T LYMPHOCYTE RESPONSE TO FLAVIVIRUS INFECTIONS.

By

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For my friends.....

"Three passions, simple but overwhelmingly strong, have governed my life: the longing for love, the search for knowledge, and unbearable pity for the suffering of mankind."

Bertrand Russell, 1967.

CONTENTS.

STATEMENT.

With the exception of the following, all the work for the experiments presented in this thesis was performed by me.

Chapter 4 and 5: The FACS analysis of class I MHC antigen expression on mouse embryo fibroblasts was performed by Dr N. J. C. King. Purified virus used in chapter 4 was prepared with the assistance of Mr M. Lobigs. The experiment described in the addendum to Chapter 5 was ~~perfomed~~ performed by Dr A Mullbacher.

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ABBREVIATIONS.

AMD	actinomycin D
B cell	bone marrow-derived lymphocyte
C	constant
cDNA	deoxyribonucleic acid complementary to mRNA
c.p.m.	counts per minute
Cs	supernatant from conconavlin A stimulated splenocytes
D	diversity
DMEM	Dulbecco's modified eagles medium
EMEM	Eagles minimal essential medium
E:T	effector : target
F1	first generation hybrid between P1 and P2
FACS	fluorescence-activated cell sorter
Fc	fragment crystallizable
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	gavity
h	hours
H-2	histocompatibility-2 complex
Ig	immunoglobulin
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon (α, β, γ)
IL-1	interleukin-1
IL-2	interleukin-2
Ir	immune response (gene)

irr	irradiated
J	joining
JBE	Japanese B encephalitis virus
LCM	lymphocytic choriomeningitis
MAb	monoclonal antibody
MEF	mouse embryo fibroblast
MHC	major histocompatibility complex
min	minute
ml	millilitre
m.o.i.	multiplicity of infection
mRNA	messenger ribonucleic acid
MVE	Murray Valley encephalitis virus
NMS	normal mouse serum
P1, P2	parent 1, parent 2
p.f.u.	plaque forming unit.
poly I:C	poly inosinic-poly cytidylic acid
RAMIg-FITC	rabbit anti-mouse Ig conjugated to FITC
s	seconds
T cell	thymus-derived lymphocyte
TCR	T cell receptor
Tc	cytotoxic T cell
Th	helper T cell
Ts	suppressor T cell
TNP	trinitrophenyl
WNV	West Nile virus
w/v	weight / volume
W	watts

ABSTRACT.

I developed a
~~A~~ protocol for obtaining cytotoxic T cell responses to the flavivirus West Nile (WNV) *in vivo*. CBA/H (H-2^k) mice were immunized with 10^6 plaque forming units (p.f.u.) WNV intravenously and their spleen cells used directly in cytotoxic assays. This method reliably produced WNV-immune Tc cells which showed WNV-specific cytotoxic activity on infected L929 (H-2^k) target cells.

Cytotoxic activity against WNV-infected target cells was first detected 4 days after immunization, peaked on day 5 and declined rapidly after day 7. An immunizing dose of 10^3 p.f.u. of WNV was adequate for significant cytotoxicity to be detected. However, the cytotoxic response increased with increasing immunizing doses to plateau levels when 10^6 p.f.u. WNV was used. The cells responsible for lytic activity were H-2 restricted, Thy 1^+, $CD8^+$, $CD4^-$ and virus-specific with respect to WNV and influenza. Only L929 cells infected for 16 hours with WNV at a multiplicity of infection of 100 were suitable targets for the primary WNV-immune spleen cells.

Investigation of MHC restriction necessitated the generation of a more potent secondary *in vitro* murine cytotoxic response. Cytotoxic activity was obtained from spleen cells of mice primed 7 days previously with 10^6 p.f.u. WNV and boosted *in vitro* for a further 5 days with WNV-infected stimulator spleen cells. The cells responsible for lysis of WNV-infected target cells were restricted by class I H-2 antigens. The *K* region of the *H-2* ^{k} haplotype and both *K* and *D* regions of the *H-2* ^{d} haplotype were permissive. The cytotoxic cells were virus-specific with respect to WNV and Influenza. The phenotype of the cells which mediated cytotoxicity was Thy 1^+, $CD8^+$ and $CD4^-$, but the precursors of the cytotoxic T cells were $CD8^-$ or expressed low levels of this surface antigen. A $CD4^+$ helper population was required for the optimal generation of the cytotoxic response *in vitro*.

Cross-reactivity of flavivirus-immune Tc cells was investigated. Secondary flavivirus-immune Tc cells reactive to WNV, Kunjin, Murray Valley encephalitis (MVE) or Japanese B encephalitis (JBE) were generated *in vitro* following priming *in vivo*. These four

flavivirus- immune Tc cell populations were cross-reactive at the level of target cell lysis as they lysed target cells infected with WNV, Kunjin, MVE or JBE significantly more than they lysed uninfected targets. Furthermore, cross-reactivity was also detected at the level of induction of the secondary flavivirus-immune Tc cells. Cold target competition experiments confirmed the cross-reactivity of flavivirus-immune Tc cells and no virus-specific subset was demonstrated.

Flavivirus infection of tertiary mouse embryo fibroblasts (MEF) resulted in significantly increased expression of class I MHC antigen (H-2K and H-2D). This was first detectable 8 hours after infection and continued to ~~increse~~ increase for at least another 88 hours, causing increases in the individual K or D antigens of 6-fold or more. This correlated with increased susceptibility to lysis by both flavivirus-immune and allo-immune Tc cells. The mechanism(s) by which flaviviruses increased class I MHC antigen expression is not fully elucidated, but appears to be mediated by a mechanism partly independent of β-interferon secretion as anti-α/β interferon antibodies partly inhibited the WNV-induced increase but totally prevented the increase caused by addition of pure β-interferon, β-interferon containing supernatants from WNV-infected MEF or poly inosinic-poly cytidylic acid. Actinomycin D treatment of MEF which inhibited mRNA synthesis by greater than 90% as determined by ^{3}H-uridine incorporation, totally inhibited the increased MHC expression by WNV-infection. Thus the increase in class I MHC antigen expression is dependent upon cellular mRNA synthesis.

As a corollary, increased MHC antigen expression on uninfected MEF induced with γ-interferon leads not only to increased lysis by allo-immune Tc cells but also by secondary WNV-immune Tc cells. This increased lysis of uninfected γ-interferon-treated MEF by secondary WNV-immune Tc cells is independent of the expression of viral antigens. This observation is under dispute. A virus-specific subset of Tc cells is present ~~withtin~~ within the secondary WNV-immune Tc cell population as demonstrated by cold-target competition experiments. The avidity of interaction between secondary WNV-immune Tc cells and WNV-infected or recombinant γ-interferon treated targets is greater than the avidity of

interaction with uninfected targets as determined by blocking of lysis with anti-CD8 monoclonal antibody supporting the existence of a low avidity and high avidity self-reactive Tc cells. It is postulated that these findings have important implications for virus-induced autoimmune phenomena.

CHAPTER 1.

GENERAL INTRODUCTION.

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SECTION 2.

SECTION 1

The T Cell.

T cells mediate significant effector functions to a wide range of antigens and are pivotal in controlling the magnitude of the immune-response by both T and B cells. T cell responses to a specific antigen are defined in functional terms because the interaction of a T cell with its appropriate antigen can be recognized only by the effects that this interaction produces. These functions include the proliferation of T cells, the secretion of lymphokines and target cell lysis. T cells are central to the immune-response of an individual. T cells, like B cells, respond to a wide range of antigens. However, unlike B cells which respond to free antigen, T cells respond to antigens presented on the surface membranes of cells. T cells have clonally distributed receptors capable of recognizing foreign antigen in association with self major histocompatibility complex (MHC) antigens. The T cell antigen receptor (TCR) is made from polypeptides coded for by variable, diversity, junctional and constant gene segments (see below). The rearrangement of these gene segments during T cell ontogeny allows for the generation in diversity of the T cell repertoire ~~repetoire.~~ However, some selection procedure must occur so that the mature T cell pool does not respond to self antigens but does respond to foreign antigens in association with self MHC antigens.

T Cell Tolerance.

"I shall always regard the differentiation between self and non-self as crucial to all immunological theory." (F. M. Burnet).

Tolerance is especially complex with regard to T cells because they recognize foreign antigen only when associated with cell surface molecules encoded for by the genes of the MHC i.e.MHC restriction. During T cell ontogeny, rearrangement of variable, diversity and junctional gene segments which make up the α and β chain of the TCR results in a population of mature T cells expressing a diverse array of possible idiotypes. Recent

evidence has shown that the TCR binds to a determinant comprising a MHC molecule associated with a peptide fragment derived from the foreign antigen (Townsend *et al.*, 1985; Maryanski *et al* ., 1985a; Dembic *et al.*, 1986; Saito *et al.*, 1987). Thus, while mature T cells do not react avidly with self MHC molecules, they do react with self MHC molecules when they are associated with foreign antigen. There is evidence suggesting that "thymic education" is important in generating a functional pool of T cells. The mechanism(s) are not understood by which T cells reacting with self MHC molecules per se are depleted and / or suppressed while selecting those reacting with foreign antigen in association with the self MHC molecules to generate a mature functional T cell pool. Evidence has been presented that thymic antigen presenting cells (APC) i.e. thymic macrophages and dendritic cells, have a role in determining T cell specificity. Longo and Shwartz (1980) found that in F1→P radiation-induced bone marrow chimeras, APC of donor bone marrow phenotype will with time begin to appear in the host thymus. The mature host-restricted T cells were then depleted by treatment with cortisone and anti-thymocyte serum after the host thymus had been populated with donor APC. The resulting chimeric thymus, containing host epithelial cells and donor APC, influenced the specificity of the repopulating T cells such that they exhibit donor-type MHC-restriction. These findings were extended by Longo and Davis (1983) who showed that donor APC were detected in the host thymi of radiation-induced bone marrow chimeras 3 weeks after irradiation if a dose of 1200 rads rather than the more conventional 900 rads was used. In these F1→P chimeras the APC of the donor haplotype positively selected the T cell population such that the mature class II MHC-restricted helper T (Th) cells exhibited the MHC-restriction specificity of the donor, not the host. Von Boehmer and Schubiger (1984) studied cytotoxic T (Tc) cell function in mice in which C57Bl/6 (H-2^b) thymi had been transplanted into BALB/c (H-2^d) nu/nu (T cell deficient) animals after the elimination of APCs from the thymi by treatment with 2-deoxyguanosine. They demonstrated that class I H-2^b MHC antigens on the thymus epithelium did not suppress the development and responsiveness of H-2^b-immune Tc cells while H-2^b antigen expression on APC cells

did. Thus ~~tolerane~~ tolerance to class I MHC antigens was imposed by the MHC haplotype of the APC implying some mechanism of negative selection of self reactive clones by thymic macrophages and dendritic cells. These experiments did not exclude an extrathymic pathway for maturation of T cells but showed that the lymphocytes within the thymus were responsive to MHC antigens expressed by the thymic epithelium.

~~In experiments were~~ Experiments in which chimeric thymuses were generated *in vitro* Jenkinson *et al.* (1985) showed that tolerance to MHC anigens was induced within the thymic cultures but was directed to the donor haplotype of the colonizing stem cells and not to the MHC haplotype of the "host" thymic epithelium. ~~They~~ authors postulated that while dendritic cells are potent stimulators of allo-immune mature T cells, contact with dendritic cells may induce tolerance in immature T cells. Thus, it was not necessary to postulate extrathymic maturation of T cells to explain development of tolerance to self MHC antigens.

Lo and Sprent (1986) extended these studies and demonstrated that induction of tolerance and MHC-restriction in Th cells were controlled by different cells within the thymus. In their experiments F1→P chimeras showed strong proliferative responses to antigen presented by cells with parental haplotype. Similarly, chimeras constructed by parental thymus graft into F1 mice followed by irradiation to eradicate thymic APC and F1 bone marrow reconstitution also showed strong proliferative response to parental thymus haplotype. Taken together these results show that thymic epithelial cells rather than APC are responsible for imprinting MHC-restriction on Th cells while the APC induce tolerance *in vivo* (Von Boehmer and Schubiger,1984) and also *in vitro* (Jenkinson *et al.*, 1985). The results of Jenkinson *et al.* (1985) and Lo and Sprent (1986) disagree with the results of Longo and Schwartz (1980) and Longo and Davis (1983). This contradiction cannot be presently explained.

Lo and Sprent (1986) postulated that "receptor-bearing thymocytes make inital contact with the MHC determinants on epithelial cells in the cortex, a region largely free of APC. After "learning" self-MHC-restricted specificity the T cells then migrate from the cortex to the cortico-medullary junction. Here contact with APC deletes those cells expressing high

affinity for self MHC determinants. Low affinity T cells then pass through this filter and migrtate into the periphery."

The studies described by Lo and Sprent (1986) in bone marrow chimera, thymus-grafted mice provided evidence that class II MHC-restricted Th responses are restricted to thymic ~~hapotype~~ haplotype. However, class I MHC-restricted Tc cell responses appear to be more complex and show complete restriction to the thymic haplotype in some studies (Zinkernagel *et al.*, 1978) but incomplete restriction in others (Bevan,1977; Matzinger and Mirkwood, 1978; Blanden and Andrew, 1979).

Zinkernagel *et al.* (1978) demonstrated in F1(stem cells) →P chimeras an absolute restriction to donor haplotype in primary *in vivo* virus-immune Tc cell responses. In contrast, secondary *in vitro* reponses to minor H antigens (Bevan, 1977; Matzinger and Mirkwood, 1978) and viral antigens (Blanden and Andrew, 1979) showed host type bias but not absolute restriction.

Blanden *et al.* (1987) proposed that activation of a T cell was an "all or none " event. The T cell was activated when the strength of the signal delivered to it exceeded the threshold strength required for activation. This signal is generated when the T cell receptor (TCR) binds to a ligand on a target cell and the strength of the signal generated is directly proportional to the number of TCR-ligand interactions. The latter are in turn partly dependent upon the concentration of ligands on the target cell as well as the number of TCR available on the interacting T cell and the affinity of interaction between the TCR and ligand.

The generation of the mature T cell repertoire necessitates the deletion of those T cell clones which are activated by binding to self antigens. Blanden *et al.* (1987) suggested that the concentration of the TCR on the maturing T cells and / or self antigen expression on thymic epithelium are significantly higher than the levels encountered in the extrathymic environment. Thus, even T cell clones with very low affinity for self would be triggered and expanded in this environment. They extended the ideas of Lo and Sprent (1986) and postulated that as the T cell matures in the thymus those clones with affinity to self would

first be expanded by contact with thymus epithelium. Then selection of the mature T cell repertoire ~~repetoire~~ is completed by deletion of those clones which exceed the signal threshold by interacting with self antigen expressed at extrathymic concentrations. Thus, the resulting mature T cell population would be tolerant to self antigens at the level of expression in the extrathymic environment.

These concepts can be used to explain the results of Blanden *et al*'. (1981) and Gallagher *et al.* (1986). Blanden *et al.*, (1981) reported some apparently paradoxical results with murine irradiation chimeras. When (P1 X P2)F1 →P1 spleen cells were restimulated *in vitro* with virus-infected mitomycin C-treated (P1 X P2)F1 cells approximately 16% of the mice gave high lysis of uninfected P2 targets as well as infected P1 targets. This cytotoxicity directed against P2 required 5 days *in vitro* culture to be evident. It was not present upon immediate removal of the spleen cells from the mouse. Gallagher *et al.* (1986) showed that low expression of P2 MHC antigen expression correlated with the the high lysis of uninfected P2 targets in individual mice. Blanden *et al.* (1987) suggested that in (P1 X P2)F1→P1 chimeras with low P2 MHC antigens there could be self tolerant T cells capable of responding to higher levels of the same MHC antigens expressed in normal (P1 X P2)F1 or P2 cells. Thus, "T cell self tolerance would be a quantitative phenomenon determined in part by the self ligand concentration on the cells concerned with self tolerance imposition and not simply related to the affinity between the receptor and ligand."

Billingham *et al.* (1953) first demonstrated actively aquired immune tolerance in neonatal animals. These investigators injected cells from male adult ~~B10.A~~ A/J mice into CBA foetal mice on day 15 to 16 of gestation. At age 6 weeks post-delivery these CBA mice were challenged with a ~~B10.A~~ A/J donor skin graft. In three of the five mice the allograft was intact 50 days later. Since these initial experiments investigators have provided evidence for clonal deletion or active suppression of allo-reactive cells.

Suppressor T (Ts) cells were first dmonstrated by Gershon and Kondo (1970). Dorsch and Roser (1977) showed that neonatally tolerized rats have specific suppressor cells

circulating in their thoracic duct lymph during adulthood. These rats were identified by adoptive transfer experiments. Sublethally irradiated adult recipients syngeneic with the neonatally tolerized chimeric host received host thoracic duct lymphocytes and donor type skin grafts as well as third party skin grafts. The survival of the donor skin grafts was significantly longer than that of the third party grafts. These results were interpreted to support the existence of Ts cells in neonatally tolerized animals. The theoretical possibility that the adoptively transferred thoracic duct lymphocytes contained chimeric F1 lymphocytes which induced tolerance in the irradiated recipients was considered unlikely because small numbers of adoptively transferred cells had no effect on graft survival in irradiated recipients. Furthermore, large doses of F1 cells, equivalent to 100% chimerism in the tolerant cell donors, only induced long-term graft survivial in 1 out of 10 recipients. However, the possibility that the persisting 5% chimeric cells which are present in the tolerized donors had somehow been selected for tolerance-inducing activity cannot be absolutely excluded. If this were correct then the experiment testing the dose of F1 cells necessary to induce tolerance would be invalid.

Evidence for early clonal deletion in neonates tolerized to histocompatibility antigens was provided by Nossal and Pike (1981). Using limiting dilution techniques these investigators were able to demonstrate a significant reduction in frequency of precursor Tc cells which when cultured *in vitro* developed into allo-immune Tc cell clones reactive against the tolerizing allo-antigen. Neonatal CBA (H-2^k) mice were tolerized with (CBA X BALB/c) F1 (H-$2^{k/d}$) spleen cells on the day of birth and the frequency of allo-immune Tc cell clones which lysed H-2^d targets was measured. By day 5, the allo-immune Tc cell clone frequency for thymus was reduced in 3 out of 4 experiments. This deficit of clones became more marked thereafter, being 3-8% of control values at 4 weeks. The reduction in allo-immune Tc cell clone frequency in the spleen was first detected on day 8 and was equally profound. Nossal and Pike (1981) suggested that clonal deletion occurred in the thymus and was detected a little later in the spleen when the spleen had been populated with thymic emigrants purged of precursor allo-immune Tc cells. The possibility that tolerance

was due to the presence of Ts cells was investigated. Daily injections of anti-IJ^k serum were give to the tolerized mice from birth until the day of assay to detect any possible effects of Ts cells acting *in vivo*. The *in vivo* treatment with anti-IJ^k serum partially increased the frequency of the precursor allo-immune Tc cells in the thymus and spleen during the early stages of tolerance induction. However, treating the tolerant thymus and spleen cell populations with anti-IJ^k serum and complement just prior to *in vitro* culture had no effect on the frequency of allo-immune Tc cell clones detected. This data was interpreted in the light of experiments by Gorczynski and McRae (1979a, 1979b) who described two types of Ts cells. Nossal and Pike (1981) invoked this model of Ts cell activity and argued that the suppressor activity they demonstrated at least partially prevented the creation of precursor allo-immune Tc cells from more primitive prethymic precursors. However, a second Ts cell population inhibiting the conversion of precursor Tc cells into allo-immune Tc cell clones *in vitro* could not be detected. They concluded that suppressor T cells capable of inhibiting the differentiation of precursor Tc cells into Tc cell clones may have been present but because this had only a partial effect, functional clonal elimination must have been equally important in the functionally tolerant state.

Stockinger (1984) further investigated the functional deletion of precursor Tc cells in neonatally tolerized animals. She argued that under conditions of numerical excess of Ts cells *in vitro*, limit-dilution analysis may not necessarily reveal a dissociation of precursor Tc cells from the influence of Ts cells. Only experiments which selectively perturb the function of Ts cells would reveal interactions of this type. Monolayers of blast spleen cells immune to the tolerizing antigen were generated in a mixed lymphocyte reaction and subsequently inactivated with 3000 rads of irradiation to avoid their participation in the subsequent culture. These monolayers were then incubated with spleen cells from tolerized animals to adsorb out anti-idiotypic regulatory Ts cells prior to *in vitro* culture. Adsorption on the blast monolayer caused a substantial shift in reactivity of the spleen cell population from tolerized animals to a precursor Tc cell frequency that was within the range of the precursor Tc cell frequency of normal mice. Testing the role of Ts cells *in vivo* ,

Stockinger (1984) used spleen cells of tolerized mice in which tolerance has beeen demonstrated by their ability to retain an allogeneic skin graft syngeneic with the tolerizing antigen. The spleen cells from these mice, first adsorbed on blast monolayers immune to the tolerizing antigen then had an increase in frequency of precursor Tc cells immune to the tolerizing antigen compared with the non-adsorbed control spleen cells. The persistence of low numbers of precursor Tc cells always detected in tolerized animals, indicated that suppression was not complete. Stockinger explained incomplete suppression in the light of data by Goronzy *et al* . (1981) who had demonstrated two sets of precursor Tc cells in polyclonally activated T cells. Stockinger (1984) argued that there were two populations of precursor Tc cells, a frequent population amenable to the action of Ts cells and a rare, non-suppressable population that was always demonstrable.

T Cell Ontogeny.

The thymus is the major if not the only site of maturation of T lymphocytes from committed haemopoietic precursor cells. The haemopoietic precursor cells do not express TCRs and therefore cannot discriminate between self and altered-self. They do not express surface markers characteristic of T lymphocytes.

During maturation of T cells it is assumed there is rearrangement and expression of genes encoding the α/β heterodimer of the TCR. In the murine thymus this occurs first during day 17 of gestation (Pardoll *et al.*,1987). Following expression of the TCR there is positive selection for cells recognizing foreign antigenic peptides when presented by self-MHC antigens as well as negative selection against cells expressing TCR which recognize self-MHC antigens alone or with self peptides and would thus be self reactive. How this T cell differentiation occurs intra-thymically to generate the repertoire of the mature peripheral T cell pool from the haemopoietic precursor cells has been a subject of great interest and controversy.

Mature functional peripheral T lymphocytes can be divided into subsets according to their expression of CD4 and CD8. The expression of CD4 and CD8 on mature T cells is

mutually exclusive (Dialynas *et al.*,1983a, 1983b). Approximately two-thirds of peripheral T cells are $CD4^+,CD8^-$ and are almost always class II MHC-restricted while one-third are $CD4^-,CD8^+$ and are usually class I MHC-restricted (see T Cell Accessory Molecules - CD4 and CD8). A very small proportion of peripheral T cells do not express CD4 or CD8 and will not be discussed further.

Thymic lymphocytes are believed to be immature T cells at various stages of development from precursor to mature T cells. Attempts have been made to identify subpopulations of thymocytes defined by cell surface antigens and then by using this information order these subpopulations into a sequence representing intra-thymic maturation. For the purpose of this discussion only CD4 and CD8 will be considered.

Flow cytometric analysis of thymocytes stained with anti-CD4 and anti-CD8 monoclonal antibodies define 4 subpopulations. The $CD4^+,CD8^+$ (double positive) population are the largest group and include small cortical cells and thymic blasts. These cells are mostly located in the thymic cortex and most of them die within the thymus. The significance of this population is unknown (Scollay *et al.*, 1984). The two groups of single positive cells, $CD4^-,CD8^+$ and $CD4^+,CD8^-$, have the same size profile determined by flow cytometry as the medullary cells. Other studies have confirmed that most medullary cells are either $CD4^+,CD8^-$ or $CD4^-,CD8^+$. Furthermore, the ratio of the two subgroups of single positive medullary cells is similar to that found in mature peripheral T lymphocytes (Scollay and Shortman, 1983) The cells leaving the thymus seem to be functionally competent and committed to the $CD4^+,CD8^-$ or $CD4^-,CD8^+$ lineages but with a few maturational changes to undergo (Scollay, 1982). The emerging thymocytes are very similar to the medullary thymocytes, however it is not absolutely certain whether they derive from the medullary cells or from a small phenotypicaly similar population in the cortex (Scollay *et al.*, 1984).

The fourth population of thymocytes lack both the function associated antigens, CD4 and CD8, seen on the mature T cells. Fluorescence labelling of the thymi selectively labelled outer cortical cells (Scollay and Weissman, 1980). Many but not all $CD4^-,CD8^-$

cells were labelled, suggesting that a proportion were located in the subcortical region. In other studies where mice were injected with ^{3}H-thymidine and their thymi removed 1 hour later 31% of double negative cells were labelled with the ^{3}H-thymidine indicating that this double negative population was rapidly dividing (Scollay *et al* .,1984).

