 1978). Furthermore, stimulation of T cells with isolated haemagglutinin molecules does rule out the possibility that X antigens chemically alter H-2 antigens (Zweerink et al 1977). Results reported in Chapter 2 further impinge on this problem. (TNP-modified Sendai)-immune Tc cells could be induced and caused specific lysis of (TNP-modified Sendai)-sensitized targets. The data obtained by Bevan (1975), showing H-2 restricted lysis due to minor H antigens, also creates conceptual difficulties, as it is hard to imagine that the large number of minor H antigens are each able to specifically modify either K or D antigens.

There is one obvious exception where X does indeed modify H-2. Forman et al (1977a) showed that chemical modification of all surface antigens using TNP produced H-2K and D antigens that carry TNP molecules. Such modification, which creates true altered self, may be recognized by
Tc cells in a somewhat different manner from Tc cells in H-2 restricted responses to viral or minor H (including H-Y) antigens. The experiments described in Chapter 2 support this idea. TNP-immune Tc cells showed a general lack of specificity for self, that is, they crossreacted on allogeneic TNP-modified targets, whereas virus-immune Tc cells did not crossreact on allogeneic infected targets.

The converse of the model just mentioned is the idea that H-2 antigens modify viral antigens. A model envisaging H-2-coded glycosyl transferase enzymes modifying X antigens so that they appear partly self has been proposed by Blanden et al (1976b). Three findings have made this hypothesis untenable. Firstly, peptide mapping and sequencing data have shown that H-2 mutations recognized by H-2 restricted Tc cells affect the polypeptide chain of the H-2 molecule rather than the sugar moiety (Brown and Nathenson 1977). Secondly, inhibition of glycosylation by 2-deoxyglucose does not inhibit H-2 restricted lysis of influenza-infected targets by a part of the influenza-immune Tc cell population (Braciale 1977) and, thirdly, the cross-priming experiments performed by Bevan (1976) for minor H and by Simpson and Gordon (1977) for H-Y, which envisage as explanation a macrophage-mediated antigen-processing and presentation step in vivo cannot easily be explained by such a model.

The complex altered self model, in contrast to modified altered self, can, with modification, accommodate most experimental data and no definitive experiment refuting it exists. There is evidence suggestive of close association between some viral antigens and H-2K and/or D antigens on the cell surface. In a tumor virus system, co-capping of H-2 molecules with antiviral serum has been reported (Schrader et al 1975) and further indirect evidence comes from Blank et al (1976) and Blank and Lilly (1977). They have shown firstly that H-2b Friend virus (FV)-immune
To cells lyse only targets carrying H-2D^b antigens, but not K^b. Furthermore, when FV virus particles grown in H-2^b mice were tested for incorporation of host protein into the viral envelope, H-2D^b was preferentially present, but K^b was absent. We have observed a similar phenomenon when alphaviruses were grown in C3H.OH (H-2K^dD^k) mice. Weak viral neutralization was possible with anti H-2D^k serum but not with anti H-2K^d serum (Chapter 4). Little progress has been achieved in biochemical isolation of a complex H-2-virus antigen, except a report by Inada and Uetake (1978) in the adenovirus system. It must be mentioned that close association does not directly argue against a dual recognition model, but it is not an absolute prerequisite for it as it is for a complexed altered self model. Two experimental observations very much in accord with this model are adsorption data (Chapter 3) and cold target competition data (Chapters 2,6). The adsorption data argue strongly that either self antigens or X antigens alone are not recognized i.e. there is no demonstrable affinity or, for that matter, avidity of Tc cell receptors for either self H-2 or viral antigens alone.

Recently, IR gene involvement in the Tc cell response to Sendai, vaccinia or influenza virus has been proposed to explain high and low responsiveness associated with a particular H-2 antigen (Doherty et al 1978, Zinkernagel et al 1978d,e) but no detailed mechanism has been formulated. A less complicated explanation can account for at least some of the low/high responder phenomena, such as in the tumor virus system (Blank et al 1976) and Tc cell responses to the male antigen (H-Y) (Matsunaga and Simpson 1978). The idea is that there is variation in the ability of X antigen to associate (form a complex) with different H-2K or D molecules. The experiments described in Chapters 4 and 5 lead to a similar conclusion for the alphavirus Tc cell response. In this instance
a single haplotype (H-2^k) was found to be able to mount a significant specific Tc cell response, which was further found to be restricted to the D end. We could exclude I region influences as F_1 hybrid Tc cells between non-responder and responder parents only lysed responder parent targets but not non-responder. Furthermore, the chimera studies described in Chapter 5 gave good evidence that non-responder (H-2^d) stem cells can recognize H-2D^k plus alphavirus when allowed to differentiate in H-2D^k recipients, i.e. they do not have a germ line defect in ability to see alphavirus-coded antigens.