Until recently, there has been no way to define a precursor product relationship between thymocyte subpopulations. There has beeen much debate about attempts to order the thymic subsets into a developmental pathway on the basis of phenotypic similarities. Most debate has centred around whether the double negative cells give rise to double positive cells (most of which die intrathymically) and that these double positive cells give rise to single positive cells or whether the double negative cells give rise to the three different subpopulations directly i.e. the double positives and the two subpopulations of single positive cells.

More recent evidence supports the view that mature CD4^{+} and CD8^{+} T cells derive from double positive precursors. Smith (1987) injected anti-CD8.1 and anti-CD8.2 monoclonal antibodies into irradiated bone marrow reconstituted mice. The mice were reconstituted with a 1:1 mixture of cells expressing Thy 1.2, CD8.1 and Thy 1.1, CD8.2. After 4 to 6 weeks of treatment with the anti-CD8 monoclonal antibodies the percentage of Thy 1.1^{+}, CD4^{+} and Thy 1.2^{+}, CD4^{+} was determined for each group. The Thy 1.1^{+}, CD4^{+} T cells were significantly depleted in chimeras injected with anti-CD8.2 likewise Thy 1.2^{+}, CD4^{+} T cells were significantly depleted in chimeras injected with anti-CD8.1. This data supports the hypothesis that CD4^{+}, CD8^{-} T cells arise from CD4^{+}, CD8^{+} thymocytes *in vivo*. Furthermore, in irradiated bone marrow reconstituted chimeras injected with anti-CD4 monoclonal antibody double positive and both subpopulations of single positive thymocytes were deleted suggesting that double positive precursors give rise to CD8^{+} cells.

Kisielow *et al.* (1988) constructed C57BL/6 transgenic mice expressing and α/β TCR specific for H-Y antigen in the context of class I MHC H-2D^{b} antigens. They demonstrated that both male and female transgenic offspring had T cells which expressed

this α/β TCR but the frequency of cells was higher in the female than in the male mice. Furthermore, the phenotype of the male (but not the female) T cells was abnormal. Over 90% of peripheral T cells in the male transgenic mice were $CD4^-$, $CD8^-$ or expressed low levels of CD8 and the number of $CD4^+$, $CD8^-$ T cells was small. By examining the male thymuses, Kisielow *et al* showed that the unusual peripheral T cell phenotype was the result of the deletion of autospecific thymocytes expressing high levels of CD8 i.e. the $CD4^+$, $CD8^+$ thymocytes. The intrathymic deletion of $CD4^+$, $CD8^+$ T cells spared cells expressing low levels of CD8 but affected the precursors of $CD4^+$, $CD8^-$ cells which were not male specific. This observation provided strong evidence that $CD4^+$, $CD8^+$ thymocytes contain the precursors for single positive $CD4^+$, $CD8^-$ and $CD4^-$, $CD8^+$ T cells and supported the observations of Smith (1987).

Kappler *et al.* (1987a) made the ~~serendipidous~~ serendipitous observation that a $V\beta$ gene of the TCR, $V\beta 17a$, the product of which is bound by the monoclonal antibody KJ23a, is expressed at a very high frequency in T cells which respond to allogeneic forms of the IE class II MHC protein. Kappler *et al.* (1987b) observed that the mature peripheral T cells of mouse strains which do not express IE molecules because of deletion of the E_α gene (e.g. SJL mice) expressed the $V\beta 17a$ gene while C57BR mice which do express IE did not express $V\beta 17a$ on their T cells. They concluded that because $V\beta 17a^+$ T cells react with various allelic forms of IE molecules, T cells expressing $V\beta 17a$ are eliminated in the C57BR mice and not the SJL mice during induction of tolerance to self-MHC molecules. Roehm *et al.* (1984) demonstrated that the level of expression of the TCR was very low, 10-20% of that of peripheral T cells, on immature cortical thymocyte and that the level increased to that expresed by peripheral T cells in medullary thymocytes. Thus, the level of expression of the TCR is a measure of maturity of the T cells. Kappler *et al.* (1987b) used the monoclonal antibody KJ23a to detect the expression of the $V\beta 17a$ gene in immature and mature thymocytes. They demonstrated that SJL thymocytes which expressed both CD4 and CD8 (i.e. double positive cells) had low fluorescence for KJ23a as measured by flow cytometry indicating a low expression of the $V\beta 17a$ gene while single positive

thymocytes i.e. $CD4^+$, $CD8^-$and $CD4^-$,$CD8^+$ had high fluoresence for KJ23a indicating a high level of $V_\beta 17a$ gene expression. Kappler *et al.* (1987b) then studied thymocytes from C57BR mice to determine whether the expression of IE molecules resulted in the elimination of $V_\beta 17a^+$ thymocytes and if they were eliminated at what stage of maturation this occurred. These results showed that immature $CD4^+$,$CD8^+$ thymocytes from SJL and C57BR mice had a similar percentage of KJ23a bright thymocytes. However, the percentage of KJ23a bright thymocytes was significantly greater in single positive SJL thymocyte than in C57BR thymocytes. They concluded that in mice expressing IE molecules, $V_\beta 17a$ expression was normal in the immature thymocyte population and that the $V_\beta 17a^+$ clones are eliminated at some time during thymocyte maturation. Therefore, tolerance to self-MHC antigens is due to clonal deletion in the thymus either during or before thymocytes mature into single positive cells.

There remains a major unsolved problem. In normal animals it is not known to what extent T cell tolerance is induced during intrathymic maturation rather than following the emigration of the T cell from the thymus. Is tolerance the result of elimination of self-reactive T cell clones within the thymus or is it maintained by suppression of their activation without their actual elimination? Mullbacher *et al.* (1983) demonstrated that the presence of K^k in B10.4R (K^k, D^b) mice or in F1 hybrids greatly reduced the vaccinia-immune-D^b T cell response, while the presence of K^b, K^d and K^q alleles did not. The presence of precursors for vaccinia-immune-D^b T cells in the nonresponder mice was demonstrated by transfer of (B10 X B10.4R) F1 nonresponder spleen cells into infected lethally irradiated B10 responder recipients. The resultant chimeras ~~generayed~~ generated vaccinia-immune-D^b effector T cells. They concluded that the effect of K^k on vaccinia-immune-D^b T cell response was the result of self tolerance mechanisms which cross reactively suppressed both K^k-immune and vaccinia-immune-D^b T cell precursors.

Tolerance to self-antigens involves tolerance not only to MHC molecules but also to other self-antigens which may be presented in association with MHC antigens. Many of these non-MHC self-antigens are not expressed intrathymically and therefore it may be

assumed that tolerance to these non-MHC self-antigens is established after emigration of the T cell from the thymus. Some early experiments by Dresser (1962) and Chiller *et al.* (1971) demonstrated that foreign γ-globulin protein antigens injected into mice induced a state of T cell tolerance to subsequent injections with immunogenic forms of the same antigen. The mechanism by which these experiments induced a state of tolerance is unknown but tolerance must be induced extrathymically because T cells ~~were~~ where exposed to tolerizing antigen after passage through the thymus.

The T Cell Receptor (TCR).

One of the major challenges in immunology over the last few years has been to characterize the structures on the T cell which enable it to recognize foreign antigens in the context of class I or class II MHC molecules. The first isolation of the TCR was indirect. A monoclonal antibody, reactive with a tumour-specific epitope on a murine T cell lymphoma, was found to precipitate a cell surface molecule that was composed of two disulphide-linked polypepties, (Allison *et al.*, 1982). However, because antigen specific-functions for these T cell specific surface proteins could not be demonstrated it was not initially recognized as the TCR. Meuer *et al.* (1983a; 1983b) provided the first information about the structure of the functional TCR using clonotypic monoclonal anibodies reactive with $CD4^+$ and $CD8^+$ T cell clones. They demonstrated that these antibodies could block antigen-specific cytolysis and proliferation. Two peptides were identified from immunoprecipitates prepared with these monoclonal antibodies. When the TCR peptides were isolated from the T cells one was designated α and one β (Kappler *et al.*, 1983a; 1983b). Both the α and β chains were shown to contain variable and constant regions.

In both the mouse and the human, the TCR complex for Th and Tc cells is composed of at least five polypeptide chains. (Borst *et al.*, 1982; Meuer *et al.*, 1983c). Two of these are the highly variable disulphide-linked α and β subunits, each assembled by somatic rearrangement from pools of separate gene segments (see below). This αβ heterodimer

mediates antigen recognition when antigen is associated on the surface of a cell with either class I or class II MHC products. The αβ heterodimer is associated non-covalently with the three monomorphic subunits CD3 (T3) that are believed to mediate signal transduction when the the αβ heterodimer binds to antigen.

Hedrick*et al.* (1984a) and Yanagi *et al.* (1984) isolated cDNA encoding the β-chain by using a research strategy based on the following assumptions:

1) the TCR would be expressed in T cells not B cells;

2) the mRNA for the TCR would be found on membrane-bound polysomes; and

3) the TCR genes would be rearranged and have variable,V and constant, C regions in a manner analagous to immunoglobulin genes.

The next gene which was identified by Saito *et al.* (1984a) was thought to be that of the α-chain. However, the predicted amino acid sequence of the protein did not correspond with the second peptide expressed on human T cell tumour lines. (Hannum *et al.*, 1984, Jones *et al.*, 1985). Shortly after this a third cDNA encoding a peptide consistent with that of the α-chain already characterized was isolated. The gene which had been isolated by Saito *et al.* (1984b) was designated γ. The α and β chain make up the T cell receptor heterodimer on the mature T cell. The role of the γ-chain chain is discussed below.

The α and β chains of TCR, like both the light and heavy chains of immunoglobulin, have variable (V) and constant (C) regions (see figure 1) (Kappler *et al.*, 1983a; McIntyre and Allison, 1983). The variable portion of β genes consists of a variable (V_β), a diversity (D_β) and a junctional (J_β) gene segment selected from the repertoire. These gene segments rearrange during T cell ontogeny to form a functional V segment (reviewed in Davis, 1985; Kronenberg *et al.*, 1986). Similarly the V_α genes rearrange V_α and D_α segments but no J_α gene segments have been demonstrated (Chien *et al.*, 1984b; Saito *et al.*, 1984b; Arden *et al.*, 1985; Hayday *et al.*, 1985). Somatic mutation has been demonstrated to occur in TCR genes which gives rise to further diversification of receptor specificities (Augustin and Sim,1984).

A recent study by Rupp *et al.* (1987) showed that a cytotoxic T cell clone and a helper T cell clone have identical V_α andV_β regions and thus have a similar idiotype on their TCRs, but do not exhibit any cross reactivity. It therefore seems that the antigen and MHC specificity of the TCR lies in the combination of expressed$V$, D , J and C gene segments.

The Beta Chain.

The variable portion of the β chain consisits of a variable (V_β), diversity (D_β) and junctional (J_β) segments. The expressed V_β gene repertoire appears to be quite small. There are about 20-30 V_β gene segments located upstream of the two clusters of one D, seven J (six of which are functional) and any V_β–J_β–D_β combination may be possible except that $D_{\beta 2}$ only rearranges with $J_{\beta 2}$ (reviewed in Davis, 1985; Kronenberg *et al.*, 1986).

There are two similar C regions, $C_{\beta 1}$ and $C_{\beta 2}$ (Chien *et al.*, 1984a), which differ by four residues in the mouse (Gascoigne *et al.*, 1984) and by five residues in the human (Tunnecliffe *et al.*, 1985). Both Th cells and Tc cells can use the same C_β gene (Hedrick *et al.*, 1985) and they are not analogous to immunoglobulin genes where each of the multiple constant regions of the heavy chain defines an immunoglobulin class and effector function.

The genes encoding the C regions are arranged in four exons interrupted by introns of nearly identical length which are located in similar positions to those found in immunoglobulin genes. (Gascoigne *et al.*, 1984, Malissen *et al.*, 1984). The exons appear to encode an external domain with an internal disulphide bridge, a small hinge-like region containing cysteine residues capable of forming a disulphide bond with the other chain of the heterodimer as well as a transmembrane region (not homologous with any other immunoglobulin genes), and a cytoplasmic tail.

The Alpha Chain.

The TCR α chain was discovered in a way similar to the β chain. The cDNA clones encoding the a chain have a predicted amino acid sequence that indicates it has regions corresponding to leader, variable, joining, constant, transmembrane, and intracytoplasmic regions. (Chien *et al.*, 1984b). There is only a single constant region in the a chain which is shorter than that of other TCR genes. At the present there are thought to be 50-100 V_α gene segments separated by an unspecified distance from at least 20 J region segments. The presence of a D region in the a chain remains controversial and any V_α-J_α combination appears possible (Arden *et al.*, 1985, Hayday *et al.*, 1985, Winoto *et al.*, 1985, Yoshiakai *et al.*, 1985).

The Gamma Chain.

The gene encoding the γ chain was the second gene to be identified in the search for the TCR structure (Saito *et al.*, 1984a). Initially it was mistaken for the a chain until it was realized that the gene that had been isolated could not encode the a chain polypeptide because of the absence of sites for N-glycosylation. The γ chain gene, identified by Saito *et al* ., (1987) shares several characteristics with the a and b genes:

1) it is expressed only in T cells;

2) it is assembled from gene segments which resemble the C, J and V regions of immunoglobulins;

3) it has structures resembling the transmembrane and intracytoplasmic regions of integral membrane proteins and

4) it has a cysteine residue in the position expected for interchain disulphide bridging (Helig *et al.*, 1985).

Until recently there was no evidence that the expression of the γ chain gene was associated with any functional subset of T cells (the αβ heterodimer is associated with Th cells and Tc cells). Furthermore, in most mature T cells, it is abortively rearranged and the transcript cannot be translated into a protein product.

Several groups used a strategy based on the tenet that any antigen receptor on a T cell would be associated with the CD3 complex. After isolating lymphocytes which express CD3 in the absence of the αβ heterodimer, four groups independently reported a TCR complex which is composed of a dimer containing a product derived from the γ gene in association with CD3 (Brenner *et al.*, 1986; Moingeon *et al.*, 1986; Bank *et al.*, 1986; Weiss *et al.*, 1986a). This was the first evidence that the γ gene coded for a cell surface product.

The Delta Chain.

A fourth TCR gene termed "x" was identified by Chien *et al.*, (1987a). Protein sequencing (Born *et al.*, 1987) and analysis with anti-peptide antisera (Loh *et al.*,1987) have shown that "x" encodes the δ chain of the χδ TCR. The TCR δ chain variable region can be assembled from four distinct gene segments V, D_1, D_2, J which is more than any other antigen receptor gene (Chien *et al.*, 1987b). Both *D* region genes can be used in a VD_1D_2J joining event. It is of interest that the three types of antigen-receptor pairing (heavy and light chains of immunoglobulin, αβ and γδ heterodimers of the TCRs) combine D region containing polypeptides with non-D species. This may reflect some use for antigen recognition in such pairings.

Two distinct types of of TCR have thus far been identified: the αβ heterodimer which recognizes antigen in association with MHC molecules, and the γδ receptor which is first detected approximately two days before the αβ heterodimer during T cell ontogeny on $CD4^-$, $CD8^-$ thymocytes (Pardoll *et al* .,1987; Bluestone *et al* .,1987). The γδ receptor bearing cells show MHC-unrestricted cytotoxicity (Borst *et al.*, 1987, Moingeon *et al.*, 1987)

In the human there are at least two forms of the "new" TCR. The molecular mass of the γ chain gene product identified by Brenner *et al.* (1986) is significantly larger than the γ chain gene product identified by Borst *et al.* (1987). The γ chain described by Brenner *et al.*, (1986) is a 55-60 kilodalton polypeptide non-disulphide-bonded to a 40 kilodalton

non-γ product. This receptor is clearly quite different from the receptor described by Borst *et al.,* (1987) which contains a 36-40 kilodalton γ chain linked to the non-γ chain by a disulphide bond. There is strong evidence to support both the difference in size and disulphide linkages of the two γ chains by the differential usage of the the two C_γ gene segments, $C_\gamma 1$ and $C_\gamma 2$ (Littman *et al.,* 1987). The functional significance of these two forms of the γ chain is unknown.

The role for this new γδ TCR is not clear. It appears to be present on cells with generalized cytotoxicity because Borst *et al.* (1987) have reported that cells bearing this receptor have cytotoxicity against a broad range of targets and Brenner *et al.* (1986) demonstrated that cytotoxic activity of clones bearing γ chain containing receptors are not blocked by antibodies to MHC products. Furthermore, this receptor does not appear to be the receptor for either the natural killer (NK) or suppressor subsets of cells (Moingeon *et al.,* 1986, Robertson, 1987).

Figure 1.

SCHEMATIC REPRESENTATION OF THREE CELL SURFACE INTERACTION MOLECULES.

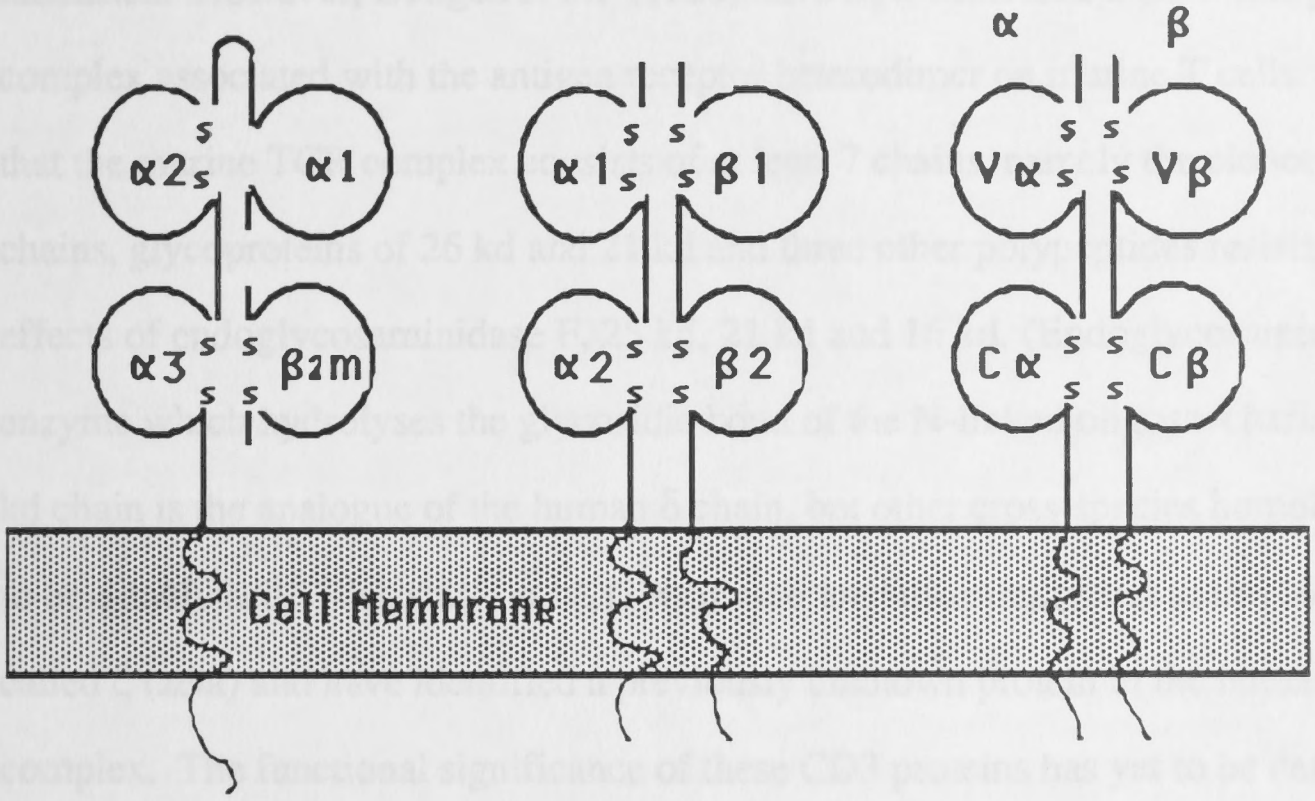


Diagram showing the domain structure of class I and class II MHC molecules and the T cell receptor. The association of β_2 microglobulin with the class I MHC molecules is also shown.

Adapted from Hunkapiller and Hood (1986).

The CD3 Complex.

The CD3 complex is composed of several proteins, a γ chain with a molecular weight of 28-25 kd, and δ and ε chains of 20 kd each. The γ and δ chains are glycoproteins, whereas the ε chain does not contain detectable oligosaccharides (Weiss *et al.*, 1986a).

Until recently, a murine complex homologous to the human CD3 had not been identified. However, Oettgen *et al.* (1986) have now described a CD3-like protein complex associated with the antigen receptor heterodimer on murine T cells. They showed that the murine TCR complex consists of at least 7 chains, namely the clonotypic α and β chains, glycoproteins of 26 kd and 21 kd and three other polypeptides resistant to the effects of endoglycosaminidase F, 25 kd, 21 kd and 16 kd. (Endoglycosaminidase F is an enzyme which hydrolyses the glycosidic bond of the N-linked oligosaccharides). The 26 kd chain is the analogue of the human δ chain, but other cross-species homologies have yet to be established. Weissman *et al.* (1986) used an antiserum raised to the 16 kd chain called ζ (zeta) and have identified a previously unknown protein in the human TCR complex. The functional significance of these CD3 proteins has yet to be determined.

T Cell Activation.

T cells exist in two states, a "resting" state, when they do not contribute to an ongoing immune-response, and an "active" state, when they proliferate, secret lymphokines and / or are cytotoxic. T cell activation is therefore essential to immune responses. In physiological circumstances a lymphocyte will become "active", i.e. express its latent functional properties, and undergo cell division, only when it encounters an antigenic determinant that it can bind. Its daughter cells will express receptors with the same binding site. This is a tenet of the clonal selection theory of Burnet.

In vivo and *in vitro,* T cells may be activated not only by antigen presented on antigen presenting cells and by anti-idiotypic antibodies (Ertl and Finberg, 1984) but also by ligands which interact with the TCR complex, (the αβ heterodimer and CD3). Thus, not only mitogens but also some monoclonal antibodies directed against non-idiotypic epitopes

of the αβ heterodimer or CD3 can activate T cells.

In 1975, Lafferty and Cunningham proposed a two-signal model for *in vitro* T cell activation by alloantigen. T cell activation occurred when the responding cell bound antigen through its surface receptor (signal 1) and simultaneously received a second signal, "lymphocyte costimulator", from a metabolically active stimulator cell. Neither signal 1 nor signal 2 alone is sufficient for T cell activation. There has been some controversy surrounding the nature of the second signal required for T cell activation. This accessory cell-mediated second signal can be replaced by soluble factor(s) - lymphocyte activating factor (LAF) which is produced by stimulated macrophages or by phorbol myrisate acetate (PMA) (Rosenstreich and Mizel, 1979). Both IL-1 (Williams *et al.,* 1985 and Manger *et al.,* 1985) and IL-2 (Rosenberg *et al.,* 1984) have been implicated as providing the costimulator activity.

Williams *et al.,* (1985) investigated the early steps for primary T cell activation by measuring increased RNA synthesis. Using a monoclonal antibody directed against CD3, Williams *et al* demonstrated that activation was dependent upon the presence of accessory cells when the antibody was in a soluble form but IL-1 replaced the accessory cell requirement when the monoclonal antibody was bound to the surface of sepharose beads. They concluded that activation of normal resting T cells required not only a signal delivered through CD3 complex but "cross-linking" to the accessory cell membrane or to the solid phase sepharose beads as well as a second signal IL-1.

The availability of purified recombinant IL-1 (rIL-1) with the same biological properties as macrophage derived IL-1 had allowed Kiode *et al.* (1987) to investigate the effects of rIL-1 on dendritic cells and T lymphoblasts. Their results indicated that rIL-1 does not act as a lymphocyte activating factor (signal 2) but it amplified the effects of dendritic cells when they were treated with rIL-1 prior to use as accessory cells. It appeared that IL-1 acted by some mechanism which enhanced clustering of Th cells with dendritic cells before the onset of mitogenesis. The ~~dicotomy~~ dichotomy between these results and those of Williams *et al.*, (1985) is not easily explained. Williams *et al.* (1985) used highly purified supernatants

from human *Staphlococcus albus* -stimulated adherent mononuclear cells. The supernatants were initially concentrated 20-fold, purified by specific immunoadsorption followed by two stages of gel-filtration and ion exchange chromatography (Rosenwasser and Dinarello, 1982). This purified supernatant gave a single stained band on 7.5% sodium dodecyl sulphate polyacrylamide gels. However, the possibility that another unidentifed compound which mediates signal 2 was co-purified with IL-1 cannot be excluded. The identification of the soluble factor which mediates signal 2 in T cell avtivation is still not known.