One other option of explaining low responsiveness to a given virus associated with a particular H-2K or D allele is to postulate idiotype specific help i.e. D^k in the H-2^k haplotypes gives low responses to Sendai, vaccinia and ectromelia virus but high responses with LCM, influenza and alphaviruses (Doherty et al 1978, Blanden et al 1975, Zinkernagel et al 1978d,e, Chapters 4,5). The K^k region, on the other hand, gives low response to alphaviruses but high response to the other viruses mentioned above. If T cell help is implicated in the generation of responsiveness, then it must be Tc cell idiotype-specific that in turn favours an altered self model of Tc cell recognition.

A difficulty with the complexed altered self model is the lack of Tc cell responses restricted to other cell surface antigens, such as the minor H antigens. There is no known reason why these should not be able to form new altered self complexes with X antigens. One would have to argue that either H-2K and D antigens are particularly suited to form complexes and other cell surface antigens are not, or that such complexes between X antigens and minor H do occur but T cell receptors have a germ line bias for H-2K and D antigens. Physical linkage between H-2K and H-2D, such as a killer channel as proposed by Langman (1978), would also
overcome this difficulty.

As mentioned before, most available data on H-2 restricted Tc cell responses can be accommodated by both a complexed altered self and a dual receptor hypothesis, but there does exist some evidence that, at face value, can be more easily explained with dual recognition models. These centre mainly on specificity studies and, to some extent, also on antibody blocking data.

An example of exquisite specificity for self in the viral systems has been highlighted by experiments using mutant mice, especially the K^b mutants. These mutants (i.e. K^{ba}) are not recognized serologically but with ectromelia virus, vaccinia and LCM (Blanden et al 1976a, Zinkernagel 1976a) immune Tc cells of wild type recognize wild type (K^b) but not mutant (K^{ba}) targets and vice versa. Even so, allogeneic Tc cells e.g. H-2^d anti H-2K^b crossreact on mutant targets. This specificity of virus-immune H-2 restricted Tc cells is exemplified by no known breakdown of H-2 restriction so far (Chapter 2). The converse is true for TNP-immune Tc cells which crossreact extensively on allogeneic TNP modified self and the specificity with K^b mutants obtained in the virus systems is not observed (Forman and Klein 1977). The excellent specificity for self stands somewhat in contrast to specificity for X antigen in at least some instances. For example, we show in Chapter 6 extensive crossreactivity of Tc cells on target cells infected with serologically distinct alphaviruses, but specificity is apparently observed between Tc cells recognizing serologically different influenza haemagglutinin molecules. Two clonally independent receptors (for X and H-2) are easier to reconcile with observations of different specificities for self and X antigen. For an altered self model, the V region has to be divided into two areas which would make it dual recognition by definition.
Blocking data with both antiviral antibodies and anti H-2 sera (Chapters 6, 7, 8) can be explained both by dual recognition and complexed altered self. In the latter, blocking must be due solely to steric hindrance, as no strain specificity or species specificity was observed with antiviral antibodies and all blocking activity of such sera could be adsorbed out with allogeneic infected cells, which argues against antibody recognition of a complexed altered self determinant.

Finally, data of Zinkernagel (1978a, b) who used F1→Pl chimeras and thymus grafting, together with data from neonataly tolerant mice (Zinkernagel et al 1978a, Kees personal communication) favour the idea that H-2 restriction is learned in the thymus and is independent of X antigen presentation, as F1 cells responded against infected cells of the type carrying the H-2 antigens present on thymic epithelium. Negative selection experiments (Wilson et al 1977, Doherty and Bennink 1979) and experiments with irradiation chimera F1→Pl (Blanden and Andrew 1979, Bevan 1977) indicate however that specificity for Pl is not absolute and lysis of P2 targets can also be observed. This suggests that the thymic influence on restriction specificity is not absolute.

Models other than the ones discussed are a distinct possibility, at least, receptors on precursors may be qualitatively and/or quantitatively different. We discussed briefly in Chapter 9 an option of a multi-potential T cell precursor initially displaying a large number of receptors of different specificities.

The present knowledge of H-2 restricted Tc cell responses offers very few definitive approaches to differentiate between the various models of Tc cell recognition.

The most obvious one would be a direct biochemical approach of isolation and characterisation of the Tc cell receptor(s) analogous
to those used to characterise antibodies.

Another approach which could be used to differentiate at least between altered self and dual recognition is by employing anti-idiotype antibodies or anti-idiotype Tc cells (Binz and Wigzell 1977a,b). One can postulate that monoclonal antibodies to a T cell receptor would be strain and X antigen specific if altered self models are closer to the truth, but would be either strain or X antigen specific if dual recognition models turn out to have been the better bet. Both models are conjectures which will ultimately be refuted.
PUBLICATIONS


PUBLICATIONS


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