Once activated, T cells both express receptors for, and secrete, IL-2 (formerly T cell growth factor). A continuous supply of IL-2 is required to maintain activated T cells *in vitro* or to clone T cells (Schreier *et al.,* 1980). With the ability to grow and maintain clones of T cells, it became possible to undertake more detailed studies of T cell activation. Meuer*et al.* (1984) demonstrated that T cell activation and proliferation required several steps. Resting T cell clones show few IL-2 receptors but display a maximal number of TCR. However, TCR receptor triggering i.e., activation, by either antigen presented in the context of the appropriate MHC, or by sephrose-bound anti-clonotypic monoclonal antibodies, resulted in a decrease in the number of surface antigen receptors and a rapid induction of the expression of IL-2 receptors. Furthermore, such activation led to endogenous IL-2 production, secretion and binding to the IL-2 receptors of the clones. Once a "critical" number of IL-2 receptors have bound IL-2, then DNA synthesis and mitosis occurs. Finally, in the absence of continued antigenic stimulation, a reciprocal situation occurs with reduction in the number of IL-2 receptors and a concomitant increase in antigen-specific receptor expression and a return to the resting state.

Williams *et al.* (1985) and Manger *et al.* (1985) demonstrated a fundamental difference in the accessory cell requirements for the reactivation of antigen-primed T cell clones as compared with the primary activation of resting T cells by mitogenic anti-CD3 monoclonal antibody. Activation of resting cells required two signals. In contrast, many T cell clones will generate IL-2 receptors and proliferate upon stimulation of the T cell antigen

receptor complex by monoclonal antibodies bound to sepharose beads and directed against the TCR complex without the apparent need for a second signal.

Studies with highly purified murine T cells have demonstrated that lectins in the absence of macrophages or PMA can induce a proliferative response. This proliferation is dependent on an exogenous source of IL-2 and is restricted to the $CD8^+$ subset of T cells (Erard *et al.*, 1985a; 1985b; 1985c; Vohr and Hunig,1985). In contrast, proliferation of purified $CD4^+$ cells requires the presence of macrophages or PMA but not IL-1in addition to a lectin. (Erard *et al.*, 1985c; Czitrom *et al.*, 1983). These data suggest that the minimal requirements for activation differ between the two major subsets of T cells. The $CD8^+$ subset can be induced to repond to IL-2 by triggering of the TCR complex in the absence of accessory cells, whereas activation of the $CD4^+$ subset is accessory cell dependent. Furthermore, the inability of IL-1 to replace the accessory cell or PMA requirement in activation of $CD4^+$ T cells is consistent with the data of Kiode *et al.* (1987) that IL-1 does not provide the second signal. The reason for differences in the activation requirements for the $CD4^+$ and $CD8^+$ T cell subsets is unknown.

Activated T cells can be induced to secrete lymphokines by stimulation of their TCR without the requirement of a second signal. This interaction was used to study the interaction of the activated T cell with its priming antigen by Hodgkin (personal communication). He demonstrated that for a given activated T cell - target cell interaction the efficiency of lymphokine release is determined by the probability of triggering and this in turn is determined by the nature of the target cell. He showed that, under conditions of activated T cell excess, the order of reaction does not vary with the target cell but is dependent upon the stimulator cell used to prime the T cell *in vitro*. Thus, CBA anti-BALB/c spleen cells had a reaction order of 1 indicating that one T cell - target cell interaction was necessary to release lymphokine, while CBA anti-P815 cells ~~reqired~~ required 2 T cell - target cell interactions for lymphokine release using the same P819 target cell population. However, by varying the assay conditions to conditions of target cell excess designed to test T-T cell interactions no difference in the order of reaction by CBA

anti-BALB/c or CBA anti-P815 could be detected when P815 cells were used to trigger lymphokine release. In both situations the order of reaction was one indicating that T-T cell interactions are not required. Thus, the conditions under which T cell activation occur clearly influence the nature of the interactions necessary to activate T cells. Careful assessment of the conditions used in assays is essential before any comparisons of T cell activation can be made between studies.

More recently, biochemical indicators have been used to study the early events of T cell activation. The mechanism by which the cell surface receptor transmits a signal that activates the cell has been studied extensively. Increases in cytoplasmic free calcium play a prominent role in signal transduction by a variety of cell-surface receptors. The observation that calcium ionophores and PMA are mitogenic for T cells and that lectins increase intracellular calcium suggests that changes in intracellular calcium are important in T cell activation (Weiss *et al.*, 1984, Imboden and Stobo, 1985 and Oettgen *et al.*, 1985). Receptor mediated increases in intracellular calcium can be due to an uptake of extracellular calcium, to a release of intracellular calcium stores or a combination of both. The initial increase in free intracellular calcium is due to a release of calcium from intracellular stores. This release of the calcium in turn appears to be mediated by inositol triphosphate, the putative mobilizer of intracellular calcium triggered by hormone receptors. The process of transmembrane signalling links the TCR-CD3 complex to a potent transmembrane transducting mechanism, because the hydrolysis of polyphosphoinositides generates a number of intracellular messengers. (See Berridge,1984, for a review of intracellular messengers).

The Major Histocompatibility Complex.

Throughout vertebrate evolution the major histocompatibility complex (MHC) has been conserved so that in mammals it is a multigene family whose members encode polypeptides which are glycosylated and expressed on cell surface membranes. Class I and class II MHC molecules, immunoglobulins and the T cell receptor are all members of the immunoglobulin supergene family. [A supergene family is a set of multigene families and

single-copy genes related by sequence, which implies a common ancestry, but which are not necessarily related in function (Hood *et al.*, 1985)]. Class I and class II MHC glycoproteins are of fundamental importance to the recognition of foreign antigens by T cells. The MHC (H-2 complex in mice and HLA complex in humans), which regulates the immune response, was first recognized in mice using inbred strains. By grafting skin between these mice and studying acceptance or rejection of the grafts, it was possible to map the rejection of non-self to a region of chromosome 17 of the mouse and this was denoted the histocompatibility-2 (H-2) complex (Gorer *et al.*, 1936a, 1936b).

Organization of MHC Class I Genes.

The class I genes of the murine MHC complex, often referred to as the major transplantation antigens, are encoded in the *K* and *D* regions. Another group of class I genes is located telomeric of *H-2D* and encodes the Qa and Tla antigens (see figure 2). The antigens of the *K* and *D* regions are present on almost every cell in the body, albeit at varying concentrations (Klein 1975), and are involved in the recognition of virus-infected or neoplastic cells by self Tc lymphocytes, as well as provoking rejection of grafted foreign tissues (Zinkernagel and Doherty, 1979).

The *K* region encodes two molecules, K1 and K2 as determined by serological methods (Ivanyi and Demant,1981; Tryphonas *et al.*, 1983). The presence of these two *K* region molecules has been supported by DNA cloning in the $H\text{-}2^{d}$ haplotype by Steinmetz *et al.*,(1982a). Serological and molecular genetic techniques have yielded conflicting results regarding the number of molecules encoded by the *D* region. Five D^{d} class I molecules have been identified by serological techniques - the D molecule (Gorer and Mikulska,1959 and Hansen *et al.*, 1981), L molecule (Lemmonier *et al.*, 1975; Neauport-Sautes *et al.*, 1977; 1978 and Hansen *et al.*, 1977;1981), M molecule (Ivanyi and Demant, 1981.), R molecule (Hansen *et al.*, 1981) and L2 molecule (Ivanyi and Demant, 1982). Only D^{d} and L^{d} have been cloned so far and it is not clear whether the R^{d}, M^{d} and $L2^{d}$ molecules are encoded by separate D^{d} region genes some of which have

not yet been cloned or, alternatively, are splicing products or post-translationally modified products of the known *D* region genes.

Several of the class I genes have been cloned and sequenced (L^d Evans *et al.*, 1982 and Moore *et al.*, 1982; K^d Kvist *et al.*, 1983; K^b Weiss *et al.*, 1983; K^k Arnold *et al.*, 1984a and D^d Sher *et al.*, 1985). These class I genes all have a common structure. The first exon encodes the signal sequence, the 2nd, 3rd and 4th exons encode the α_1, α_2 and α_3 regions respectively, the 5th exon encodes the transmembrane region of the molecule and the remaining 3 exons encode the cytoplasmic tail and the 3' untranslated region.

In contrast to the class I antigens encoded in the *H-2* region of the MHC (H-2K, H-2D and H-2L), the antigens encoded in *Qa* region and the *TLa* regions are less polymorphic and their expression is limited to certain tissues (Boyse and Old, 1969; Hood *et al.*, 1983). The Qa and TL antigens are not involved in antigen presentation for recognition by Tc cells and their function is unknown. However, they can act as target molecules for alloreactive Tc cells (Forman, 1979, Kastner *et al.*, 1979, Wernet and Klein, 1979). The number and organisation of the Qa and Tla antigens varies depending on the haplotype. Some of these molecules are expressed on various tissues during ontogeny and may serve as markers of differentiation (Klein *et al.*, 1983).

More recent studies with the K^b mutants suggest that some of the genes of the *Qa* region may have acted as donors of gene sequences in genetic recombinations to generate these mutant class I genes. These observations have led to the hypothesis that genetic interaction between class I genes may be the driving force behind the production of the K^b mutants and may be a mechanism by which the polymorphism of MHC molecules is generated. (Weiss *et al*, 1983, Pearse *et al*, 1983, Klein *et al.*, 1983). No gene of the *Tl* region has been found to act as a donor in studies with the K^b mutants. (Pontarotti *et al*, 1986).

Organization of MHC Class II Genes.

The *I* region lies between the *K* and *D* regions of the *H-2* complex. Only four *I* region antigens (Ia) have been identified by serological and biochemical analysis (Jones,1977 and Uhr *et al.*, 1979). The *I-A* region encodes three of these antigens, A_β, A_α and E_β. The fourth antigen, E_α, is encoded in the *I-E* region (Jones *et al.*, 1978). However, Steinmetz *et al* ., (1982b) have mapped 7 class II gene sequences in the $H\text{-}2^d$ haplotype. Centromere to telomere they are $A\beta_3\text{-}A\beta_2\text{-}A\beta\text{–}A\alpha\text{–}E\beta\text{–}E\beta_2\text{-}E\alpha$.. Similar class II genes have been identified in the $H\text{-}2^b$ haplotype by Devlin *et al.* (1984) with an additional locus, $E\beta_3$, located telomeric to $E\alpha$.. The $A\beta_2$ and $E\beta_2$ genes have been shown to produce mRNA, but it is not known whether this mRNA is translated into functional polypeptides (Wake and Flavell, 1985). Class II antigens are expressed preferentially by certain cells of the lymphoid and monocyte lineages e.g. B cells, macrophages, Langerhans cells in the skin, splenic and thymic dendritic cells and some T cells.

The phenomenon of class II restriction of T cell recognition of foreign antigens implies that mice of different haplotypes show immunologically significant structural polymorphism in the products of the *I* region genes which have also been well characterized by serological methods. Allelic forms of the $E\alpha$ gene have been cloned for the *k* (Mathis *et al.*, 1983) and *d* (McNicholas *et al.*, 1982) haplotypes. The $A\alpha$ gene has been cloned from 6 haplotypes; *k, d, b, u, f,* and *q* (Benoist *et al.*, 1983a; 1983b). Four alleles of the $E\beta$ gene have been cloned, *k,d, b* and *u*. (Saito *et al.*, 1983, Mengle-Gaw and McDevitt,1983, Widera and Flavell, 1984); and the $A\beta$ alleles have been cloned from the *k, d* and *b* haplotypes (Larhammer *et al.*, 1983; Choi *et al.*, 1983). Comparison of the nucleotide sequence for the allelic forms of the *Ia* genes shows that the nucleotide variability is extensive and clustered in the external domains of $A\alpha$, $A\beta$ and $E\beta$. In contrast, the two alleles of the $E\alpha$ show little difference in nucleotide sequence.

The *I-J* region has been identified serologically and is found to be expressed on a subset of Ts cells. However, there is some controversy surrounding the exact nature of the

I-J region. Firstly no product from this region has been identified biochemically [although anti-J sera have been raised (Murphy, 1978) and J-specific monoclonal antibodies produced (Waltenbaugh, 1981)]. Secondly, the region which encodes the putative I-J molecule may lie outside the *H-2* complex. (Steinmetz *et al.,* 1982b).

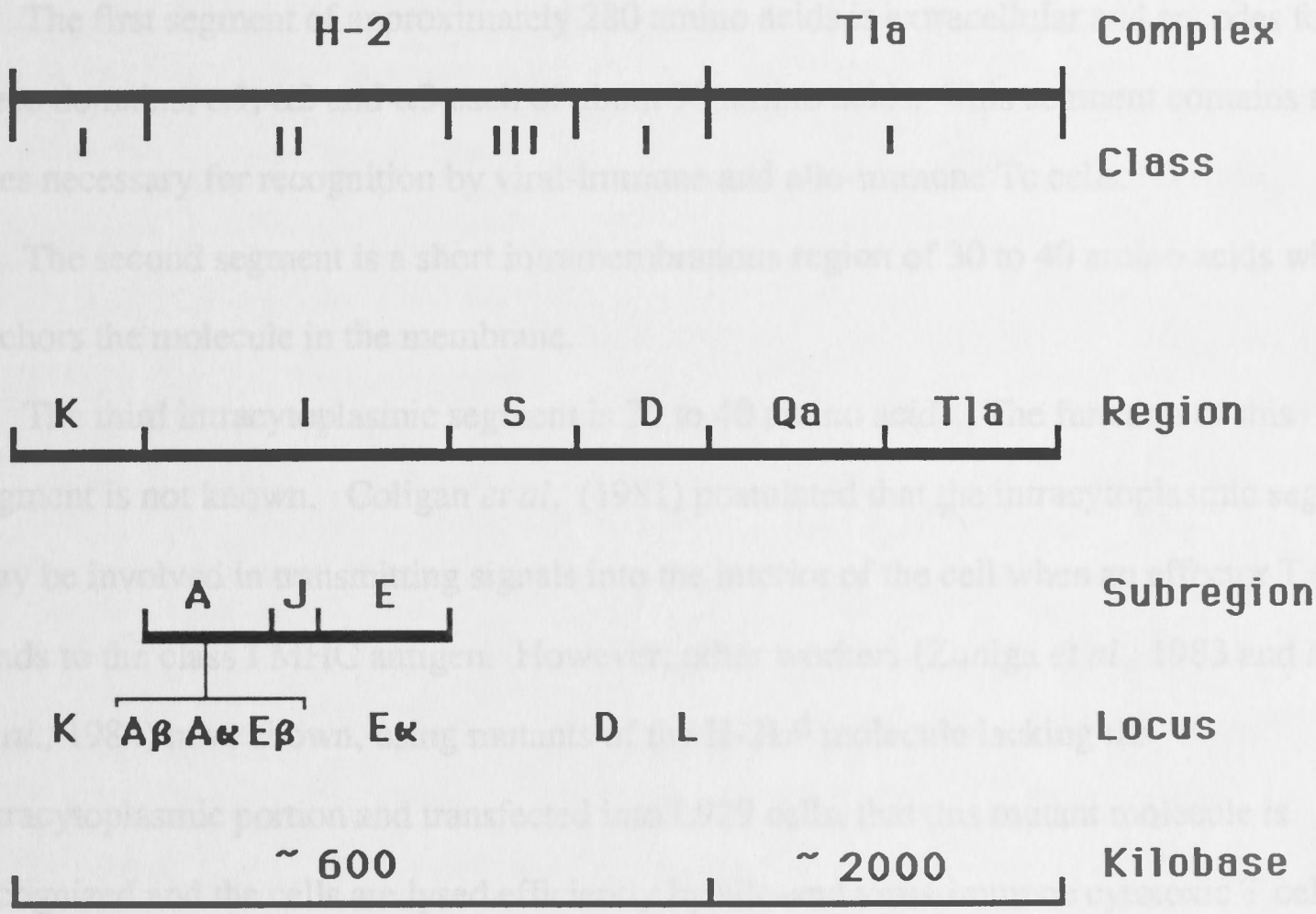
Organization of MHC Class III Genes.

The class III genes in the *S* region of the *H-2* complex encode polypeptides which are present in serum and absent from lymphocyte membranes. They encode certain components of the complement proteins C2, Bf, Slp, C4 and two genes encoding 21-hydroxylase which is involved with steroid biosynthesis (Chaplin *et al.,* 1983, White *et al.,* 1984). They bear no known structural or functional relationship to the class I and II genes and as such are outside the scope of this review.

Figure 2.

Region of Murine Chromosme 17 Including the

Major Histocompatibility Complex.



Map of a region of murine chromosome 17 showing the H-2 and Tla complexes showing regions, subregions and loci. A fuller desciption is given in the text.

Adapted from Hood *et al.* (1983).

Structure and Function of MHC Class I Antigens.

The Heavy Chain.

The class I antigens are glycoproteins composed of two chains (see figure 1). The heavy or H chain of approximately 45 kilo daltons molecular weight and a chain length of 340-350 amino acids, is integrated into the cell surface membrane and has three functional segments (Coligan *et al.*, 1981).

The first segment of approximately 280 amino acids is extracellular and encodes for the three domains, $\alpha 1$, $\alpha 2$ and $\alpha 3$ each of about 90 amino acids. This segment contains the sites necessary for recognition by viral-immune and allo-immune Tc cells.

The second segment is a short intramembranous region of 30 to 40 amino acids which anchors the molecule in the membrane.

The third intracytoplasmic segment is 30 to 40 amino acids. The function of this segment is not known. Coligan *et al.* (1981) postulated that the intracytoplasmic segment may be involved in transmitting signals into the interior of the cell when an effector T cell binds to the class I MHC antigen. However, other workers (Zuniga *et al.*, 1983 and Murre *et al.*, 1984) have shown, using mutants of the H-2L^d molecule lacking the intracytoplasmic portion and transfected into L929 cells, that this mutant molecule is recognized and the cells are lysed efficiently by allo-and virus-immune cytotoxic T cells.

The $\alpha 1$ and $\alpha 2$ domains of the class I H-2 antigens contain sites recognized by Tc cells, both during viral infection and allogeneic responses (Ozato *et al.*, 1983, Reiss *et al.*, 1983, Allen *et al.*, 1984, Arnold *et al.*, 1984b,1985, Bluestone *et al.*, 1985 and Scholler *et al.*, 1986). Maloy and Coligan (1982) compared the sequences of six molecules for which extensive information exists, K^b, D^b, K^d, L^d, D^d and Qa(27.1) and showed that the variability in the extracellular part of the molecules appears to be clustered in three regions. Studies of the sequence data for *K* and *D* region products by Coligan *et al.*, (1981) revealed a greater homology between the *K* region or *D* region products of the same haplotypes than between different alleles of the *K* region. It was accordingly suggested that gene duplication was more likely to have occurred after the allelic divergence

of the species from a common ancestral gene rather than before. The K alleles are not more related to each other than they are to the D alleles, thus there is no "K-ness" or "D-ness" in these molecules.

The α_1 and α_2 domains are also the regions in which amino acid changes are detected in K^b mutants (Nathenson *et al.*, 1986). It has been proposed that the generation of mutants arises from genetic interaction of the K^b gene with other class I genes. The amino acid sequences of the K^b mutants have allowed evaluation of structural and functional relationships in class I molecules. This work also provided a model for studying a possible underlying mechanism of gene diversification leading to the extensive polymorphism exhibited by this region of the MHC.

The spontaneously arising *in vivo* MHC mutants provide a useful model to study the role of the MHC gene products in immune recognition. The mutant mice which were identified by skin graft incompatibility with mice expressing wild-type antigen, a measure of T cell function, have alterations in their class I and/or class II MHC molecules (reviewed in McKenzie *et al.*, 1977; Kohn *et al.*, 1978). The most widely studied group are the K^b mutants, where gain-loss mutations could be detected by T cells without any significant recognition of the altered MHC product by B cells (Blanden *et al.*, 1976). Meleif *et al.* (1975) demonstrated using skin graft rejection and mixed lymphocyte reactions that the K^{bm1} mutant shows greater antigenic difference to wild type K^b than either K^{bm8} , K^{bm5} or K^{bm6}. This ~~heirachy~~ hierachy of antigenic differences between the K^b mutants and the wild type was also seen by Blanden *et al.* (1976) using recognition of virus-infected macrophage targets by virus-immune Tc cells. Taken together these data suggested that the antigenic sites on class I molecules recognized by syngeneic virus-immune T cells were also those recognized by alloimmune T cells. Furthermore, (K^{bm1} X K^{bm8})F1 mice did not show complementation with respect to target cell lysis thus indicating that the K^{bm1} and K^{bm8} mutations are in the same genetic element (Melief *et al.*, 1975, Melvold and Kohn, 1976).

Bjorkman *et al.* (1987a, 1987b) have recently determined the structure of a human class I molecule, HLA-A2, using X-ray crystallography. Their model shows that the

membrane- proximal end of the glycoprotein has two domains, $\alpha 3$ and β_2-microglobulin (see below), with tertiary structures resembling immunoglobulin domains but paired by a novel interaction not seen in immunoglobulin structure. The $\alpha 1$ and $\alpha 2$ domains are very similar to each other in structure and are not like immunoglobulin constant or variable domains. The domains, $\alpha 1$ and $\alpha 2$ form a platform composed of a single β–pleated sheet topped by α–helices with a long groove between the helices. This groove provides a binding site for peptides derived from processed antigen. Most of the highly polymorphic amino acid sequences of class I MHC molecules are clustered on top of the molecule in this groove, as would be expected from the functional studies over the preceeding decade or so reviewed above. The α helices and the associated peptide form the epitope bound by MHC class I restricted Tc cells via their TCR.

The Light Chain.

Beta$_2$-microglobulin, a 12 kilodalton (kd) polypeptide of 99 amino acids encoded by a non-MHC region on chromosome 2, is associated non-covalently with the third extracellular cytoplasmic domain, α_3, of the heavy chain and is not membrane bound. β_2-microglobulin contains a central disulphide linkage i.e. it is a single globular domain. In the mouse, β_2-microglobulin is dimorphic involving a single amino acid substitution at position 85 (Gates *et al.*, 1981). Although β_2-microglobulin and class I heavy chain genes are located on different chromosomes they are coordinately regulated, and regulation appears to be controlled transcriptionally (Hood *et al.*, 1983). Several studies have provided evidence suggesting that the surface expression of class I antigens is controlled by the expression of β_2-microglobulin in a manner analogous to the control of Ig heavy chain expression by light chain (Siden *et al.*, 1981). Studies by Potter *et al.* (1985) demonstrated the expression of H-2D^b on the surface of a mutant cell line EL4/Mar in the absence of any detectable cell surface β_2-microglobulin. They postulated that a glycosyl unit attached to the $\alpha 3$ domain of D^b could replace the requirement for β_2-microglobulin. However, the possibility that β_2-microglobulin dissociates from D^b shortly after insertion

into the cell membrane could not be excluded.

Structure and Function of MHC Class II Antigens.

The class II or Ia antigens are composed of two protein subunits (figure 1), a heavier α chain (molecular weight approximately 34 kilo daltons) and a lighter β chain (approximately 28 kilo daltons). Both the α and β chains are composed of 5 functional regions:

1) a leader sequence of approximately 25 amino acids which serves to guide and insert the polypeptide into the membrane whereupon it is enzymatically cleaved;

2) N-terminal extramembrane domains, α_1 and β_1, of 84-88 amino acids in the α chain and 96 amino acids in the β chain;

3) a second extramembrane domain, α_2 and β_2, approximately 95 amino acids;

4) a hydrophobic transmembrane region; and

5) a cytoplasmic tail.

Both external domains, β_1 and β_2 of the β chain, and the α_2 domain of the α chain contain disulphide linkages. The α and β subunits of the class II antigens are associated intracellularly with a third glycoprotein, the invariant chain, I_i, not coded for by the MHC (Day and Jones,1983). I_i is known to associate with the α and β chains in the endoplasmic reticulum but it has not been detected in association with cell surface expression of Ia (Sung and Jones, 1981). The exact role of this protein is unknown but it is thought to be involved with insertion of the class II molecules into the cell membrane.

Studies of structure-function relationships have demonstrated that both class I and class II molecules consist of four extracellular domains, three of which have internal disuphide bonds and two of which have the membrane proximal domains showing considerable homology to immunoglobulin constant regions. It is tempting to speculate that the clustered nature of the allelic polymorphism of the α1 and α2 domains of class I (Nathanson, 1986) and the α1 and β1 domains of the Aα, Aβ and Eβ chains of the class II MHC molecules suggests that these domains are functionally similar and are recognized by

the binding site of the T cell receptor. Mengle-Graw and McDevitt (1983) suggested that folding of the class II molecule may allow for juxtaposition of the three or four variable regions at the surface of the folded molecule where they could interact with antigen and/or the T cell receptor binding site.

Regulation of Class I and Class II MHC Antigen Expression.

Murine class I MHC antigens (K and D) are expressed on almost all cells during postnatal life (Klein *et al.*,1983). K and D antigens are not expressed on fetal cells until approximately day 7 of gestation but their cell surface concentration increases until adult levels are achieved (Klein, 1975). The level of class I MHC antigen expression can be influenced by both MHC and non-MHC genes (O'Neill and McKenzie, 1980, King and Parr, 1982) as well as by cell type (King and Parr, 1982, Parr *et al.*, 1982).

The Qa and Tla class I MHC antigens are expressed on lymphoid cells. The Tla antigens are expressed only on thymocytes at certain stages of differentiation and on certain leukaemias. The Qa antigens are expressed at varying levels on T and B cells however, most B and some T cells only express very low levels of these antigens (Klein *et al.*,1983). Clearly, there is regulation of expression of class I MHC however, little is known about the mechanisms of this regulation. The level of cell surface class I MHC antigen expression is dependent upon several variables including production of the MHC proteins, their insertion into the cell membrane, shedding and intracellular catabolism.

The level of class I MHC antigen expression can be increased *in vitro* by exposing the cell to interferons (IFN) α, β and γ (Kim *et al.*, 1983, Wong *et al.*, 1983) or IFN-containing solutions (King *et al.*, 1985). The IFNs increase the MHC antigen expression by increasing transcription of MHC genes and have a differential effect in their ability to increase the level of MHC antigen expression. γ-IFN is the more potent and increases the level of MHC antgien expression at lower concentrations than α– and β-IFN (Wallach *et al.*, 1982). Furthermore, the level of H-2K and H-2D class I MHC antigen expression can be independently regulated as demonstrated by King *et al.* (1985) on mouse embryo

fibroblast cell surfaces.

Studies by Machy *et al.* (1987) demonstrated that class I MHC antigens are internalized via coated pits in T cells but remain on the cell surface of B cells. Thus, class I MHC antigens are differentially regulated in T and B lymphocytes. The shedding of K antigens is different from that of D antigens in murine spleen cells. These different rates of shedding are influenced by the *K* and *D* alleles of the MHC as well as by non-MHC genes (Emerson *et al.*,1980).

Viral infection can also affect the level of MHC antigen expression with adenovirus (Paabo *et al.*,1986) ectromelia virus (Gardner *et al.*,1975) and measles virus (Rager-Zisman *et al.*, 1981) all documented to decrease the level of class I MHC antigen expression while murine hepatitis virus (Suzumura *et al.*,1986, Massa *et al.*, 1986), Epstein-Barr virus (McCune*et al.*, 1975) retrovirus (Flyer *et al.*, 1985) ~~Thelers~~ Theiler's murine encephalomyelitis virus (Rodriguez *et al.*, 1987) and flaviviruses (King and Kesson, 1988) all increased class I and / or class II MHC antigen expression.

Class II MHC antigens are expressed mainly on cells of the lymphoreticular system. Thus, their pattern of distribution is different to that of class I MHC antigens and it follows that class II MHC antigens are regulated independently of class I MHC antigens. Wong *et al.*, (1985) demonstrated that all IFNs (α, β and γ) increased the expression of class I MHC antigens while only γ–IFN increased the expression of class II MHC antigens on astrocytes *in vitro*.

Class I MHC antigens act as restriction elements for Tc cell responses while class II MHC antigens act as restriction elements for Th cell responses (Klein *et al.*, 1983). The increased level of class I MHC antigen expression causes a marked increase in susceptibility of the cells to lysis by Tc cells (O'Neill and Blanden, 1979, Kesson unpublished observation), while increased levels of class II MHC antigen expression caused a marked increase in T cell proliferation and IL-2 release (Liu *et al.*, 1989). The increased level of MHC antigens which can occur following treatment of cells with IFNs or infection with viruses may have a significant effect on self tolerance if T cell self tolerance

is determined quantitatively as proposed by Blanden *et al.* (1987). The up-regulation of MHC antigen expression could possibly break T cell self tolerance probably due to recognition and triggering of low affinity T cell clones (see "Tolerance").

DNA sequence elements involved in the regulation of class I MHC genes are starting to be identified. Transcriptional promoters and enhancers and DNA sequence elements involved in the interferon response have been identified in murine class I MHC genes (Israel *et al.*, 1986). IFN regulation of class I gene expression involves at least two mechanisms, one dependent upon sequences upstream and the other on sequences downstream of the H-2 promoter region. (Israel *et al.*, 1986; Korber *et al.*, 1987; Sugita *et al.*, 1987). Israel *et al.* (1986) showed that the promoter for the *H-2K^b* gene can be induced by the IFNs α,β and γ and that the IFN response sequence is necessary for induction to occur. However, the IFN response sequence is only active when associated with a functional enhancer sequence found in the promoter of H-2K^b and other class I genes. Korber *et al.* (1988) demonstrated that the magnitude of response to IFNs and the requirement for different elements in the promoter of the class I H-2D^d gene were cell specific and dependent on the type of IFN used. These studies suggest that different mechanisms are used to control the IFN response of H-2 promoters in different cells and that transcriptional regulation and IFN induction of H-2 antigen expression of class I MHC antigens is multifactorial. Since the quantitative variation in class I MHC antigen expression influences the aquisition of tolerance, MHC restriction and the efficiency of cell-mediated immune responses, class I promoters may have evolved multiple DNA sequence elements responsive to different regulatory pathways to ensure their specific and sensitive regulation.

Antigen Presentation.

T and B lymphocyte populations show a marked difference in their response to antigen, although similar rearrangements of gene segments are involved in generating the antigen receptors used by these two subsets of lymphocytes (Kronenberg *et al.*, 1986). B

lymphocytes express cell surface membrane immunoglobulin receptors which bind free antigen. In contrast, T cells express the αβ heterodimer as a receptor and respond to antigen i.e. peptides of 12-20 amino acids presented on the surface of cells in association with either class I or class II MHC antigens.

A critical feature in the development of modern concepts of antigen presentation was the discovery of the restriction imposed on T cell-macrophage interactions by the products of the MHC. Rosenthal and Shevach (1973) demonstrated that stimulation of T cells by soluble protein required the presentation of "antigen" by histocompatible macrophages. Furthermore, the interaction between T cells and macrophages could be blocked with antisera directed against the MHC antigens of the macrophage. This was interpreted as supporting an association event between the T cell and macrophage mediated by cell surface products of genes linked to the MHC. Later, Zinkernagel and Doherty (1974a) using lymphocytic choriomeningitis virus, and Shearer (1974) using TNP modified lymphocytes, showed that sharing of class I MHC antigens between donors of T cells and target cells was necessary for target cell lysis to occur. It has become apparent that activation of Th cells is often important for immune responsiveness to protein antigens. This activation requires the presentation of a "processed" antigen by an antigen presenting cell (APC) which displays class II MHC gene products. This antigen processing is a metabolically active, time dependent process where the protein is endocytosed by the APC (Zeigler and Unanue, 1981). Furthermore, intracellular processing of the antigen can be blocked with either ammonia or chloroquine, both of which interfere with lysosomal protein degradation by depressing the activity of acid hydrolases (Ziegler and Unanue, 1982). The antigen seen by the Th cells is not the intact native protein but a "determinant" of the APC, the specificity of which depends upon self MHC and a peptide derived from the foreign protein. Shimonkevitz *et al.* (1983) observed that a small tryptic peptide enzymatically degraded *in vitro* from ovalbumin could be presented by metabolically inactive cells bearing the appropriate class II MHC molecule, thus providing indirect evidence that APC process antigen.

This view of antigen presentation by APC to class II-restricted T cells diverged markedly from the previously held views about the method of antigen recognition by class I-restricted Tc cells. Class I-restricted T cell responses were thought to be specific for integral cell-membrane proteins i.e. viral coded glycoproteins and minor histocompatibility antigens. Studies by Schrader and Edelman (1977) demonstrated that target cells were susceptible to specific lysis by sensitized Tc cells 30 minutes after coating with UV-inactivated Sendai virus and there was no evidence of the synthesis of new viral proteins. These results were supported by Hapel *et al.* (1978, 1980) using UV inactivated ectromelia and vaccinia viruses. These viruses were able to form antigenic complexes with host cell membrane MHC molecules within 10 minutes of exposure and indicated that productive infection was not required for induction of Tc cells. Taken together these results suggested that cells did not have to synthesize viral protein to serve as targets for class I-restricted viral-immune Tc cells and that antigen processing was not a requirement for class I-restricted responses.

These apparent major differences in antigen presentation to class I and class II restricted T cells are not supported by more recent evidence. Firstly, class I and class II-restricted T cell clones have been demonstrated to be able to use identical V_α and V_β gene segments in their TCRs (Kronenberg *et al.*, 1986; Rupp *et al.*, 1987) thus making it unlikely that there are marked differences in antigen which interact with the two classes of MHC molecules.

Kees and Krammer (1984) demonstrated influenza-immune memory Tc cells recognized antigenic epitope contributed to by internal viral proteins, not the surface glycoproteins haemagglutinin and neuraminidase. Townsend *et al.* (1984a) showed that many cross-reactive influenza-immune Tc clones recognized determinants dependent upon the viral nucleoprotein. This observation was confirmed by demonstrating that L cells which had been transfected with the influenza nucleoprotein gene became targets for class I-restricted influenza-immune Tc cells (Townsend *et al.*, 1984b). These results were of crucial importance because nucleoprotein is not glycosylated, accumulates in the nucleus of

infected or transfected cells, and has none of the characteristics of an integral membrane protein. The question then arose as to how this protein can be expressed on the cell surface membrane.

More recent studies of influenza-immune class I-restricted Tc cells demonstrated that they recognize degraded forms of the nucleoprotein antigen. Townsend *et al.* (1985) showed that murine influenza-immune Tc cells recognize targets expressing a variety of truncated influenza nucleoprotein genes. The influenza-immune Tc cell recognition of these influenza nucleoprotein fragments did not correlate with antibody detection of nucleoprotein on the cell surface. Furthermore, antibodies directed against the influenza nucleoprotein were unable to block influenza-immune Tc cell lysis of these targets. These results together with the lack of evidence for a signal sequence for membrane transport of nucleoprotein suggested that influenza-immune Tc cells recognized determinants dependent upon peptides derived from degraded forms of the nucleoprotein antigen (Townsend *et al.*, 1985). This information suggested that viral antigen presentation was associated with a normal mechanism for protein degradation in the cell and that the resulting peptides must somehow reach the MHC class I site for recognition by Tc cells on the cell surface membrane. Thus, antigen recognition by class I-restricted Tc cells has analogies with class II-restricted antigen recognition by Th cells.

The hypothesis that Tc cells could recognize degraded fragments of non-membrane viral proteins presented by infected cells was tested by Townsend *et al.* (1986a) in a manner analogous to that used by Shimonkevitz *et al.* (1983). Townsend *et al.* (1986a) initially identified a segment of 59 a.a. in the nucleoprotein molecule as containing epitopes recognized by the Tc cells. They then added appropriate concentrations of short synthetic peptides corresponding to this linear region of the nucleoprotein to target cells *in vitro* and demonstrated lysis by Tc cells. Townsend *et al.* (1986b) then went on to test if proteins which are normally inserted into the plasma membrane required any "processing" for recognition by Tc cells. Using influenza haemagglutinin, a transmembrane protein of the virus which is recognized by a subset of influenza-immune Tc cells, they were able to

determine that surface expression of the intact molecule was not a necessary requirement for lysis by influenza-immune Tc cells. They generated a recombinant vaccinia virus which expressed haemagglutinin deleted of its signal sequence. Cells infected with the recombinant vaccinia did not express the signal deleted haemagglutinin on their surface but this protein could be detected in the cytoplasm of the cells by antibodies. However, influenza haemagglutinin-immune Tc cells could recognize and lyse target cells expressing the signal deleted haemagglutinin as efficiently as cells expressing the intact haemagglutinin molecule. These findings suggest that haemagglutinin can be expressed on the cell surface in a form recognized by Tc cells which does not depend upon insertion of the intact molecule into the cell membrane. Furthermore, the inability of anti-haemagglutinin antibodies to detect the signal deleted haemagglutinin on the cell surface suggests that the the haemagglutinin molecule recognized by the Tc cell has a disrupted tertiary structure. These results are consistent with the concept that class I MHC expressing cells are capable of degrading and presenting newly synthesized viral proteins.

Further studies by Maryanski *et al.* (1986a) extended this concept of antigen processing for class I-restricted responses from viral proteins expressed internally to antigens normally expressed as integral self membrane proteins. Class I MHC antigens are integral membrane proteins expressed on a wide range of cells. HLA class I gene products expressed on murine cells are recognized by allo-immune human Tc cells (Maryanski *et al.*, 1985). They also act as restriction antigens for HLA class I-restricted virus-immune Tc cells (Gomard *et al*, 1986) and as nominal antigens recognized by class I-restricted murine Tc cells (Maryanski *et al.*, 1986b). Maryanski *et al.* (1986a) demonstrated that a synthetic peptide derived from a class I MHC molecule could serve as a nominal antigen for two murine class I-restricted HLA-immune Tc cell clones. Previous studies (Maryanski *et al.*, 1985, 1986b) demonstrated that anti-HLA antibodies could block lysis of HLA-transfected murine cells by allo-immune Tc cells but not class I-restricted Tc cells. Taken together these results suggest that the allo-immune and class I-restricted Tc cells recognize different forms of the same molecule: the allo-immune Tc cells and the class I HLA-restricted

viral-immune Tc cells recognize the membrane bound HLA molecule while the murine class I-restricted HLA-immune Tc cells recognize a "processed" fragment of the HLA antigen.

However, Morrison *et al.* (1986) have provided evidence that the antigen processing pathways for class I and class II-restricted cells are different. Morrison *et al.* (1986) showed that an influenza-immune Tc cell clone specifically recognized target cells in which new haemagglutinin protein synthesis had occurred, in contrast to their class II-restricted clones which only recognize target cells that have been exposed to soluble haemagglutinin in the surrounding medium. Furthermore, only class II-restricted antigen presentation is sensitive to the effects of chloroquine suggesting the presence of two differing metabolic pathways for the production of immunogenic peptides. The pathway for processing antigens from endogenously synthesised proteins and presentation with class I MHC molecules appears to differ from the degradation of endocytosed soluble antigens whtch are processed and presented with class II MHC molecules (Morrison *et al.*, 1986).

Taken together these results support the presence of at least two pathways for antigen presentation to T cells, one which processes proteins synthesized within the cytoplasm of the cell and leads to class I MHC and another which deals with endocytosed antigens and leads to class II MHC. The evidence presented above which showed that antigen is processed and peptide fragments derived from the native protein can act as nominal antigen for class I-restricted Tc cells can explain the observations of Schrader and Edelman (1977); Hapel *et al.* (1978, 1980). The virus stocks used in these studies were grown in cells. These cells were disrupted to release virus and would presumably also contain antigenic peptides as well as the live virions. These antigenic peptides could act as nominal antigen for class I MHC-restricted virus-immune Tc cells in a manner analogous with the studies of Townsend *et al.*, (1986a). Alternatively, Yewdell *et al.* (1988) showed using ultraviolet light inactivated virus, that fusion of the viral envelope with cell membrane can also lead to structural viral proteins entering the class I MHC processing pathway.

T Cell Accessory Molecules-CD4 and CD8.

Cantor and Boyse (1975) described mutually exclusive subsets of T cells in the mouse. In bulk cell populations, the helper subset was found to express Lyt 1, while the cytotoxic- suppressor subset was found to express Lyt 2, Lyt 3 (CD8). However, the validity of Lyt 1 as the phenotype for murine helper cells was challenged when Ledbetter *et al.*,(1980) demonstrated that all murine T cells expressed Lyt 1, albeit at varying concentrations. In 1983, Dialynas *et al.* (1983a) described a murine T cell surface molecule designated L3T4 (CD4), which is a more satisfactory marker because expression of CD4 and CD8 by mature T cells appears to be mutually exclusive. Using cloned T cells, these cell surface markers were found to correlate closely with the class of MHC antigen recognized by the T cell and only coincidentally with the effector function of the T cell (Swain, 1981) although some exceptions to this have been documented (e.g. Spits *et al.*, 1983). T cells reacting with class I MHC alloantigens or restricted by self class I MHC antigens usually, but not always, express CD8 while T cells reacting with or restricted by class II antigens usually express CD4 (Dialynas *et al.*, 1983b).

A functional role for these molecules is suggested by experiments using monoclonal antibodies directed against them. Antigen-specific cytolysis mediated by $CD8^+$, $CD4^-$ T cell clones was inhibited by monoclonal antibodies directed against CD8 (MacDonald *et al.*, 1981, 1982). Conversely, antigen-specific cytolysis by $CD4^+$ cells was inhibited by anti-CD4 monoclonal antibodies (Dialynas *et al.*, 1983b). The appropriate monoclonal antibody has also been demonstrated to block antigen-induced proliferation and lymphokine release (Dialynas *et al.*, 1983a, Marrack *et al.*, 1983, Wilde *et al.*, 1983). However, studies by Beekoff *et al.* (1985) and Bank and Chess (1985) demonstrated that anti-CD4 antibodies delivered an inhibitory signal when the $CD4^+$ T cells were being stimulated with Ia antigen negative cells. Thus, the CD4 molecule can under some conditions deliver a negative signal to T cells.

The gene segments which are rearranged to form the α and β chains of the TCR appear to do so randomly. Thus, there is no evidence that gene segments of either chain can be

divided into class-specific subsets. It therefore seems unlikely that the TCR alone determines the class of MHC antigen required. That the MHC class is recognized by structures other than the antigen-specific receptor was proposed by (Swain *et al.*, 1983) because presence of CD8 and CD4 correlate much more closely with class I and class II MHC associated antigen presentation respectively. It seems unlikely that these molecules are involved in the direct recognition of foreign antigen as they are not clonally distributed and are non polymorphic (Littman, 1987). It has been proposed that these molecules increase the binding and/or signalling efficiency of the TCR (Governman *et al.*, 1986; Blanden *et al.*, 1987) by binding to non-polymorphic sites of the class I and class II MHC molecules respectively. However the binding of MHC molecules by CD8 and CD4 does not in itself explain the phenomenon of MHC restriction because some T cell clones recognize MHC molecules without their involvement (MacDonald *et al.*, 1981). Furthermore, MHC-restricted recognition of the K^b mutants is highly specific for the variable regions of the class I MHC molecule (Blanden *et al.*, 1976).

MHC Restriction.

The MHC as a functional genetic unit has been retained during vertebrate evolution which suggests that it has a pivotal role in the immune response or other biological functions essential for survival of the animal to reproductive age. However, the biological role of the MHC had been a puzzle to immunologists since the discovery of alloreactivity. Immunologists had known for a long time that the antibody response of an animal to antigenic stimulation generally required cooperation between B and T lymphocytes (e.g. Taylor, 1969). Using adoptive transfer of thymocytes into nude mice, Kindred and Shreffler (1972) demonstrated that adoptively transferred thymocytes provided help for anti-SRBC antibody production by B cells only when the donor of the T cells expressed the same MHC haplotype as the recipient animal. A similar requirement for MHC matching was found when antigen-presenting guinea pig macrophages were used to stimulate proliferation of T cells (Rosenthal and Shevach, 1973).

Katz and coworkers, using the hapten-carrier model of T-B cell cooperation which had previously been studied by Mitchison (1971a and 1971b) demonstrated both *in vivo* and *in vitro* that matching of the MHC genes (of the donors of T and B cells) (Katz *et al.*, 1973a, 1973b) but not the background genes (Katz *et al.*, 1973c) was an essential requirement for T-B cell cooperation for the generation of an anti-hapten antibody. Later they found that matching of only the *I* region of the H-2 complex was necessary and sufficient for T-B cooperation and antibody production (Katz *et al.*,1975). These experiments were interpreted in terms of a "physiological interaction" model which essentially implied that the same MHC molecules needed to be expressed on T and B cells and were involved in a "like-like" interaction.

The finding that immune Tc cells have dual specificity for MHC and foreign antigen was reported simultaneously by two laboratories. Zinkernagel and Doherty (1974a) studied the lysis of LCM virus-infected targets and Shearer (1974) worked with TNP-modified target cells. Both groups showed that the immune Tc cells were specific for the stimulating antigen and secondly, that the donors of the immune Tc cells and target cells had to express the same MHC antigens. Further studies showed that the antigens to which MHC-restricted Tc cells could be primed were not only viral or haptens but also minor transplantation antigens (Bevan, 1975a, 1975b) including H-Y antigens (Gordon *et al.*, 1975, 1976). This important experimental advance ultimately led to a new conceptual framework to explain the phenomena of T-B interaction and MHC-linked *Ir* gene effects.

Blanden *et al.* (1975) demonstrated using ectromelia- and LCM- immune murine spleen cells in cytotoxic assays that only the *K* or *D* region needed to be shared by the donors of T cells and target cells for target cell lysis to occur. There was no need for *I* region homology. *In vivo* studies, with adoptive transfer of ectromelia-immune T cells into mice which had been infected with virulent ectromelia, demonstrated that reduction in viral titres in target organs occurred when the *K* or *D* regions, but not the *I* region, were shared by the immune T cell donor and the recipient (Kees and Blanden, 1976) and that the K^{bm1} mutation completely changed the specificity of recognition by *K* region-restricted

antiviral T cells. This was also shown *in vitro* at the level of target cell lysis by Tc cells (Blanden *et al.*, 1976). The same requirement for commonality in the *K* or *D* regions was demonstrated to be essential for *in vitro* stimulation of virus-immune memory cells by virus-infected stimulator cells (Pang and Blanden, 1977).

The basic facts of the MHC-restriction phenomenon were clear by 1974. A T cell-mediated immune response required recognition of either class I or class II MHC molecules (depending on the nature of the response) and "antigen". However, controversy remained as to the interpretation of the phenomenen. The "physiological interaction" model proposed a single T cell receptor specific for viral antigen as well as physiological interaction of the antigens encoded by the H-2 complex. If this model were correct a T cell from an F1 hybrid would possess a receptor recognizing viral antigen and would express both types of parental H-2 antigens thus allowing it to ~~interavt~~ interact with virus-infected target cells of either parent strain. This "physiological interaction" model of Katz *et al.* (1973a, 1973b) was tested by Zinkernagel and Doherty (1974b). They demonstrated that LCM-immune (P1 X P2)F1 spleen cells transferred into irradiated P1 mice were only able to recognize and lyse LCM-infected targets of P1 and not P2 haplotype. They argued that proliferation of the (P1 X P2)F1 LCM-immune spleen cells able to lyse P1 and not P2 LCM-infected targets disproved the physiological interaction model. If this model were correct, then F1 T cells should proliferate equally in either parental type and lyse both P1 and P2 LCM-infected targets. Zinkernagel and Doherty (1974b) speculated that a single antigen-specific receptor was not specific for viral antigen (viral protein inserted in the membrane) but rather for unique modifications of the MHC-encoded molecules that were induced by the viral proteins during the process of viral infection i.e. "altered self".

Further studies by Zinkernagel and Doherty (1975) showed tht the cytotoxic activity of (P1 X P2)F1 LCM-immune T cells was inhibited only when LCM-infected cold target competitors were syngeneic with the target cells i.e. the presence of excess P1 LCM-infected target cells caused no specific decrease in killing of P2 LCM-infected target cells and vice versa. This was interpreted as demonstrating two specificities of

LCM-imune Tc cells in F1 mice, each associated with altered-self characteristics of one parental H-2 type. Davidson *et al.* (1975) confirmed and extended the results of Zinkernagel and Doherty (1975) by demonstrating that the H-2K and H-2D antigens did not behave as clonally expressed physiological interaction structures. If the experiments by Zinkernagel and Doherty (1974b, 1975) described above were compatible with the physiological interaction model then the H-2K or H-2D region gene products responsible for the putative physiological interaction would be clonally expressed on virus- immune Tc cells (i.e. there would be cells expressing H-2K plus TCR or H-2D plus TCR but not both H-2K and H-2D plus TCR). Using recombinant A.TH ectromelia-immune Tc cells (K^s,D^d), they were able to cause a significant decrease in lysis of $H\text{-}2^s$ ectromelia-infected targets by pre-incubating the T cell population with allo-immune Tc cells directed against the $H\text{-}2D^d$ antigen. Therefore, the A.TH ectromelia-immune Tc cells which lysed infected $H\text{-}2K^s$ targets through shared $H\text{-}2K^s$ were also displaying $H\text{-}2D^d$. Thus, the physiological interaction model was disproved and the altered self hypothesis gained acceptance.

Immune Response (Ir) Genes.

In 1943 Fjord-Scheibel described quantitative differences in the antibody responses of different individual random-bred guinea pigs to immunizations with diphtheria toxin. A more rigorous investigation of this phenomenon was first undertaken in Benacerraf's laboratory (Levine *et al.*, 1963a,1963b), when his team exploited the use of synthetic polypeptides to study the genetic control of the immune response in outbred and inbred guinea pigs. Using hapten conjugates of poly-L-lysine as simple antigens, Levine and Benacerraf (1965) were able to demonstrate genetic control of the response by selectively mating homozygous non-responder guinea pigs with heterozygous responder guinea pigs. Of these matings, approximately half the offspring were responders and half were non-responders. The researchers accordingly concluded that the response to the antigen was probably mediated by a single Mendelian- dominant gene. Although other genetic

interpretations of the data could be made, the important concept to emerge from this work was that the immune response to simple antigens was controlled by only a few genes. Similar studies of the immune responses of inbred CBA and C57BL mice to synthetic antigens were undertaken by McDevitt and Sela (1965) who confirmed that a genetic region controlled this responsiveness. These same investigators (McDevitt and Sela, 1967) then demonstrated that responsiveness was determined by a single autosomal dominant gene. In a critical experiment, McDevitt *et al.* (1972) showed that this gene mapped to the *H-2* complex of the mouse between the *K* region and the *S* region; the new region was designated the *I* region because it contained so-called immune response (*Ir*) genes.

The immune response to an antigen consists of many steps, any of which can fail and lead to nonresponsiveness. However, for the immune response to fail with respect to a specific antigen (not a generalized immune defect), we now know that the defect must lie in the recognition by T cells of specific antigen in the context of the MHC molecules of the individual. The *I* region genes of the murine MHC encodes the class II MHC antigens. These are the *Ir* genes which control the immune responses dependent on class II-restricted T cells. Similarly, the murine *K* and *D* regions encode class I MHC antigens and these are *Ir* genes controlling class I-restricted responses.

In principle, *Ir* gene effects may be due to one of two kinds of defects. One of these would be at the level of the interaction between the foreign antigen and the MHC antigen and the other at the level of the T cell receptor needed to recognize this complex.

The first mechanism requires that the MHC molecules of a non-responder individual are unable to "bind" with the foreign antigenic peptides derived from the nominal antigen and therefore this individual is unable to provide an appropriate MHC-antigen complex on its antigen presenting cells.

Babbitt *et al.* (1985) demonstrated that the peptide containing amino acids 46 to 61 of hen egg-white lysozyme (HEL 46-61) is immunogenic and induces a T cell response in $H\text{-}2^{k}$ haplotype mice but fails to do so in $H\text{-}2^{d}$ mice. The immunogenicity of this peptide correlates with its *in vitro* ability to bind purified $I\text{-}A^{k}$ class II MHC molecules. However

consistent with the non-responder *H-2*^{d} haplotype HEL 46-61 does not bind to I-A^{d} molecules. Babbitt *et al.* (1986) extended this observation and generated a number of synthetic peptide derivatives. They found a direct relationship between the capacity of a peptide to inhibit the binding of HEL 46-61 to I-A^{k} and the reduction in antigen presentation as determined by IL-2 release. Thus, these data provide strong support for the concept that immunogenicity is a function of peptide binding to MHC molecules.

However, a self peptide was generated which bound to I-A^{k} molecules with a similar affinity as the foreign peptide HEL46-61 and a T cell response was not elicited indicating that binding of a peptide to an MHC molecule is not in itself sufficient to generate an immunogenic epitope. Taken together these results support the hypothesis that immune-response gene defects can exist both at the level of binding to the MHC molecule and at the level of the T cell repertoire.

The second mechanism requires that the T cell receptor repertoire of an individual is incomplete in that it lacks the ab heterodimer that can bind to a certain combination of MHC molecules and antigen. This may occur because the individual lacks the gene segments required to generate an ab heterodimer with the idiotype required for recognition of the foreign antigenic determinant. Alternatively, the T cell repertoire may not contain the required ab heterodimer as this may have been deleted by necessity for an individual to develop self-tolerance during T cell ontogeny.

Mullbacher (1981a) proposed a model whereby self tolerance may interfere with certain H-2 restricted responses. Mullbacher (1981b) tested his hypothesis using H-Y immune Tc cells which cross-react on allogeneic targets. Knowing that some individual CBA/H female mice generate H-Y-immune Tc cells which cross-react with allogeneic B10 female targets, he proposed that two populations of Tc cells can be generated, one population which cross-reacts with B10 alloantigens and a second which does not. Female CBA/H mice were made neonatally tolerant to (CBA X B10) F1 female spleen cells. Then the spleen cells from these neonatally tolerant mice as well as from CBA/H control mice were cultured *in vitro* with CBA/H male spleen cells and tested on CBA/H male and female targets. Only

the spleen cells from the CBA/H control mice killed the male targets. This was interpreted to show that in (CBA/H X B10) F1 mice, any H-Y-immune Tc cells which cross-react with B10 antigens would be autoreactive and would be deleted or suppressed. In this situation it is proposed that the "H-2^k-H-Y complex" mimics B10 antigens (H-2^b) and tolerance to B10 deletes or suppresses Tc cells which react to the "H-2^k-H-Y complex". Thus, Mullbacher provided indirect evidence for natural self tolerance being the mechanism by which *Ir* genes control Tc cell responses.

This mechanism of natural tolerance could be used to explain haplotype preference, a phenomenon in heterozygous mice where a Tc cell response to a given antigen is associated with only one parental haplotype, although Tc cells responses associated with the other haplotype are permissive in the homozygous parent. This has been demonstrated in (H-2^k X H-2^b) F1 mice described by Mullbacher *et al.* (1981) and Brenan *et al.* (1981).

The same ~~principal~~ principle applies to *Ir* gene control of virus-immune Tc cells responses. Mullbacher *et al.* (1983) studied the murine Tc cell response to vaccinia virus in which the alleles of the *K* region of the H-2 complex influence the strength of the anti-vaccinia response associated with the D^b allele. In particular the presence of K^k in H-2 recombinants or F1 hybrids leads to a decreased response to D^b-vaccinia. The defect does not lie in antigen presentation as vaccinia-infected 4R (K^k D^b) cells can stimulate vaccinia-immune-D^b Tc cells *in vitro*. Furthermore, the non-responder animals possess Tc cell precursors for vaccinia-immune-D^b-restricted responses as transfer of non-responder spleen cells into lethally irradiated responder recipients allows the generation of vaccinia-immune D^b-restricted Tc cells. Evidence in favor of the cross-tolerance hypothesis was obtained using B10 mice which were neonatally tolerized to K^k antigens. Nineteen of the 26 tolerant mice gave significantly lower responses to vaccinia-infected D^b targets than control mice. They proposed that as a consequence of self-tolerance to K^k, the suppression of K^k-immune Tc cells resulted in cross-reactive suppression of vacccinia-immune-D^b Tc cells.

SECTION 2.

The Flaviviruses.

The family Flaviviridae as presently constituted consists of 65 viruses of which yellow fever is the prototype ("flavus" is Latin for yellow). Historically, yellow fever was the first infectious disease of humans shown to be due to a filterable agent or virus and yellow fever was the first viral illness whose transmission by a blood-sucking arthropod was demonstrated. Other arthropod-transmitted viruses were subsequently identified and these became known as the Group A and Group B arboviruses (Porterfield *et al.*, 1978). They belonged to the family Togaviridae.

The family Togaviridae was defined by Fenner *et al.* (1974) as viruses of "single-stranded RNA, 3-4 X 10^6 daltons, having isometric, probably icosahedral, nucleocapsids surrounded by a lipoprotein envelope containing host cell lipid and virus-specific polypeptides including one or more glycopeptides. Virions yield infectious RNA." This family of viruses initially contained two genera, Alphaviruses and Flaviviruses being the names given to the Group A and Group B arboviruses respectively.

The family of Togaviradea as described by Matthews (1982) included four genera - Alphavirus, Flavivirus, Rubivirus and Pestivirus-and five additional possible members. In 1984 the Togavirus study group proposed that the genus Flavivirus be removed from the family of Togaviridae and be recognized as a family in its own right. The Togaviridae family had originally been defined in terms of morphological criteria but it later became clear that flaviviruses, while morphologically similar to alphaviruses, do not share the same gene sequence, replication strategy or morphogenesis. Flaviviridae is accordingly now recognized as a family in its own right (Westaway *et al.*, 1985).

Many flaviviruses, including all those with medical or veterinary importance, have arthropod vectors. These arthropods, blood-sucking insects such as ticks or mosquitoes, transmit the virus in one of two ways. In mechanical transmission, the vector simply functions as an "infected needle" which transports the virus from a viraemic individual to

an uninfected individual. In biological transmission, the virus goes through a cycle of replication in the arthropod host before infecting an individual.

A number of members of the flavivirus family are human pathogens and cause diseases with significant morbidity and mortality throughout the world. Most viruses of medical importance are contained in three of the subgroups:

a) the West Nile subgroup: West Nile, Japanese B encephalitis, Murray Valley encephalitis and other related encephalitides;

b) the tick-borne subgroup: tick-borne encephalitis, Kyasanur Forest disease, Omsk haemorrhagic fever and others; and

c) the dengue subgroup: dengue 1-4.

Yellow fever virus has been demonstrated to be in a distinct group because it does not cross-react with any other members of the Flaviviridae family in antibody neutralization tests. Neutralizing antibody cross-reactivity among subgroup members relates to *in vivo* cross-protection seen in some studies (Hamman and Sather, 1956, Casals, 1963). In these studies sequential infection with two or more of the viruses resulted in broadened serological responses and enhanced cross-protection, especially if members of the same subgroup were used.

In humans, three main clinical syndromes caused by infection with flaviviruses can be recognized:

1) acute encephalitis from the West Nile subgroup;

2) a febrile illness with fever, arthralgia and rash e.g. from West Nile fever or dengue

3) haemorrhagic fever e.g. from yellow fever or dengue haemorrhagic fever and shock syndrome (Sanford,1987, Monath,1985). Although there is considerable overlap in these syndromes, they do reflect differing tissue tropisms and virulence in this family of viruses.

Most information regarding flavivirus pathogenesis is derived from experimentation on laboratory rodents. These animals provide a good model of flavivirus encephalitis but not of the other syndromes associated with human flavivirus infection (i.e. 2 and 3 above).

Viruses which produce these syndromes in humans, including both dengue and yellow fever, cause acute encephalitis in laboratory rodents.

Flavivirus Structure.

Flaviviruses contain three structural proteins. The genome RNA is contained within a central core structure, the nucleocapsid, which contains a single capsid protein, C. The symmetry of the ~~nucleocapsid~~ nucleocapsid (which has not been rigorously defined) is thought to be icosahedral (Brinton, 1986). The nucleocapsid is surrounded by a lipid bilayer containing the envelope protein, E, which is usually but not always glycosylated (Wright, 1982). The E protein forms the observed projections from the lipid envelope and it is not known whether these spikes are monomers, dimers or trimers of the E protein (Brinton. 1986). The nonglycosylated membrane protein, M, is also associated with the viral envelope.

The Genome.

The flavivirus genome is a single plus-stranded RNA approximately 11 kilo-bases in length (Westaway, 1980, Deubel *et al.*,1983). The purified RNA is infectious, indicating that the RNA genome alone is sufficient to initiate the complete replication cycle. No subgenomic mRNA has been identified and it is thought that the virion RNA is the only flavivirus-specific RNA. This suggests that the RNA is not cleaved prior to translation. The nucleotide sequence of yellow fever virus (YFV) was determined by Rice *et al.* (1985) from complementary DNA clones (cDNA). The RNA contains a single long open reading frame of 10,233 out of 10,862 nucleotides. The long open reading frame has also been identified in Kunjin virus (Speight *et al.*,1988), WNV (Castle *et al.*,1986) and JBE virus (Sumiyoshi *et al.*, 1987). The partial sequence of cDNA clones of Murray Valley encephalitis virus (~~Delgarno~~ Dalgarno *et al.*, 1986) also supports the identification of a long open reading frame encoding the polypeptides analogous to those in YFV.

The flavivirus genome contains a type 1 cap at the 5' end but lacks the polyadenylated tract at the 3' end frequently seen in eukaryotic mRNA. Instead, a fairly stable secondary

structure, a complex stem and loop, is found (Rice *et al.*,1985, Brinton *et al.*,1986). The 3' stem and loop structure, which differs in sequence between different flaviviruses but not in form, may be important for initiating minus-strand RNA synthesis (see below) or involved in interactions between the genome RNA and the capsid protein.

The flavivirus genome is organized into two sections. The 5'-terminal quarter encodes the structural proteins while the 3'-terminal three-quarters encodes the non-structural proteins. The open reading frame begins with the coding sequence for the nucleocapsid protein C. The C protein has a high percentage of basic amino acids which Rice *et al.* (1985) suggested partly neutralize the negative charges of the RNA. The protein, prM (precursor to M) follows protein C. The sequence for the mature protein, M, is contained within the C-terminal end of prM. M has not been found within infected cells but only in extracellular virions. It appears that prM is cleaved to yield M during virus maturation (Shapiro *et al.*, 1972, Westaway, 1980). The RNA sequence encoding M is followed by that encoding the major envelope protein, E, which contains both haemagglutinin and neutralizing epitopes (see "Flavivirus antigens determined by antibody response" below). Thus the RNA encoding the structural proteins is adjacent. The non-structural proteins are encoded in the remainder of the reading frame. Some of these proteins must be active in the replication of the viral RNA. The number and relative positions of these non-structural proteins remain controversial matters. However, Rice *et al.* (1985) provided a hypothetical gene map for the 17D YFV. The gene order they postualated was 5'-C-prM(M)-E-NS1-ns2a-ns2b-NS3-ns4a-ns4b-NS5-3'. Gene products for these genes denoted in upper case had been previously identified but the genes designated in the lower case were non-structural proteins hypothesised on the basis of the presence of putative cleavage sites used for the non-structural proteins NS3 and NS5 (Rice *et al.*, 1986a). More recently, Speight *et al.* (1988) provided positive identification of not only NS3 and NS5 of Kunjin virus but also for three previous hypothetical non-structural proteins of the flavivirus, ns2a, ns2b and ns4b. The identity of ns4a remained unknown. The gene order for Kunjin virus non-structural proteins is homologous with that of the 17D YFV, but

cleavage sites were different from those which had previously been postulated (Speight *et al.*,1988). The gene sequence of flaviviruses where structural proteins are encoded at the 5' end of the RNA is similar to that of the picornaviruses and not that of the alphaviruses.

Viral Replication.

The genome plus-strand RNA is the initial template from which a complementary minus-strand RNA is synthesized. The minus-strand then becomes the template from which the progeny plus-strand RNA is synthesized. This newly synthesized plus-strand RNA is used as mRNA for the translation of proteins, as templates for further minus-strand RNA production to allow viral replication to proceed and as molecules for encapsidation into progeny virions (Brinton, 1986).

Considerable controversy surrounds the mechanism for translation of the flavivirus genome. Westaway (1977) and Westaway *et al.* (1984) have suggested that flavivirus RNA is translated by multiple internal initiation events. This would allow for the translation of the non-structural proteins early in the replication cycle when enzymes are necessary for RNA replication. However, now that the complete nuceotide sequence of several flaviviruses is known, YFV (Rice *et al.*, 1985), WNV (Castle *et al.*,1986), and Kunjin virus (Speight *et al.*,1988), logical interpretation suggests that translation is initiated at the first methionine codon near the 5' end and a polyprotein is generated which is then rapidly cleaved posttranslationally to yield the final proteins. Furthermore, high molecular weight proteins have been detected by pulse-label experiments in Dengue-2 virus and JBE virus-infected cells supporting the hypothesis of Rice *et al.* (1985). These polypeptides contained the sequence for smaller viral proteins (reviewed in Rice *et al.*, 1986a).

The possibility that some flavivirus proteins are translated from internal initiation sites has not been totally excluded. In short pulse experiments NS5 is strongly and rapidly processed compared with the other non-structural proteins suggesting that it may be translated independently. However, this observation may also be explained by slower cleavage and release of the other non-structural proteins rather than by internal initiation of

translation. Further evidence supporting internal initiation of translation was the appearance of NS5 in gels when translation occurred in the presence of protein cleavage inhibitors. Furthermore, experiments with ultraviolet irradiation suggested that NS5 had a smaller size rather than the largest size which would be predicted if it was the last protein translated from a single long open reading frame (reviewed in Westaway, 1987).

Flaviviruses can replicate in a wide variety of cultured vertebrate and arthropod cells. However, replication in vertebrate cells is considerably slower than for alphaviruses (Brinton, 1986). Maximal titres of virus are not produced until 24 hours after infection (Trent and Naeve, 1980). Electron microscope studies by Gollins and Porterfield (1984) of WNV entry into the P388D1 cell line demonstrated that single virions were endocytosed in coated vesicles. Uncoating and degradation of the virus occurred in lysosomal vacuoles after which viral RNA was released into the cytoplasm of the cell where replication begins (reviewed above).

The ~~inital~~ initial latent phase of the flavivirus replication cycle is approximately 8 to 12 hours following which progeny virions are readily observed within the cisternae of the endoplasmic reticulum (Murphy, 1980, Westaway, 1980). Hypertrophy of rough and smooth endoplasmic reticulum and some areas of the Golgi is characteristic of flavivirus infection. Cytoplasmic membranes often proliferate in the perinuclear region in the infected cells to form meshlike structures located near the intracellular progeny virions. Westaway (1980) suggested that these perinuclear membranes may somehow be involved in virus maturation. Mature virions accumulate within the Golgi complex and within vesicles with electron dense particles, presumably ribosomes, along their outer surface. There is no direct evidence for the envelopment of flaviviruses by an intracellular budding process and the origin of the envelope around mature flaviviruses is not known. The mechanism by which assembled mature virions leave the infected cell is a subject of controversy. Westaway (1980) described lamellae of smooth membranes in Kunjin infected cells opening directly to the exterior while Filshie and Rehacek (1968) and Dalton (1972) observed virus containing vesicles move to the peripheral cytoplasm and fuse with the

plasma membrane. Finally, Murphy (1980) suggested that virions "packaged" in smooth or rough membranes may be released when infected cells lyse (Murphy *et al.*,1968, Sriurairatna *et al.*,1973).

Flavivirus Antigens Determined by Antibody Respose.

Flaviviruses can be divided into multiple serocomplexes by the plaque reduction neutralization test. All viruses within a complex are called serotypes and the serotypic determinant appears to reside on the E glycoprotein as determined by the competitive antibody-binding assay (Trent, 1977). Flavivirus-group-reactive and complex-reactive determinants are also identified on the E glycoprotein, which appears to be the only structural protein which can elicit neutralizing antibody. The capsid protein has flavivirus-group- reactive determinants. Studies of St Louis encephalitis virus (SLE) by Roehrig *et al.* (1983) identified 8 epitopes on the E glycoprotein. Monoclonal antibodies to one of the type specific epitopes, (E-1^{C}), had very high plaque reduction neutralization and haemagglutination inhibition titres. Roehrig *et al*, postulated that this epitope was located in a domain on the glycoprotein which was central for viral adsorption.

The 8 E-glycoprotein epitopes are clustered into three domains on tick-borne encephalitis (Heinz *et al* .,1983). Domain A is defined by three haemagglutination ~~haenagglutination~~-inhibiting antibodies, two of which are flavivirus group-reactive while a third is tick-borne virus sub-type specific. Within this domain only the sub-type specific antibody is involved in virus neutralization. This observation explains why neutralizing antibodies show higher serologic specificity than haemagglutination inhibition tests and that haemagglutination inhibition tests can be made type and sub-type specific by antibody adsorption. Domain B contains three tick-borne encephalitis-complex reactive epitopes and the corresponding antibodies inhibit haemagglutination and neutralize the virus. Domain C consisted of one subtype-specific epitope.

Mathews and Roehrig (1984) used passive antibody transfer to determine the efficiency of monoclonal antibodies directed to each epitope in protecting animals from lethal infection

with SLE. In large doses most anti-SLE monoclonal antibodies were marginally protective, however an anti-E-1^c monoclonal antibody was extremely efficient in protecting the animals from lethal virus challenge. Combination of monoclonal antibodies to cross-reactive E glycoprotein epitopes provided a synergistic protective effect.

Immune Responses to Flaviviruses.

Three general approaches have been taken to investigating the immune responses to flavivirus infections: 1) direct measurement of the immune response *in vivo* or*in vitro* in specimens taken at various times during the course of infection; 2) adoptive transfer studies, where either cells or serum from infected hosts are transferred into a recipient host; and 3) subtractive studies, in which the course of the infection is altered by induced immuno-suppression.

Humoral Immunity.

In murine models of flavivirus encephalitis, both IgM and IgG with neutralizing and haemagglutination-inhibition function appear simultaneously, 4 to 6 days after peripheral inoculation (Webb *et al.,* 1968; Monath and Borden,1971; Bhatt and Jacoby,1976) but as in most viral infections, IgM predominates early in the course of infection. Detection of these antibodies is associated temporally with termination of viraemia and the appearance of virus in the brain of the animal. Weiner *et al.* (1970) studied the effect of varying doses of WNV or Powassan virus inoculated intramuscularly (i.m.) or intraperitoneally (i.p.) into mice of varying ages. Younger mice were more susceptible to infection and mortality was higher in the younger mice. Their data suggested that there is early viral replication in extraneural tissues (muscle, liver, spleen and kidney) as determined by immunofluorescent staining. This was followed by viraemia, with subsequent spread to the brain. Weiner *et al* found that the day when virus was first detected in the brain correlated with the outcome. Virus was first detected in the brain on day 8 in abortive infections, and on days 3 to 5 in lethal infections. They suggested that if CNS invasion is sufficiently delayed,

host defence mechanisms may be able to contain the virus and abort the the infectious process.

Studies of systemic and local CNS antibody responses in humans infected with Japanese B encephalitis have shown correlations between survival and an earlier and stronger antibody response (Burke *et al* ., 1985a, 1985b). Camenga *et al* . (1974) were able to convert a subclinical WNV encephalitis in mice which had been inoculated with 10^6 LD_{50} WNV i.p. into a lethal infection by a single injection of 150mg/Kg cyclophosphamide i.p. 24 hours later. [Cyclophosphamide is an immunosuppressive agent with predominant effects on T and B lymphocyts. Doses of 150mg/Kg in mice have been demonstrated to significantly decrease B lymphocyte response (Shand, 1979)]. The cyclophosphamide-potentiated infection was characterized by suppressed reponse of neutralizing antibody, prolonged viraemia and enhanced titres of virus in the brain. A single i.p. injection of WNV-immune serum provided neutralizing antibody titres similar to that seen in WNV-infected control mice. Injection of this immune-serum into cyclophosphamide treated mice up to 6 days after infection reduced mortality from 100% to less than 20%. Adoptive transfer of immune spleen cells two days after cyclophosphamide-potentiated WNV infection reduced mortality from 88% to 13%. Mice which had received these immune sleen cells developed neutralizing antibody titres comperable to those in control WNV infected mice, while mice with cyclophosphamide-potentiated WNV infection did not develop neutralizing antibody. The significant reduction in mortality was not seen when the immune spleen cells were given later. Adoptive transfer of immune spleen cells which had been treated with anti-thymocyte serum two days after infection reduced mortality from 88% to 33%. However, the WNV-immune Tc cell response is first detected on the 4th day following infection (Kesson *et al.*,1987) so no conclusions can be drawn about the role of Tc cells from the experiments by Camenga *et al* . (1974). While these studies do not exclude a significant role for T cell-mediated mechanisms in protection against lethal WNV encephalitis they do support the role of antibody in the experimental model.

Alternatively, antibody can lead to enhancement of flavivirus infection and may contribute to the pathogenesis of the disease (Halsted, 1980). In dengue haemorrhagic shock syndrome, the presence of antibodies to one serotype of dengue virus is thought to mediate significant immunopathology when secondary infection with another serotype of dengue virus occurs. Central to the immunopathological response to the infection is the antibody-dependent enhancement of dengue viral replication in Fc-receptor bearing monocytes (Halsted *et al.*, 1973; 1976; Halsted, 1980). Preformed heterologous non-neutralizing IgG antibodies complex with the virus and promote entry into Fc-receptor bearing cells (Gollins and Porterfield, 1984), leading to increased yields of infectious virus from infected cells.

More recently, Barrett and Gould (1986) have demonstrated *in vivo* enhanced virulence using monoclonal antibodies to yellow fever virus in a murine system. They showed that i.p. injection of monoclonal antibody 24 hours prior to infection consistently resulted in a reduced survival time of up to 33%. This required a particular combination of virus and antibody. Gould *et al.* (1987) showed that the enhanced virulence resulted from specific reactions between virus and antibody in the infected brain. It was not mediated through Fc and complement receptor-bearing macrophages. There was no analogy between the mechanisms effecting increased flavivirus growth *in vitro* in the presence of specific antibody and increased neurovirulence *in vivo* after parenteral administration of antibody.

Cell Mediated Immunity.

The importance of T cells in recovery from acute viral infections such as ectromelia (Blanden, 1974) and influenza (Ada, 1981, Lin and Askonas, 1981) has been well established. In contrast, relatively little is known about the role of cell-mediated immunity (CMI) either in a protective or immuno-pathological role in flavivirus infections.

Most studies have shown that CMI appears about the same time or shortly before the appearance of antibodies. Delayed-type hypersensitivity (DTH) responses determined by

footpad swelling after antigenic challenge have been demonstrated for several flaviviruses (Hudson *et al.*, 1979; Pang *et al.*, 1982; Mathur *et al.*, 1983). The maximum DTH response was detected 6 days after virus challenge and declined rapidly thereafter. CMI may contribute to recovery and protection from flavivirus infections. Adoptive transfer of immune spleen cells within one day of infection were shown to protect against lethal encephalitis in mice challenged with Banzi virus (Jacoby *et al.*,1980). Genetically resistant C3H.RV ~~C3H/RV~~ mice can be made susceptible to a lethal challenge with the flavivirus Banzi by treating them with anti-thymocyte serum. Adoptive transfer of immune spleen cells taken 5 days after priming with Banzi virus conferred partial protection against lethal challenge, but splenic cells taken on days 7 or 10 usually gave better protection. This would suggest that memory Tc cells as demonstrated in WNV infections by Kesson *et al.* (1988) played a role in conferring ~~eonfering~~ protection as very little Tc cell activity could be detected in WNV-infected mice 10 days after infection (Kesson *et al.*, 1988). Pretreating Banzi virus-immune cells with anti-thymocyte serum and complement prior to adoptive transfer significantly increased the death rate. Furthermore, only live cells conferred protection, suggesting that preformed antibody in the donor cell suspension was not responsible for protection.

These data contrast with the results of Camenga *et al.*, (1974). They found that syngeneic WNV-immune spleen cells from donors which received 2 to 4 i.p. inoculations with WNV protected the study mice from a lethal challenge with WNV only when the immune spleen cells were transferred within 2 days of infection. Immune spleen cells transferred 4 or 6 days after infection did not confer protection. The difference in these results may reflect differences in experimental technique. In the study by Camenga *et al.* (1974) immune spleen cells were harvested after donor mice received multiple immunizations over several weeks which may favor proliferation of B cells. However, the population of cells which conferred protection was not formally identified and the role of B cells in protecting these mice from lethal flavivirus encephalitis was inferred from indirect evidence ~~evidenee~~. In the protocol for Banzi virus the donor mice were given a single dose of live virus. These latter conditions favor T cell-dependent adoptive immunization (Adler and

Rabinowitz, 1973). Selective depletion of T cells by irradiation and thymectomy followed by bone marrow reconstitution (Bhatt and Jacoby, 1976) or by anti-thymocyte serum (Jacoby *et al.*, 1980) has been shown to potentiate Banzi virus encephalitis as determined by brain virus titres, although survival time is prolonged. This suggests that T cell-mediated immune mechanisms may be detrimental to the animal and may generate immunopathology in a manner similar to that seen in lymphocytic choriomeningitis virus infection (Cole *et al.*, 1972; Zinkernagel and Doherty, 1973), though they are needed to clear the CNS. It may be that the early occurence of T cells leads to viral clearance from the brain but if the T cell response occurs after a significant number of brain cells are infected then the resulting "viral clearance" may lead to significant and dangerous immunopathology (Camenga and Nathenson,1975, Semenkov *et al.*, 1975).

The generation of Tc cells has been demonstrated for Banzi virus (Sheets *et al.*, 1979) and for Langat virus (Gajdosova *et al.*, 1981). However, the role of Tc cells in clearance of flaviviruses from the central nervous system is not well established and little work has been done to study the contribution of Tc cells to any immunopathology in these diseases.

Cytotoxic T Cells in the Immune Response to Virus Infections.

Humans with primary T cell immunodeficiencies (e.g. severe combined immunodeficiency or diGeorge syndrome) or secondary T cell immunodeficiencies (e.g. aquired immunodeficiency syndrome or patients receiving bone marrow transplantation) frequently succumb to overwhelming viral infections (Cooper and Lawton, 1987). Abnormalities of cell mediated immunity predispose the individual to disseminated viral infections particularly with the latent viruses, herpes simplex virus, cytomegalovirus and varicella zoster. T cell deficiency is probably always accompanied by ~~by~~ some abnormality of antibody response although this may not be reflected as hypogammaglobulinemia. A hypogammaglobulinemia or dysgammaglobulinemia may explain why patients with T cell defects are also subject to bacterial infections. In contrast, patients with agammaglobulinemia and normal cell mediated immunity may clear a primary viral infection

e.g. varicella zoster or rubeola. Long-lasting immunity may not develop resulting in dysgammaglobulinemia patients and this may result in recurrent infections with the same virus (Cooper and Lawton, 1987). There are exceptions to these generalizations which are recognized but T cell mediated immune mechanisms are necessary to limit viral infections and allow recovery of the host.

Murine models of viral infections generally demonstrate that maximal Tc cell responses usually occur 4-8 days after infection (Gardner *et al.*, 1974a, Yap and Ada, 1977, Kesson *et al.*,1987). It is postulated that Tc cells *in vivo* lyse infected cells before progeny virus particles are assembled. Cell-mediated immune responses have been extensively studied in mice infected with ectromelia - a poxvirus. This is a mouse pathogen and provides an excellent laboratory model for the study of viral infections. The basic features of pathogenesis of ectromelia were reviewed by Fenner (1949). Normally, mice are infected following abrasions of their skin, usually the foot pad. The virus multiplies at the site of entry after which it is transported via the blood stream to the liver and spleen. Death due to virulent strains of virus in susceptible mice is due to massive hepatic necrosis.

Blanden (1970) demonstrated 80% mortality in mice infected with ectromelia and treated with anti-thymocyte serum, which depleted the mouse of circulating T cells, compared with no mortality in the control groups. Mice injected with anti-thymocyte serum and infected with ectromelia generated neutralizing antibodies and interferon to similar levels seen in control mice, but virus persisted in the liver and spleen whereas it was cleared in control mice. In contrast, delayed hypersensitivity responses were significantly depressed by anti-thymocyte serum treatment. The role of cell-mediated immunity in recovery from ectromelia was further elucidated by Blanden (1971a) using passive transfer of immune spleen cells into infected recipients. Cells harvested between days 4 and 10 possessed significant anti-viral activity as determined by reduction of virus titres in the liver and spleen. The anti-viral activity was not attributable to interferon but some activity was seen by hyperimmune serum. The effect of hyperimmune serum was not as great as that by immune spleen cells. Ectromelia-immune spleen cells treated with anti-light chain serum

or anti-thymocyte serum prior to adoptive transfer did not possess the same anti-viral activity as control ectromelia-immune spleen cells. These data support the role of both B and T cells in viral clearance in ectromelia infection. Recovery from potentially lethal infection is dependent upon control of virus growth in the liver. If virus-induced cytopathology is too great death ensues. Viral clearance starts 4-6 days after subcutaneous infection with virulent virus and presumably depends upon the presence of activated T cells which recognize virus-induced antigens expressed on the infected cell surface. Furthermore, histology of infected livers showed that regression of infected foci was accompanied by accumulation of mononuclear cells at the infected foci triggered by T cells. These inflammatory cells appear to ingest and destroy infectious virus and necrotic tissue. In contrast, mice with graft versus host disease, which have both depressed cellular and humoral responses but intact macrophage activity, had increased mortality due to ectromelia infection. (Blanden, 1971b). Furthermore, ectromelia-immune spleen cells rigourously depleted of mononuclear phagocytes did not impair the anti-viral activity in adoptive transfer experiments. Blanden concluded that "mononuclear cell infiltration of infected liver foci, triggered by T cell-mediated immunity was of critical importance in recovery from ectromelia infection."

Effector T cells generated in ectromelia-infected mice were cytotoxic for ectromelia-infected target cells when tested in a cytotoxic assay *in vitro* (Gardner *et al.*, 1974a, 1974b). These effector T cells were shown to be virus-specific and MHC-restricted and their activity was inhibited by the action of anti-Thy serum and complement i.e. the lysis of infected targets was mediated by Tc cells. Thus, the generation of Tc cells assayed *in vitro* and protection by T cells assayed *in vivo* coincided.

While the antigenic epitopes involved in antibody reponses to flaviviruses have been studied (Heinz *et al* ., 1983, Roehrig *et al* ., 1983) no studies of the antigens required for T cell-mediated responses to flaviviruses have been undertaken in part because of the lack of a reproducible cytotoxic T cell response to flavivirus infections. The work described in this thesis was undertaken to develop reproducible cytotoxic T cell assays to flavivirus

infection. The parameters necessary to generate and assay flavivirus specific Tc cells have been characterized and the role of class I MHC antigen concentration in the antigen presenting complex has been investigated.

Further studies could now be undertaken to investigate the contribution of flavivirus antigens to the determinant recognized by flavivirus-immune Tc cells. This could be approached in two ways: 1) by using vaccinia-flavivirus constructs with flavivirus genes (which have now largely been identified, see above) inserted into a vaccinia virus vector. Thus, the flavivirus proteins which are processed to give antigenic peptides would be identified. Furthermore, this use of vaccinia-flavivirus constructs could be of use in analysis of the flavivirus-immune Tc cell response at a clonal level; 2) a second approach could be undertaken using peptide fragments derived from the flavivirus proteins. This would identify which amino acids are antigenic and contribute to the determinant along with the class I MHC molecules recognized by flavivirus-immune Tc cells. With information derived from one or both of these methods a rational approach to the design of a safe effective vaccine which stimulates the desired T cell -mediated response could be undertaken.

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CHAPTER 2.

THE PRIMARY *IN VIVO* MURINE CYTOTOXIC T CELL RESPONSE TO THE FLAVIVIRUS, WEST NILE.

SUMMARY.

A protocol for obtaining cytotoxic T cell responses to the flavivirus West Nile (WNV) has been developed *in vivo*. CBA/H (H-2^k) mice were immunized with 10^6 p.f.u. WNV intravenously and their spleen cells used directly in cytotoxic assays. This method reliably produced WNV - immune Tc cells which showed WNV - specific cytotoxic activity on infected L929 (H-2^k) target cells. There was inadequate lysis of infected targets by WNV-immune spleen cell when the m. o. i. was less than 100 p.f.u. WNV, or when tertiary mouse embryo fibroblasts, resident peritoneal macrophages or thioglycollate - induced peritoneal macrophages were used as targets. Only L929 cells infected for 16 h with WNV at a m.o.i. of 100 were suitable targets.

Cytotoxic activity against WNV-infected target cells was first detected 4 days after immunization, peaked on day 5 and declined rapidly after day 7. An immunizing dose of 10^3 p.f.u. of WNV was adequate for significant cytotoxicity to be detected; however, the cytotoxic response increased with increasing immunizing doses to plateau levels when 10^6 p.f.u. WNV were used.

The cells responsible for lytic activity were H-2 restricted, Thy-1^+, $CD8^+$, $CD4^-$ and virus-specific with respect to WNV and influenza.

INTRODUCTION.

Representatives of the flavivirus group may cause infections in humans with significant morbidity and mortality. Three main clinical syndromes can be recognised;

1) an acute encephalitis e.g. St Louis encephalitis, Japanese B encephalitis or Murray Valley encephalitis;

2) fever, arthralgia and rash e.g. West Nile fever or dengue and

3) a haemorrhagic fever e.g. yellow fever or dengue haemorrhagic fever and shock syndrome (Sanford ~~1983~~ 1987). In dengue haemorrhagic shock syndrome, the presence of antibodies to one serotype of dengue virus is thought to mediate significant immunopathology when secondary infection with another serotype of dengue virus occurs (Halsted 1982). However, the role if any, of T cells in dengue haemorragic shock syndrome is not known.

The importance of T cells in recovery from acute viral infections such as ectromelia (Blanden,1974) and influenza (Ada,1981; Lin and Askonas,1981) has been well established. Acute and persistent lymphocytic choriomeningitis virus (LCMV) infection can also be cleared by T cells (Mims and Blanden, 1972; Oldstone *et al.*,1986) even from the substance of the brain, without direct vascular access. (Oldstone *et al.*,1986) However in some circumstances T cell mediated immune mechanisms may be detrimental to LCMV-infected mice. Cole *et al.* (1972); Zinkernagel and Doherty (1973) have provided evidence that T cell- mediated injury of the brain in an acute infection with LCMV encephalitis is central to the immunopathological process.

In contrast relatively little is known about the role of cell mediated-immunity (CMI) either in a protective or immunopathological role in flavivirus infections, although antibody responses have been studied in great detail (De Madrid and Porterfield 1974). There are reports on the ability of flaviviruses to induce T cell-mediated delayed type hypersensitivity (Pang *et al.*, 1982; Allan and Doherty, 1986) and cytotoxic T cells (Tc) (Sheets *et al.*, 1979, Gajdosova *et al.*, 1981). However, little has been done to characterise the

conditions necessary to generate and assay flavivirus-immune Tc cells so that the response and its functions can be investigated in detail.

In this report the conditions necessary to generate *in vivo* and to assay a primary flavivirus-immune cytotoxic cell population are described and we characterise the cell responsible for the lytic activity.

MATERIALS and METHODS.

Mice:

Mice were bred under pathogen free conditions at the Animal Breeding Establishment of the John Curtin School of Medical Research. Female mice of the strains CBA/H (H-2^k), BALB/c (H-2^d), C3H.OH (H-2^{02}), B10.Br (H-2^k), and B10.A (H-2^a) were used between the ages of 6 -15 weeks.

Virus:

West Nile Virus, Sarafend strain (WNV) was obtained from Dr I. D. Marshall, and grown in suckling mouse brains (Taylor and Marshall, 1975). Virus was recovered after sonication of a 10% w/v of brain homogenate in gelatin saline (pH 7.2, 0.5% gelatine in borate buffered CaMg saline) for 15 s using a Branson B12 Sonifier (Branson Sonic Power Co., Danbury, Conn., U.S.A.) at 50 W and centrifugation at 1500g for 20 min at 4^o C. Aliquots of supernatant were stored at -70^o C for future use as stock virus. Titres of virus stock were determined by serial dilutions and titrations on Vero cell monolayers as described by Taylor and Marshall (1975).

Influenza A/WSN was prepared by standard methods as described by Yap and Ada (1977).

Generation of Virus- immune spleen cells:

Mice were immunized with an intravenous (i.v.) injection of 10^6 p.f.u. of WNV,

diluted in 200µL of gelatine saline at 4° C unless otherwise stated. Five days later the spleens were collected in assay medium (Eagle's Minimal Essential Medium Cat. No. 410-1500 GIBCO, Chargrin Falls, Ohio, U.S.A., supplemented with 5% foetal calf serum (FCS), 200µg /ml Streptomycin, 200 U/ml Penicillin G and 125µg/ml Neomycin Sulphate) and teased apart with sterile needles to produce a single cell suspension. Viable cells were then counted using trypan blue exclusion and cell concentrations adjusted as required in assay medium. These cells were then used directly in cytotoxic assays.

The method of generation of influenza -immune spleen cells has been fully reported by Yap and Ada (1977).

Target Cells:

1) L929 Cells. The C3H (H-2^k) fibroblast cell line, L929, was grown as described in Gardner *et al.* (1974).

2) Resident peritoneal macrophages were obtained by peritoneal lavage with 10mls of Puck's saline, followed by centrifugation at 400g for 5 minutes and then resuspended in culture medium (Eagle's Minimal Essential Medium Cat. No. 410-1500 GIBCO, Chargrin Falls, Ohio, U.S.A., supplemented with 5% foetal calf serum (FCS), 200µg /ml Streptomycin, 200 U/ml Penicillin G, 125µg/ml Neomycin Sulphate and 10^{-4} 2-mercaptoethanol).

3) Thioglycollate induced peritoneal macrophages were obtained as above from a mouse injected with 2 mls of 3% w/v thioglycollate (Difco Laboratories, Detroit, U.S.A.) intraperitoneally 5 to 10 days previously as desribed in Mullbacher *et al.* (1986).

4) Mouse embryo fibroblast cultures (MEF) were prepared as described by Sinickas *et al.* (1985a).

Cytotoxic Assay:

96-well flat bottomed microtitre trays (Nunc, Roskilde, Denmark) were seeded with $10^{4.3}$ L929 target cells per well in100µL of Dulbecco's Modified Eagles Medium (Cat. No.430 - 1600, GIBCO, Chargrin Falls, Ohio, U.S.A.), supplemented with 5% FCS, 200µg/ml Streptomycin, 200 U/ml Penicillin G and 125µg/ml Neomycin Sulphate (DMEM). Plates were left for 6 h at 37° C and 5% CO_2 to allow the cells to adhere. Each well was then drained and the cells were infected for 1 hr at 37° C with 25µL per well of virus dilution in DMEM to give the multiplicity of infection required, usually 100 p.f.u. per cell. 3µCi 0f $Na_2(^{51}Cr)0_4$ (Amersham Int. Ltd., Amersham, U.K.) in 100µL DMEM per well was then added for 16 h at 37° C and 5% CO_2. After this the medium was flicked off and the cells were washed twice with DMEM at 37° C. WNV-immune spleen cells were incubated with the target cells for 6 h at 37° C in a humidified atmosphere containing 5% CO_2 in air. Triplicate cultures were set up for each dilution of WNV-immune effector cells to give the required effector to target cell ratio (E:T). Calculations of E:T were based on the number of target cells initally added to the wells 24 hours earlier and therefore the number of target cells may have been greater at the time of assay.

^{51}Cr release from the targets was measured in a Packard Auto-Gamma counter and lysis calculated using the following formula:

% lysis of infected or uninfected targets=

$$\frac{^{51}Cr \text{ c.p.m. in the presence of effectors} - {}^{51}Cr \text{ c.p.m. released in medium}}{^{51}Cr \text{ c.p.m.water lysed targets} - {}^{51}Cr \text{ c.p.m. released in medium.}} \times 100\%$$

Data given are means of triplicates. Standard errors of the means were always less than 5%. Significance was determined by Student's t Test and P values of < 0.05 were considered significant.

Treatment with Monoclonal Antibodies and Complement:

WNV-immune spleen cells were suspended at 10^7 cells/ml in assay medium containing an optimium dilution of antibody, determined by prior titration, to give maximum cell lysis. After incubation at 4° C for 45 min the cells were washed twice in assay medium at 37° C and resuspended in 1ml assay medium containing a 1:10 dilution of low Tox-M rabbit complement (C') (Cedarlane Laboratories Ltd., Ontario, Canada). After 45 minutes incubation at 37° C, the cells were washed twice with assay medium, resuspended in 1.5 ml assay medium, titrated with 3-fold steps and utilised as effector cells in cytotoxic assays. The optimal concentrations of the monoclonal antibodies were determined by complement-dependent lysis of normal spleen cells. The CD8 antibody (31M) and CD4 antibody (172) were a gift of Dr R. Ceredig and were used at a 1:10 dilution of antibody. Thy 1.2 antibody (Serotec. Clone F7D5) was used at a 1:100 dilution of antibody.

RESULTS.

The experimental protocol was a modification of the method for Banzi virus cytotoxicity reported by Sheets *et al.*, (1979). The initial experiments were designed to determine the optimal target cell type and m.o.i. per target. Four cell types were tested, L929 cells (H-2^k) and tertiary mouse embryo fibroblasts, resident peritoneal macrophages and thioglycollate-induced peritoneal macrophages from CBA/H (H-2^k) mice. Only L929 cells infected for 16 h with WNV at a m.o.i. of 100 were suitable targets for lysis by CBA/H WNV-immune spleen cell populations obtained 5 days after i.v. injection of 10^6 p.f.u., however, all target cells were lysed by secondary WNV - immune spleen cells (Kesson *et al.*, submitted). There was suboptimal lysis of infected L929 targets by WNV-immune spleen cell when m. o. i. of less than 100 p.f.u. WNV per L929 cell were used. All experiments reported below used L929 targets infected at a m.o.i. of 100.

Kinetics of the Generation in vivo of Primary CBA/H WNV immune Cytotoxic Cells.

Over a period of 43 days, groups of 3 CBA/H female mice were injected i.v. with 10^6 p.f.u. of WNV. Their spleens were removed 43, 14, 10, 7, 6, 5, 4, 2 and 0 days after inoculation. The spleen cells from each group were harvested on the same day, pooled and assayed for cytotoxic activity on WNV-infected and uninfected L929 cells.

Cytotoxic activity against WNV-infected target cells was first detected in the spleen cell population 4 days after immunizing with WNV, high activity was evident on days 5 and 6, and activity declined rapidly after day 7. (Table 1.). Little significant lysis of uninfected L929 cells was observed.

Effect of Virus Dose on the Generation in vivo of CBA/H WNV-immune Cytotoxic Cells.

Groups of 3 CBA/H female mice were immunized i.v. with 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 or 10^7 p.f.u. WNV and their spleens used as a source of WNV-immune cytotoxic cells 5 days after immunizing. Significant cytotoxicity against WNV-infected L929 target cells above uninfected control targets was evident when immunizing doses greater than or equal to 10^3 p.f.u. were used (Table 2). The magnitude of the response was dependent on the dose of WNV used to immunize the mice and reached plateau levels at 10^6 p.f.u.. Levels of lysis of infected L929 target cells were less in this experiment than those shown in Table 1. The reason for this variation between experiments is unknown, but the data in Table 2 is more generally representative of the level of lysis seen with 5 day immune cells.

H-2 Restriction Pattern of Primary In Vivo WNV-Immune Cytotoxic Spleen Cells.

Groups of 3 mice of the strains CBA/H, BALB/c, C3H.OH, and B10.A were immunised with 10^6 p.f.u. WNV and their spleen cells used in a cytotoxic assay on L929 target cells 5 days later. (Table 3, Expt.1.)

Significant lysis of WNV-infected L929 cells above lysis of uninfected, control L929 cells was mediated by WNV-immune spleen cells from CBA/H, which share all the *H-2*

complex with L929 target cells, and from B10.A mice which share the *K* and *I* regions. In contrast, there was no significant lysis by BALB/c WNV-immune spleen cells which do not share any *H-2* genes with L929 cells or by C3H.OH WNV-immune spleen cells which only share the *D* region. These data suggest that the primary anti-WNV cytotoxic response is H-2 restricted and that with respect to the H-2^k haplotype the response is associated with the *K* region. *I* region sharing is not necessary since L929 cells do not express *I* region gene products (Lechler *et al.*, 1985). The BALB/c and C3H.OH effector population could not be tested for H-2^d restricted cytotoxic activity as no suitable targets were available. Since the lysis by B10.A (K^k D^d) immune cells was lower than that caused by CBA (K^k D^k) in experiment 1, a second experiment was performed to determine if a similar difference ~~occured~~ occurred between ~~B10.Br~~ B10.BR and B10.A which share the B10 background. No difference was seen, (Table 3 Expt.2) thus confirming that K^k rather than D^k is the main restriction antigen for primary anti-WNV cytotoxicity against infected L929 target cells.

Phenotype of Primary CBA/H WNV-Immune Cytotoxic Cells.

Six CBA/H mice were immunized i.v. with 10^6 p.f.u. WNV and their spleen cells harvested 5 days later and prepared as a single cell suspension to be used as WNV-immune cells in a cytotoxic assay. The cells were divided into 5 equal aliquots and treated with Thy 1.2 antibody and C', CD8 antibody and C', CD4 antibody and C', C' alone or left untreated prior to cytotoxic assay. The viable cell recovery after treatment as determined by trypan blue exclusion was 59%, 73%, 80%, 94% and 100% respectively. Cytotoxicity was tested on WNV-infected or uninfected L929 targets.

Thy 1.2 and CD8 antibodies and C' significantly reduced cytotoxic activity below the level of the C' control whereas CD4 antibody plus C' did not (Table 4).

These data and the H-2 restriction shown in Table 3 indicate that the anti-WNV cytotoxic activity was vested in cytotoxic T lymphocytes (Tc).

Viral Specificity of Primary In Vivo CBA/H WNV-Immune Tc Cells.

Two groups of 3 CBA/H mice were injected i.v. with 10^6 p.f.u WNV or 10^2 HAU of influenza A/WSN virus and 5 days later their spleens were used as a source of virus-immune Tc cells and tested on influenza- infected, WNV-infected and uninfected L929 cells (Table 5). WNV-immune Tc cells significantly lysed WNV - infected targets but did not lyse influenza - infected or uninfected L929 target cells. In contrast, influenza - immune Tc cells significantly lysed influenza - infected targets but did not lyse WNV - infected or uninfected L929 target cells. Thus the cytotoxic response was virus-specific with respect to WNV and Influenza A/WSN.

DISCUSSION.

This paper describes both the generation and assay of a primary anti-WNV Tc cell population. A virus-specific Tc cell population can be generated in the spleens of WNV-infected mice provided that the conditions are optimised. Maximal activity was obtained when immunizing doses of $\geq 10^6$ p.f.u. WNV were given intraveneously and the spleen cells assayed 5 days after immunization. Activity rapidly declined thereafter. Similar temporal profiles have been seen with the cytotoxic activity of other murine virus-immune spleen cells e.g. ectromelia (Gardner *et al.*,1974), influenza (Yap and Ada, 1977) and the flavivirus, Langat "14", (Gajdosova *et al.*,1981). In all these situations the mice were immunized i.v.. In contrast, Sheets *et al.* (1979), using the flavivirus, Banzi, demonstrated that cytotoxic activity was first detectable on day 6 after intraperitoneal (i.p.) immunization of C3H/RV mice, the cytotoxic activity peaked on day 8 and did not return to control levels until 16 days after immunization. These data suggest that the route of immunization used i.e. i.v. verses i.p., may determine the time of maximal cytotoxic activity in virus-immune spleen cells.

Additional experiments were designed to optimise the target cells for detection of viral-specific lysis. Only L929 cells, infected with WNV at a m.o.i. of 100 for 16 hours,

gave adequate lysis when primary WNV-immune spleen cells were used as a source of Tc cells. Resident or thioglycollate- activated peritoneal macrophages or MEF proved unsatisfactory as targets for primary WNV-immune spleen cells were lysed ~~but could detect lysis caused~~ by more potent secondary Tc cell populations (Kesson *et al.*, 1988). King *et al.* (1986) have shown that the cross-sectional area of the target cell and the H-2 antigen concentration on the cell surface membrane to be determinants of target susceptibility to Tc cells. Thus, in a WNV cytotoxic assay, L929 cells may be superior targets because they are larger and/or express more viral-H-2 "complexes" than the other infected target cell types tested under the conditions used here.

The phenotype of the WNV-immune spleen cell population responsible for target cell lysis is Thy 1^+, $CD8^+$ and $CD4^-$. This is the conventional phenotype of virus-specific Tc cells as described by Pang *et al.* (1976), Mullbacher and Blanden (1978) and Sinickas *et al.* (1985b). Further investigation of the roles of $CD8^+$, $CD4^-$ and $CD8^-$, $CD4^+$ T cells in the generation of anti-WNV Tc responders is now possible with the development of secondary responses *in vitro* (Kesson *et al.*, 1988).

Primary WNV-immune Tc cells were virus specific with respect to WNV-infected and influenza A/WSN-infected L929 target cells. Further studies are in progress to investigate the cross reactivity of flavivirus- immune Tc cells within the flavivirus group.

H-2 restriction studies were limited because of the finding that only heavily-infected L929 targets ($H\text{-}2^k$) gave significant lysis with primary anti-WNV Tc cells. However, the available data show that K^k is a restriction allele for primary anti-WNV Tc cells as WNV-infected L929 cells are lysed significantly by both CBA/H (K^k, D^k) and B10.A (K^k, D^d) WNV-immune Tc cells. These data also showed that primary CBA/H WNV-immune Tc cells lysed WNV-infected L929 targets better than primary B10.A WNV-immune Tc cells. These results raise the possibility that D^k may also be a restriction antigen for WNV-immune T cells. However this seems unlikely because C3H.OH (K^d, D^k) anti-WNV Tc cells did not lyse WNV-infected L929 target cells, implying that D^k is not a good restriction antigen for WNV. An alternative explanation, viz, that D^d adversely ~~adversly~~

affects the strength of the K^k-restricted anti-WNV Tc cell response in B10.A mice also seems unlikely, since B10.A (K^k,D^d) and B10.Br (K^k,D^k) gave similar anti-WNV Tc cell responses. Thus the differences between CBA/H and B10.A could be due to non-H-2 genes such as is the case for alphaviruses (Mullbacher *et al.*, 1983). More extensive comparison of various H-2^k strains with different backgrounds is needed to investigate this possibility.

This discussion is limited to lysis of WNV-infected L929 (H-2^k) target cells because these were the only target cell type that could be lysed by primary anti-WNV Tc cell responses. A following paper describes more potent secondary responses which are capable of lysing WNV-infected MEF target cells, thus allowing investigation of the effects of other H-2 haplotypes.

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TABLE 1

Effect of Priming Interval on the Generation of Primary *In Vivo* WNV-Immune Spleen Cells*

Priming Interval (Days)	E/T+	% Specific Lysis of L929 Target Cells.† WNV Infected	Uninfected
0	30	7.0§	5.9
	10	7.2	2.3
	3	6.9	0.7
2	30	13.7	11.8
	10	8.2	7.2
	3	6.7	2.8
4	30	48.7	7.4
	10	27.8	4.1
	3	20.4	1.5
5	30	76.7	7.1
	10	57.4	3.8
	3	30.8	2.2
6	30	59.0	6.7
	10	45.7	4.6
	3	24.2	1.4
7	30	41.8	6.5
	10	19.8	4.3
	3	14.2	1.2
10	30	13.1	7.7
	10	11.1	4.4
	3	3.4	1.4
14	30	12.5	3.6
	10	7.0	2.0
	3	3.8	-0.7
43	30	6.0	1.9
	10	4.7	1.6
	3	3.0	0.2

*) CBA/H female mice greater than 6 weeks old primed with 10^6 pfu WNV i.v. and spleen cells used as effector cells.

†) Infected with 100 pfu WNV/target for 16 hours.

+) Effector to target cell ratio.

§) Means of triplicates given with standard errors of the means less than 5%.

TABLE 2.

Effect of Immunizing Dose on the Generation of Primary

In Vivo WNV-Immune Spleen Cells.*

Immunizing Dose (p.f.u. WNV)	E/T+	% Specific Lysis of L929 Target Cells.† WNV Infected	Uninfected
10^1	90	-1.4	0.6
	30	4.0	0.1
	10	0.2	-0.7
10^2	90	2.4	1.1
	30	3.8	0.8
	10	0.7	-0.1
10^3	90	9.6	1.0
	30	7.9	0.4
	10	1.7	0.6
10^4	90	13.6	1.6
	30	12.8	1.4
	10	1.5	0.6
10^5	90	17.1	1.2
	30	25.0	2.3
	10	9.5	1.7
10^6	90	32.8	4.4
	30	23..5	4.0
	10	12.4	1.7
10^7	90	30.2	4.5
	30	22.9	2.9
	10	11.0	1.6

*)

†) As for table 1.

+)

§) Means of triplicates given with standard errors of the means less than 2.4%.

TABLE 3.

H-2 Restriction pattern of Primary *In Vivo* WNV-Immune Spleen cells.*

Tc Cells.+	E:T Ratio.	% Specific Lysis of L929 Targets (kkk)† WNV Infected.	Uninfected.
Experiment 1.			
CBA/H WNV-immune (kkk)	30	35.9§	-1.5
	10	14.6§	-1.6
	3	5.7	-0.5
BALB/c WNV-immune (ddd)	30	-1.5	0.0
	10	1.8	-0.1
	3	-4.0	1.5
C3HOH WNV-immune (ddk)	30	4.1	-3.6
	10	1.8	-1.6
	3	-1.6	-1.6
B10A WNV-immune (kkd)	30	18.0§	-1.1
	10	4.3	-2.6
	3	-2.8	-1.6
Experiment 2.			
B10.BR ~~B10B1~~ WNV-immune (kkk)	90	28.7§	1.4
	30	24.1§	1.9
	10	11.1	2.9
B10A WNV-immune (kkd)	90	26.7§	0.9
	30	22.8§	1.5
	10	11.8	2.1

*) Immunized with 10^6 p.f.u WNV i.v. and spleen cells used as effector cells 5 days post priming.

†) H-2 map corresponding to *K*, *I* , and *D* regions.

+) Means of triplicate cultures with standard errors of the means less than 2.3%.

§) Significant specific lysis of infected target cells.

TABLE 4.

Phenotype of Primary *In Vivo* CBA/H WNV-Immune Cytotoxic Cells.*

Treatment.†	Aliquot§	% Specific Lysis of Target Cells.+ WNV Infected.	Uninfected.
Nil	1:10	36.8	2.8
	1:30	21.0	3.8
	1:90	9.8	2.9
C` alone	1:10	29.0	4.0
	1:30	12.6	3.2
	1:90	4.4	2.0
Anti Thy & C'	1:10	7.7¶	3.8
	1:30	4.3	3.9
	1:90	2.1	2.1
Anti CD4 & C`	1:10	27.1	4.3
	1:30	12.2	3.8
	1:90	5.6	1.9
Anti CD8 &C`	1:10	10.4¶	6.5
	1:30	5.1	5.2
	1:90	2.8	2.9

*) Primed with 10^6 p.f.u WNV i.v. and spleen cells used as effector cells 5 days post priming.

†) Conditions for antibody treatment and assay are described in Materials and Methods.

+) Means of triplicate cultures with standard errors of the means less than 2.7%.

§) Aliquot of cells treated. A 1:10 aliquot is equivalent to an effector to target ratio of 30:1.

¶) Significant decrease in specific lysis of infected target cells compared with C' control.

TABLE 5.

Viral Specificity of Primary *In Vivo* CBA/H WNV- Immune Tc Cells.

Tc Cells	E:T †	% Specific Lysis of L929 Targets.* WNV Infected+	Influenza Infected§	Uninfected
CBA/H WNV-immune¶	30	28.1•	0.2	0.1
	10	13.9•	0.5	-0.9
	3	3.8	0.8	0.0
CBA/H Influenza - immune#	30	-1.9	30.0•	0.1
	10	-0.7	30.5•	1.7
	3	-0.4	4.6	1.3

*) Means of triplicate cultures with standard errors of the means less than 3.7%.

†) Effector to target cell ratio.

+) Infected with 100 p.f.u WNV per target.

§) Infected with 10^{-4} HAU A/WSN per target.

¶) Primed with 10^{6} p.f.u WNV i.v. and spleen cells used as a source of Tc cells 5 days post priming.

#) Primed with 66 HAU influenza virus i.v. and spleen cells used as a source of Tc cells 5 days post priming.

•) Significant specific lysis of infected target cells.

CHAPTER 3.

THE SECONDARY *IN VITRO* MURINE CYTOTOXIC T CELL RESPONSE TO THE FLAVIVIRUS, WEST NILE.

SUMMARY.

A secondary *in vitro* murine cytotoxic response to the flavivirus, West Nile (WNV) is described. Cytotoxic activity was obtained from spleen cells of mice primed 7 days previously with 10^6 p.f.u. WNV and boosted *in vitro* for a further 5 days with WNV-infected stimulator spleen cells. The cells responsible for lysis of WNV-infected target cells were restricted by class I H-2 antigens. In the *H-2* ^{k} haplotype the *K* region but not the *D* region allowed responses. In the *H-2* ^{d} haplotype both *K* and *D* regions were permissive. The cytotoxic cells were virus-specific with respect to WNV and Influenza. The phenotype of the cells which mediated cytotoxicity was Thy 1^+, $CD8^+$ and $CD4^-$; however an $CD4^+$ helper population was required for the optimal generation of the cytotoxic response *in vitro*.

INTRODUCTION

Members of the flavivirus genus cause serious infections in humans in many countries throughout the world (Sanford, ~~1983~~ 1987) and with the exception of yellow fever and perhaps Japanese B Encephalitis, no significant advances have been made to immunize against these diseases. Much work has been done to elucidate the humoral responses to flavivirus infections (DeMadrid and Porterfield, 1984) and to study the mechanisms of viral neutralisation (Gollins and Porterfield, 1986; Phillpotts and Porterfield, 1985) but the study of T cell- mediated immunity in flavivirus infections has been much more limited (Gajdosova *et al.*, 1981, Sheets *et al.*, 1979).

The importance of T cells in recovery from certain viral and bacterial infections has been well documented (Ada *et al.*, 1981; Blanden, 1974). The generation of such cells both *in vivo* and *in vitro* has been reported for many viruses (Gardner *et al.*,1974; Mullbacher and Blanden, 1978; Pang and Blanden, 1976; Yap and Ada, 1977; Zinkernagel and Doherty, 1979). More recently use of cloned T cells has begun to reveal essential aspects of T cell function that lead to viral clearance from the CNS (Oldstone *et al.*,1986) and other tissues (Lin and Askonas, 1981).

A previous report (Kesson *et al.*, 1987) described the conditions required for the induction of a primary immune Tc cell response to WNV *in vivo*. In this paper we study the *in vitro* conditions necessary to generate a more potent secondary WNV-immune Tc cell population and use these Tc cells to study H-2 restriction and other functional aspects of the response.

MATERIALS and METHODS.

Mice:

Mice were bred under pathogen free conditions at the Animal Breeding Establishment of the John Curtin School of Medical Research. Female mice of the strains CBA/H

(H-2^k), BALB/c (H-2^d), C3H.H-2^{02} (H-2^{02}), B10.A (H-2^a), B10.AQR (H-2^{y1}) and B10.A(5R) (H-2^{i5}) were used when greater than 6 weeks of age.

Virus:

West Nile Virus, Sarafend strain (WNV) was obtained from Dr I. D. Marshall, and grown in suckling mouse brains (Taylor and Marshall, 1975). Virus was recovered after sonication of a 10% w/v of brain homogenate in gelatin saline (pH 7.2, 0.5% gelatine in borate buffered CaMg saline) for 15 s using a Branson B12 Sonifier (Branson Sonic Power Co., Danbury, Conn., U.S.A.) at 50 W and centrifugation at 1500g for 20 min at 4^o C. Aliquots of supernatant were stored at -70^o C for future use as stock virus. Titres of virus were determined by serial dilutions and titrations on Vero cell monolayers as described by Taylor and Marshall (1975).

Influenza A/WSN was prepared by standard methods as described by Yap and Ada (1977).

Target Cells:

1) The C3H (H-2^k) fibroblast cell line, L929, was grown as described in Gardner *et al.* (1974).

2) Murine resident peritoneal macrophages were obtained by peritoneal lavage with 10mls of Puck's saline, followed by centrifugation at 400g for 5 minutes and then were resuspended in culture medium (Eagle's Minimal Essential Medium Cat. No. 410-1500 GIBCO, Chargrin Falls, Ohio, U.S.A., supplemented with 5% foetal calf serum (FCS), 200µg /ml Streptomycin, 200 U/ml Penicillin G, 125µg/ml Neomycin Sulphate and 10^{-4} 2-mercaptoethanol).

3) Thioglycollate induced peritoneal macrophages were obtained as above from a mouse injected with 2 mls of 3% w/v thioglycollate (Difco. Detroit, U.S.A.)

intraperitoneally 5 to 10 days previously as desribed in Mullbacher *et al.* (1986).

4) Tertiary passaged mouse embryo fibroblast cultures (MEF) were prepared as described by Sinickas *et al.* (1985).

Generation of Secondary WNV-Immune Effector Cells In Vitro:

Unless otherwise stated mice were primed with an intravenous (i.v.) injection of 10^6 p.f.u. of WNV, diluted in 200µL of gelatine saline, pH 7.2 and 7 days later, the spleens were collected in Puck's saline and teased apart with sterile needles to produce a single cell suspension. Viable cells were then counted using trypan blue exclusion and cell concentrations were adjusted as required in culture medium. Secondary WNV-immune Tc cells were generated *in vitro* by culturing $10^{7.9}$ "responder" cells from primed mice with $10^{7.3}$ non-immune syngeneic "stimulator" cells which were infected with 1 to 5 p.f.u of WNV per nucleated cell in 1 ml of culture medium for 1 hour at 37° C and then irradiated with $10^{3.3}$ rads from a ^{60}Co source. These cells were cultured for 5 days in 50mls ($10^{6.3}$ cells per ml) of culture medium in Nunclon Delta 80^2 cm tissue culture flasks (No 53732) in a humidified atmosphere of 5% CO_2 in air at 37° C. The cells were then washed once with culture medium, viable cell numbers determined using trypan blue exclusion and cell concentrations adjusted as required. They were then diluted in three fold steps for use in cytotoxic assays.

Generation of Secondary Influenza-Immune Effector Cells In Vitro:

The method of generation of secondary influenza-immune Tc cells has been fully reported by Yap and Ada (1977). Briefly, mice were primed with 200 haemagglutination units (HAU) of influenza virus and their spleens were removed 7 days later and teased apart to produce a single cell suspension. Secondary influenza-immune Tc cells were generated *in vitro* by 5 days of culture of $10^{7.9}$ "responder" cells from primed mice with $10^{7.3}$ non-immune syngeneic "stimulator" cells which had been infected with 200 HAU

influenza virus in 1 ml of culture medium for 1 hour at 37° C.

Cytotoxic Assay:

96-well flat bottomed microtitre trays (Nunc, Roskilde, Denmark) were seeded with $10^{4.3}$ target cells per well in100µL of Dulbecco's Modified Eagles Medium (Cat. No. 430-1600, GIBCO, Chargrin Falls, Ohio, U.S.A.), supplemented with 5% FCS, 200µg/ml Streptomycin, 200 U/ml Penicillin G and 125µg/ml Neomycin Sulphate (DMEM). Plates were left for 6 h at 37° C and 5% CO_2 to allow the cells to adhere. Each well was then drained and the cells were infected for 1 hr at 37° C with 25µL per well of virus dilution in DMEM to give the multiplicity of infection required, usually 100 p.f.u. per cell with WNV or 60 HAU of influenza virus. 3µCi 0f $Na_2(^{51}Cr)0_4$ (Amersham Int. Ltd., Amersham, U.K.) in 100µL DMEM per well was then added for 16 h at 37° C and 5% CO_2. After this the medium was flicked off and the cells were washed twice with DMEM at 37° C. Secondary WNV-immune or influenza-immune spleen cells were incubated with the target cells for 6 h at 37° C in a humidified atmosphere containing 5% CO_2 in air. Triplicate cultures were set up for each dilution of WNV-immune or influenza-immune effector cells to give the required effector to target cell ratio (E:T). Calculations of E:T were based on the number of target cells initally added to the wells 24 hours earlier and therefore the number of target cells may have been greater at the time of assay.

^{51}Cr release from the targets was measured in a Packard Auto-Gamma counter and lysis calculated using the following formula:

$$\% \text{ specific lysis of infected or uninfected targets} = \frac{^{51}Cr \text{ c.p.m. in the presence of effectors} - ^{51}Cr \text{ c.p.m. released in medium}}{^{51}Cr \text{ c.p.m.water lysed targets} - ^{51}Cr \text{ c.p.m. released in medium.}} \times 100\%$$

Data given are means of triplicates. Standard errors of the means were always less than 5%. Significance was determined by Student's t Test and P values of < 0.05 were considered significant.

Treatment with Monoclonal Antibodies and Complement:

WNV-immune spleen cells were suspended at 10^7cells/ml in culture medium containing an optimium dilution of antibody, determined by prior titration to give maximum cell lysis. After incubation at 4° C for 45 min the cells were washed twice in culture medium at 37° C and resuspended in 1ml culture medium containing a 1:10 dilution of low Tox-M rabbit complement (C') (Cedarlane Laboratories Ltd., Ontario, Canada). After 45 minutes incubation at 37° C, the cells were washed twice with culture medium, resuspended in 1.5 ml culture medium, diluted in 3-fold steps and used as effector cells in cytotoxic assays. The optimal concentrations of the monoclonal antibodies were determined by complement-dependent lysis of normal spleen cells. The CD8 antibody (31M) and CD4 antibody (172) were a gift of Dr R. Ceredig and the Thy 1.2 antibody used was Clone F7D5 (Serotec).

Addition of Exogeneous IL-2 to Cultures.

IL-2 was added to cultures as a 3% vol./vol. supernatant from an IL-2 secreting EL-4 cell line and was a gift of Dr C. J. Sanderson Mill Hill, London.

RESULTS.

Effect of Target Cell Type and Virus Dose on Susceptibility to Lysis.

Four target cell types, L929 cells, resident peritoneal macrophages, thioglycollate induced peritoneal macrophages and tertiary MEF were tested to determine the optimal target cell type for assaying the secondary *in vitro* cytotoxic responses to WNV. (Table 1).

L929 cells infected with 100 p.f.u. WNV per cell gave the highest lysis over and above

uninfected control targets. Both resident peritoneal macrophages and tertiary MEF gave lower but readily detectable lysis. Thioglycollate induced peritoneal macrophages gave the lowest lysis.

L929 target cells were infected with m.o.i. of 100,10 or1 p.f.u. per cell and tested for susceptibilty to lysis by virus-immune cytotoxic cells. (Table 2). A m.o.i. of 100 gave the highest viral-specific lysis; the lower m.o.i. gave progressively decreasing susceptibility. L929 cells infected with m.o.i. of 100 were routinely used as target cells.

The reason for the variation in the amount of lysis between the experiments in Tables1 and 2 is unknown. Lysis of both infected and uninfected targets varied from experiment to experiment. In experiments where lysis of WNV-infected targets was high, the lysis of uninfected targets was also relatively high.

Kinetics of Priming for the Generation In Vitro of Secondary CBA/H WNV-Immune Cytotoxic Cells.

Groups of 3 CBA/H female mice were primed i.v. with 10^6 p.f.u. of WNV 43, 14, 10, 7, 6, 5, 4, 2 and 0 days before their spleen cells were harvested on the same day, pooled (within groups) and cultured *in vitro* with WNV-infected stimulator cells. Five days later the secondary WNV-immune cells were assayed for cytotoxic activity on WNV-infected and uninfected L929 cells.

Cytotoxic activity against WNV-infected target cells was high at all time points, but increased to a plateau on days 6 to 43 (Table 3). Lysis of uninfected targets was always lower than lysis of infected targets but varied markedly with time; it was high on days 0 and 2, lower on days 4, 5 and 6 and increased again on days 7, 10, 14 and 43. Thus the difference between infected and uninfected targets was affected by time after priming but was significant from day 2 onwards (Table 3.). A 7 day interval between *in vivo* priming and secondary stimulation *in vitro* was used hereafter.

Effect of Priming Dose on the Generation In Vitro of Secondary CBA/H WNV-Immune Cytotoxic Cells.

Groups of 3 CBA/H female mice were primed i.v. with 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 or 10^7 p.f.u. of WNV and 7 days later the spleen cells from each group were harvested and stimulated *in vitro* . After a further 5 days the secondary WNV-immune cells were assayed for cytotoxic activity on WNV-infected and uninfected L929 cells.

Significant cytotoxicity against WNV-infected L929 target cells above uninfected control targets was evident when priming doses greater than or equal to 10^3 p.f.u. were used and a plateau was reached at 10^5 p.f.u. (Table 4). Lysis of uninfected targets progressively decreased with increasing priming dose. Thus the increase in viral-specific lysis with increasing priming doses of WNV brain stock was caused by decreasing lysis of uninfected targets as well as by an increase in lysis of infected targets. Doses of 10^6 p.f.u. were routinely used for i.v. priming.

H-2 Restriction Pattern of Secondary WNV-Immune Cytotoxic Cells Generated In Vitro.

Spleen cells of groups of 3 WNV-primed mice of the strains CBA/H and BALB/c, were harvested and stimulated *in vitro* for 5 days. The secondary WNV-immune cells were assayed for cytotoxic activity on WNV-infected and uninfected CBA/H and BALB/c tertiary MEF (Table 5).

Lysis of infected CBA/H tertiary MEF significantly above lysis of uninfected control target cells was mediated by syngeneic CBA/H WNV-immune cytotoxic cells and not by allogeneic BALB/c WNV-immune cytotoxic cells. Similarly, lysis of infected BALB/c tertiary MEF was mediated by syngneic BALB/c WNV-immune cytotoxic cells and not by allogeneic CBA/H WNV-immune cytotoxic cells.

These data demonstrated that the secondary anti-WNV cytotoxic response was H-2 restricted.

Genetic Mapping of Secondary WNV-Immune Cytotoxic Cells Generated In Vitro.

Groups of 3 mice of the strains CBA and BALB/c were used as a source of secondary WNV-immune spleen cells which were assayed for cytotoxic activity on WNV-infected and uninfected C3H.H-2^{02} and B10.A tertiary MEF. (Table 6, Experiment 1).

Lysis of infected C3H. H-2^{02} MEF above uninfected controls was mediated by BALB/c WNV-immune cells which share the K^d and I^d regions and not by CBA/H WNV-immune cells which share the D^k region. Lysis of infected B10.A MEF above uninfected controls was mediated strongly by CBA/H WNV-immune cells which share the K^k and I^k regions and to a lesser extent by BALB/c WNV-immune cells which share the D^d region. Because CBA/H WNV-immune cells lysed WNV-infected B10.A MEF more strongly than BALB/c WNV-immune cells, a second experiment was done (Table 6, Experiment 2) to determine if K region alleles influenced the strength of the D^d restricted response.

BALB/c ($K^d D^d$), B10.A ($K^k D^d$), B10.AQR ($K^q D^d$) and B10.A(5R) ($K^b D^d$) secondary WNV-immune cells all lysed WNV-infected BALB/c MEF significantly more than they lysed uninfected BALB/c MEF. Thus the K^k, K^q and K^b alleles of the K region did not influence the strength of the response associated with the D^d allele. BALB/c WNV-immune cells caused less lysis of uninfected BALB/c MEF than the other strains of WNV-immune cells.

These data showed that the secondary anti-WNV cytotoxic response was associated with the K region of the $H\text{-}2^k$ haplotype and both the K and the D regions of the $H\text{-}2^d$ haplotype. The I regions are not involved in the recognition of MEF as these cells do not express I region gene products (King *et al.*, 1985).

Viral Specificity of Secondary CBA/H WNV-Immune Cytotoxic Cells.

Secondary CBA/H influenza A/WSN-immune or WNV-immune spleen cells were generated *in vitro* and assayed for cytotoxic activity on influenza-infected, WNV-infected or uninfected control L929 cells (Table 7).

WNV-immune cells lysed WNV-infected targets significantly more than they lysed influenza-infected or uninfected L929 target cells and influenza-immune cells lysed influenza-infected targets significantly better than they lysed WNV-infected or uninfected L929 target cells. Both populations of virus-immune spleen cells lysed uninfected targets and the targets infected with heterologous virus to a similar extent, but in both cases far less than they lysed the target cells infected with their homologous virus. Therefore the cytotoxic responses were virus-specific with respect to WNV and Influenza A/WSN.

Phenotype of Secondary CBA/H WNV-Immune Cytotoxic Cells.

A population of secondary CBA/H WNV-immune spleen cells were divided into 5 equal aliquots and treated with Thy 1.2 antibody and C', CD8 antibody and C', CD4 antibody and C', C' alone or left untreated prior to cytotoxic assay. The viable cell recovery after antibody treatment as determined by trypan blue exclusion was 33%, 53%, 52%, 94% and 97% respectively. Cytotoxic activity of surviving cells was tested after all the aliquots were adjusted to 1.5 ml and diluted in 3 fold steps so that1:10, 1:30 and1:90 fractions of the cell suspension were added to WNV-infected or uninfected L929 targets.

Thy 1.2 and CD8 antibodies and C' virtually eliminated cytotoxic activity on WNV-infected L929 targets whereas CD4 antibody plus C' treated cells were not significantly different from untreated or C' treated controls. (Table 8). These data show that the secondary WNV-immune cytotoxic cells are Thy 1.2 $^{+}$, CD8^{+} and CD4^{-}. Together with the H-2 restriction of cytotoxic activity, the results indicate that the cytotoxic cells were Tc cells.

Phenotype of Responder Cells Necessary for the Generation of Secondary CBA/H WNV-Immune Tc Cells In Vitro.

A population of CBA/H WNV-immune spleen cells was divided into 5 aliquots and treated with Thy 1.2 antibody and C', CD8 antibody and C', CD4 antibody and C', C' alone or left untreated prior to stimulation *in vitro*. The viable cell recovery after treatment

as determined by trypan blue exclusion was 56%, 70%, 64%, 75% and 100% respectively. The viable cell numbers were not adjusted prior to culture, but cultured at a concentration corresponding to $10^{6.3}$ original viable cells per ml. Exogeneous IL-2 was added to one of the cultures of cells pretreated with CD4 antibody and C' and a second aliquot of cells pretreated with CD4 antibody and C' was mixed with cells treated with CD8 antibody and C' prior to culture. The final cell concentration in this mixture was $10^{6.6}$ original viable cells per ml (i.e. twice the concentration of the other cultures). After 5 days viable responder cell recoveries from the 7 types of culture were respectively 27%, 51%, 36%, 55%, 56%, 62% and 51% of the original viable cell count. Cytotoxicity was tested after all the cultures were washed once, adjusted to a constant volume of 1.5 ml and diluted in 3 fold steps so that 1:10, 1:30 and1:90 fractions of the cell suspension were added to WNV-infected or uninfected L929 targets.

Thy 1.2 and CD4 antibodies and C' reduced cytotoxic activity about 10 fold below the level of the C' control whereas there was less than a 3 fold reduction of cytotoxic activity after CD8 antibody plus C' treatment. Addition of ~~exogeneous~~ exogenous IL-2 to anti-CD4 and C'-treated cells reconstituted the response to approximately control levels. Mixing CD4 antibody and C'-treated cells with CD8 antibody and C'-treated cells prior to culture increased the response above control levels. (Table 9). These results suggest that precursors of anti-WNV Tc cells express less CD8 than activated effector Tc cells, and that IL-2 producing CD4 helper T cells are needed to optimize the generation of Tc cells.

Effect of Responder Cell Concentration on the Secondary CBA/H WNV-Immune Cytotoxic Response after Culture of an CD4-Depleted Responder Population.

Because $CD8^+$, $CD4^-$ antiviral T cells are capable of producing IL-2 (Sinickas *et al.*, 1985) it was of interest to determine if increasing the concentration of the cells in culture overcame the need for exogenous IL-2 or $CD4^+$ T cells.

A population of CBA/H WNV-immune spleen cells was divided into 2 aliquots and treated with CD4 antibody and C' or C' alone prior to culture. The viable cell recovery

after treatment as determined by trypan blue exclusion was 62% and 83% respectively. The viable responder cells from each group were diluted in 2 fold steps prior to culture. Non-immune WNV-infected syngeneic spleen cells were irradiated with $10^{3.3}$ rads from a ^{60}Co source prior to addition to each culture as stimulator cells at a concentration of $10^{5.6}$ per ml. Cyotoxicity was tested 5 days later on WNV-infected and uninfected L929 cells. All the cultures were washed once, resuspended in culture medium and diluted so that for the different treatment categories, an equal number of the original viable cells prior to culture contributed to an aliquot being tested for cytotoxic activity.

In the case of responder cells treated with C' alone prior to culture, cytotoxic activity against WNV-infected target cells was generated at all three responder cell concentrations with the least activity generated at $10^{6.3}$/ml. (Table 10). In contrast with responder cells treated with CD4 antibody and C' prior to culture, cytotoxic activity against WNV-infected target cells decreased with decreasing cell concentration so that at 10^6 / ml and $10^{5.7}$ / ml activity was significantly lower than at $10^{6.3}$ /ml or in any C'-treated control cultures. These data suggest that with anti-CD4 and C'- treated responder cells, increasing the cell concentration can achieve sufficient IL-2 concentrations to support the generation of Tc cells, either because IL-2 ~~-producing~~ was produced by $CD8^+$, $CD4^-$ WNV-immune T cells (Sinickas *et al* ., 1985) or because ~~of~~ a small proportion of $CD4^+$ helper cells ~~that~~ escaped destruction by antibody and C'.

DISCUSSION.

Primary Tc cell responses against WNV in CBA/H (H-2^k) mice (Kesson *et al*., 1987) were not potent enough to lyse WNV-infected, H-2^k target cells other than L929, thus severely limiting investigations of H-2 restriction. In the studies reported here more potent secondary WNV-immune Tc cells were generated so that target cell types such as tertiary MEF and resident peritoneal macrophages could be used, thus allowing more extensive investigation of H-2 restriction discussed below.

However, L929 target cells were more sensitive than either MEF or macrophages for detection of WNV-immune Tc cells. Cross-sectional area of the target cell and the H-2 antigen concentration on the cell surface membrane have been shown to be determinants of target susceptibility to Tc cells (King *et al* ., 1986). In a cytotoxic assay for WNV-immune Tc cells expression of relevant WNV antigens would be essential. Thus, L929 cells may be superior targets because they are larger and/or express more viral-H-2 "complexes" than the other infected target cell types tested under the conditions used here. Cytotoxicity was highest when a m.o.i. of 100 p.f.u. WNV per cell was used for target cell infection. Lower lysis was observed when a m.o.i. less than 100 was used but lysis was still statistically significant at a m.o.i. of 1. Whether this reflects a requirement for input of structural virion antigens, or for successful infection leading to synthesis of non-structural flavivirus proteins has not been determined.

To generate statistically significant WNV-immune Tc cell activity after secondary stimulation of spleen cells *in vitro,* CBA/H mice required an i.v. priming dose of 10^3 p.f.u. WNV or greater. Optimal viral-specific cytotoxicity was generated when a priming dose of $\geq 10^5$ p.f.u. was used. At these priming doses the lysis of uninfected targets was consistently lower than when smaller doses were used. The dose of virus required to prime for secondary WNV-immune Tc cell responses *in vitro* is consistent with previous studies which showed that generation of primary WNV- immune Tc cell activity *in vivo* in CBA/H mice required an immunizing dose of $\geq 10^3$ p.f.u. WNV i.v. and peaked at 10^6 p.f.u. WNV, (Kesson *et al*., 1987).

Strong anti-WNV Tc cell responses can be generated over 5 days *in vitro* after a priming interval of two days *in vivo* and memory persists for at least 30 weeks (data not shown). Lysis of uninfected targets progressively decreased with increasing priming interval up to day 6 and from then on increased again thus resulting in optimal detection of viral-specific Tc cells in secondary responses on days 5 and 6 after priming. This coincides with the peak of the primary WNV-immune Tc cell response *in vivo* (Kesson *et al*., 1987). Therefore, it would be of interest to determine if anti-WNV Tc cells generated

in vitro were largely derived from further expansion of activated effector Tc cell populations, or by differentiation of precursors which have not yet acquired effector activity. These possibilities are discussed further below in the context of CD8 expression.

The relationship between killers of WNV-infected and uninfected targets requires further investigation with the limit dilution approach to obtain Tc cell clones. The data thus far can be explained if we assume that two categories of cytotoxic cells are generated *in vitro*, those which recognise antigens of uninfected target cells and thus can lyse both uninfected and infected targets and a second group of Tc cells which recognise virus-induced antigenic moieties and thus lyse only infected targets. Generation of the first type of cytotoxic cells was apparently minimized when secondary stimulation *in vitro* was begun 4-6 days after priming i.v. with at least 10^4 p.f.u. of WNV, but the reasons for this are unclear.

Boosting *in vitro* of WNV-immune spleen cells has allowed further characterization of the cytotoxic cells generated and the responder cell requirements for optimal responses. Cytotoxic activity of the secondary WNV-immune spleen cells generated by culture was attributed to T cells since it was abrogated by treatment with Thy 1.2 antibody and C' and CD8 antibody and C' but not with CD4 antibody and C'. The Tc cells generated were virus-specific in that the WNV-immune cytotoxic cells did not lyse influenza-infected targets to a greater extent than uninfected targets and vice versa with influenza-immune Tc cells. The reponse was H-2 restricted (reviewed in Zinkernagel and Doherty, 1979) as WNV-immune cytotoxic cells lysed syngeneic WNV-infected MEF targets, not H-2 incompatible WNV-infected MEF targets, in cross testing with the CBA/H ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mouse strains. This is typical of virus-specific Tc cells and has been described elsewhere (Mullbacher and Blanden, 1983; Pang and Blanden, 1976; Sinickas *et al.*, 1985).

The response of secondary WNV-immune Tc cells maps to the *K* region in the $H\text{-}2^k$ haplotype. There was no detectable activity associated with D^k. Whether the non-response associated with D^k region gene(s) is due to "a lack of association"

(Mullbacher and Blanden, 1978) or a cross tolerance mechanism (Mullbacher *et al.*, 1983) remains to be determined. In the *H-2* ^{d} haplotype both the *K* and *D* regions are permissive. Furthermore, K^{k}, K^{q} and K^{b} seemed to allow uniformly strong responses associated with the D^{d} region.

Treatment with CD8 antibody and C' of the responder spleen cell population from seven day WNV-primed mice caused less than a 3-fold reduction in anti-WNV cytotoxic activity of the effector cells generated by 5 days culture after stimulation, whereas treatment of a secondary effector population with CD8 antibody and C' decreased the cytotoxic activity by 9-fold. Furthermore, treatment of a 5 day immunized WNV-immune spleen cell population with CD8 antibody and C' abrogated cytotoxic activity of these primary WNV-immune spleen cells (Kesson *et al* ., 1987); however, culture of these cells for 5 days with WNV-infected, irradiated stimulator spleen cells generated a population of secondary WNV-immune, $CD8^{+}$ cytotoxic cells (unpublished data). These results suggest that the generation of secondary WNV-immune Tc cells *in vitro* is not solely clonal expansion of an $CD8^{+}$ T cell population. The majority of responding Tc cell precursors which give rise to anti-WNV Tc cells in culture may be $CD8^{-}$ as defined by antibody and complement mediated lysis, but may develop higher levels of CD8 as they become activated (Russell *et al.*, 1986).

Table 9 demonstrates that an $CD4^{+}$ responder population is required to generate a WNV-specific Tc cell population *in vitro*. Full cytotoxic potential was restored to an CD4-depleted responder population by either the addition of exogenous IL-2 or by addition of a WNV-immune $CD4^{+}$ cell population (i.e. cells treated with CD8 antibody and C'). Both Pang *et al.* (1976) and Ashman and Mullbacher (1979) have demonstrated that for $CD8^{+}$ cytotoxic T cells to be generated *in vitro*, Lyt 1^{+} cells are required to be present. In this work we used the CD4 surface antigen as a marker for helper T cells rather than the Lyt 1 antigen because expression of CD4 and CD8 by spleen cells appears to be mutually exclusive (Dialynas *et al* ., 1983) and the expression of CD4 differs from that of Lyt 1 in that Lyt 1 is expressed by all splenic T cells albeit at a lower density on CD8 cells (King *et*

al ., 1986).

Finally, secondary WNV-immune Tc cells can be generated by culturing a WNV-immune $CD4^-$ responder population at an optimal cell concentration (Table 10). This raises the possibility that help can be provided by a $CD8^+$, $CD4^-$ IL-2 secreting T cell population (Sinickas *et al* ., 1985) when an appropriate *in vitro* milieu is created by increasing cell concentration. Alternatively, a small contaminating population of surviving $CD4^+$ cells may be providing the necessary IL-2 if cell numbers are increased. Further analysis of the properties of defined subpopulations or clones of WNV-immune T cells will be required to resolve this issue.

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TABLE 1.

Sensitivity of Different H-2^k Target Cell Types to Lysis by Secondary CBA/H (H-2^k)

WNV-Immune Spleen Cells Generated *In Vitro.**

		% Specific Lysis of Targets†	
Target Cell.	E/T+	WNV Infected§	Uninfected.
CBA/H	30	54.9	12.8
Resident	10	51.2	6.2
Macrophages	3	37.2	2.6
CBA/H	30	24.5	2.7
Thioglycolate	10	16.3	0.9
Macrophages	3	7.9	-0.6
	30	82.2	37.9
L929 Cells	10	88.6	18.8
	3	69.6	10.3
CBA/H Tertiary	30	46.5	8.0
Mouse Embryo	10	29.9	0.6
Fibroblasts	3	14.4	0.2

*) Secondary effector cells generated as described in Materials and Methods.

†) Means of triplicate cultures with standard errors of the mean always less than 4.7%.

+) Effector to Target Cell Ratio.

§) M. O. I. of 100 p.f.u. per cell.

TABLE 2.

Effect of Multiplicity of Infection of Target Cells on Lysis.*

Multiplicity of Infection	E/T†	% Specific Lysis of L929 Targets+
100	30	45.5
	10	35.8
	3	18.7
10	30	27.0
	10	26.2
	3	16.4
1	30	16.1
	10	11.1
	3	8.9
Uninfected.	30	5.5
	10	-2.6
	3	-6.5

*) Secondary CBA/H effector cells generated as described in Materials and Methods.

†) Effector to Target Cell Ratio.

+) Means of triplicate cultures with standard errors of the mean always less than 3.3%. Spontaneous release of ^{51}Cr was between 10.4% and 9.2%.

TABLE 3.

Effect of Priming Interval on the Generation *In Vitro* of Secondary CBA/H WNV-Immune Cytotoxic Spleen Cells.*

Priming Interval (Days)	E/T+	% Specific Lysis of L929 Target Cells.† WNV Infected	Uninfected.
0	30	60.5	57.5
	10	49.5	34.1
	3	25.8	15.1
2	30	77.9	58.6
	10	73.3	41.7
	3	36.9	16.6
4	30	64.4	20.0
	10	51.3	9.0
	3	28.7	4.8
5	30	66.1	15.4
	10	45.5	9.6
	3	26.1	3.9
6	30	74.5	26.4
	10	59.9	11.1
	3	43.5	8.2
7	30	78.0	40.3
	10	75.5	24.4
	3	51.5	11.3
10	30	73.1	38.6
	10	76.1	19.3
	3	47.8	7.1
14	30	83.0	61.9
	10	74.5	19.3
	3	43.3	12.4
43	30	77.6	53.1
	10	72.6	25.1
	3	48.1	11.7

*) Female mice greater than 6 weeks old primed with 10^6 p..fu. WNV i.v. and spleen cells used as responder cells for *in vitro* boosting.

†) Means of triplicates given with standard errors of the means always less than 5%.

+) Effector to Target Cell Ratio.

TABLE 4.

Effect of Priming Dose on the Generation in vitro of Secondary CBA/H WNV-Immune Spleen Cells.*

Priming Dose (p.f.u. WNV i.v.)	E/T+	% Specific Lysis of L929 Targets.† WNV Infected	Uninfected.
10^1	30	82.5	81.8
	10	57.1	49.3
	3	25.8	16.2
10^2	30	41.7	38.8
	10	20.8	17.4
	3	8.6	5.1
10^3	30	70.3	45.1
	10	36.7	23.0
	3	9.8	5.1
10^4	30	55.7	21.0
	10	29.1	10.4
	3	12.8	2.6
10^5	30	66.7	24.8
	10	44.0	10.1
	3	17.6	2.4
10^6	30	70.4	14.3
	10	45.9	6.4
	3	18.3	2.5
10^7	30	69.9	18.5
	10	48.5	8.1
	3	25.5	3.3

*) Female mice, greater than 6 weeks old were primed with WNV i.v. and their spleen cells used as responder cells 7 days after priming.

†) and +) As for Table 3.

TABLE 5.

H-2 Restriction of Secondary WNV Immune Cytotoxic Cells.*

% Specific Lysis of Targets†

Cytotoxic Cells.	E/T	WNV Infected	Uninfected.
	CBA/H Tertiary MEF. (kkk)+.		
CBA/H	30	69.8§	21.1
	10	30.3	3.6
	3	22.7	4.2
BALB/c	30	18.7	14.3
	10	13.5	8.6
	3	8.7	6.7
	BALB/c Tertiary MEF.(ddd).		
CBA/H	30	15.4	20.5
	10	-0.7	1.8
	3	1.6	1.6
BALB/c	30	57.4§	4.1
	10	23.6	-1.6
	3	19.0	0.4

*) Primed with 10^6 p.f.u WNV i.v. and spleen cells boosted *in vitro* 7 days later

†) Means of triplicate cultures with standard errors of the means less than 4.0%.

+) H-2 map corresponding to *K*, *I*, and *D* regions.

§) Significant specific lysis of infected target cells.

TABLE 6.

Genetic Mapping of Secondary WNV Immune Cytotoxic Cell Response.*

% Specific Lysis of Targets†

Experiment 1.

C3H.H-2^{02} Tertiary MEF. (ddk)+.

Cytotoxic Cells.*	E/T	WNV Infected	Uninfected.
CBA/H (kkk)	30	13.4	14.9
	10	3.7	-1.0
	3	3.1	3.1
BALB/c (ddd)	30	68.0§	48.9
	10	30.2	6.1
	3	15.1	11.0

B10A Tertiary MEF. (kkd).

Cytotoxic Cells.	E/T	WNV Infected	Uninfected.
CBA/H (kkk)	30	53.1§	18.4
	10	23.3	6.5
	3	13.6	1.9
BALB/c (ddd)	30	22.3§	8.2
	10	6.0	2.6
	3	3.3	-0.5

Table 6 Cont...

Experiment 2.

		BALB/c Tertiary MEF. (ddd)+	
Cytotoxic Cells.*	E/T	WNV Infected	Uninfected.
BALB/c (ddd)	30	71.8§	6.7
	10	62.8	3.0
	3	42.2	-0.5
B10.A (kkd)	30	74.0§	39.5
	10	62.8	20.3
	3	29.2	8.6
B10.AQR (qkd)	30	69.8§	28.1
	10	54.8	10.7
	3	35.3	3.0
B10.A(5R) (bbd)	30	78.1§	25.5
	10	66.8	12.5
	3	45.4	4.5

*), †), +) and §) As for Table 5.

TABLE 7.

Viral Specificity of Secondary CBA/H WNV-Immune Cytotoxic Cells.

		% Specific Lysis of L929 Target Cells.*		
Cytotoxic Cells	E/T†	WNV-Infected+	Influenza-Infected§	Uninfected
WNV Immune¶	10	82.4#	19.6	17.4
	3	51.6	4.4	9.2
	1	36.5	2.1	6.2
Influenza Immune•	10	27.4	80.9#	21.1
	3	17.5	56.9	10.0
	1	8.6	23.6	4.4

*) Means of triplicate cultures with standard errors of the means less than 4.0%.

†) Effector to target cell ratio.

+) Infected with 100 p.f.u WNV per target.

§) Infected with 60 HAU A/WSN per target.

¶) Primed with 10^6 p.f.u WNV i.v. and spleen cells boosted *in vitro* 7 days later.

•) Primed with 200 HAU influenza virus i.v. and spleen cells boosted *in vitro* 7 days later.

#) Significant specific lysis of infected target cells.

TABLE 8.

Phenotype of Secondary CBA/H WNV-Immune Spleen Cells Generated *In Vitro.**

Treatment.+	Aliquot§	% Specific Lysis of L929 Targets.† WNV-Infected	Uninfected.
Nil	1:10	32.2	5.9
	1:30	16.5	3.1
	1:90	9.0	2.3
C' alone	1:10	36.5	7.9
	1:30	18.1	2.0
	1:90	8.2	0.2
Anti Thy & C'	1:10	3.6¶	2.5
	1:30	3.2	0.8
	1:90	-0.5	0.5
Anti CD4 & C'	1:10	28.1	6.5
	1:30	16.3	1.8
	1:90	5.3	0.8
Anti CD8 & C'	1:10	8.5¶	2.9
	1:30	4.2	1.5
	1:90	-0.6	0.6

*) Primed with 10^6 p.f.u WNV i.v. and spleen cells boosted *in vitro* 7 days later.

†) Means of triplicate cultures with standard errors of the means less than 4.2%.

+) Conditions for treatment and assay are described in Materials and Methods.

§) Aliquots derived as described in Materials and Methods.

¶) Significant decrease in specific lysis of infected target cells compared with C' control.

TABLE 9.

Phenotype of the Responder Population Necessary for Generation of Secondary WNV-Immune Tc Cells *In Vitro.**

Antibody Treatment·	Aliquot§	% Specific Lysis of L929 Targets.† WNV Infected.	Uninfected.
Nil	1:10	76.6	19.4
	1:30	48.7	7.6
	1:90	24.8	4.1
C' alone	1:10	75.7	18.5
	1:30	47.6	7.9
	1:90	22.8	4.3
Anti Thy & C'	1:10	18.4¶	7.1
	1:30	13.8	5.9
	1:90	8.7	1.7
Anti CD4 & C'	1:10	21.3¶	6.3
	1:30	7.1	5.8
	1:90	3.7	2.8
Anti CD8 & C'	1:10	58.2¶	11.0
	1:30	30.9	6.0
	1:90	17.4	3.7
Anti CD4 & C' and IL-2	1:10	71.2	19.9
	1:30	40.3	6.9
	1:90	18.3	2.5
Anti CD4 & C' and anti CD8 & C' treated cells.	1:10	80.2	20.2
	1:30	65.7	11.6
	1:90	37.5	4.8

*) Primed with 10^6 p.f.u WNV i.v. and spleen cells used 7 days later as responder cells for *in vitro* boosting.

†) Means of triplicate cultures with standard errors of the means less than 5.0%.

+), §) and ¶) As for Table 8.

TABLE 10.

Effect of Responder Cell Concentration on the Generation of Secondary WNV-Immune Tc Cells *In Vitro.**

Responder Cell Concentration	Aliquot§	% Specific Lysis of L929 Target Cells.† WNV Infected	Uninfected.
C'-Treated Responder Cells+			
2 X 10^6 / ml.	1:40	31.5	4.9
	1:120	12.2	1.3
	1:360	3.6	0.2
1 X 10^6 / ml.	1:20	48.6	11.3
	1:60	27.0	1.2
	1:180	10.8	2.0
0.5 X 10^6 / ml.	1:10	33.9	8.2
	1:30	38.4	11.6
	1:90	28.3	12.9
CD4 Antibody and C'-Treated Responder Cells+			
2 X 10^6 / ml.	1:40	34.2	4.2
	1:120	16.5	0.1
	1:360	6.1	-1.2
1 X 10^6 / ml	1:20	17.7¶	1.2
	1:60	7.1	0.2
	1:180	2.3	-1.3
0.5 X 10^6 / ml	1:10	6.4¶	-0.2
	1:30	2.0	-1.7
	1:90	2.2	1.5

*) Primed with 10^6 p.f.u WNV i.v. and spleen cells used 7 days later as responder cells for *in vitro* boosting.

†) Means of triplicate cultures with standard errors of the means less than 5.0%.

+) Conditions for antibody treatment and assay are described in Materials and Methods.

§) and ¶) as for Table 8.

CHAPTER 4

INTERFERON - INDEPENDENT INCREASES IN CLASS I MHC ANTIGEN EXPRESSION FOLLOW FLAVIVIRUS INFECTION.

SUMMARY

Infection of tertiary-passaged mouse embryo fibroblasts by four flaviviruses, West Nile (WNV), Kunjin, Murray Valley encephalitis and Japanese B encephalitis resulted in a 6-10 fold increase in the expression of individual H-2K and H-2D class I MHC antigens 16-48h after infection. The mechanism(s) by which flaviviruses increased class I MHC antigen expression ~~is~~ was not fully elucidated, but appears to be mediated by a mechanism partly independent of β-interferon (β-IFN) secretion as anti-α/β-IFN antibodies partially inhibited the WNV-induced increase but totally prevented increases caused by the addition of 1) pure β-IFN, 2) ß-IFN -containing supernatants from WNV-infected MEF, or 3) Poly inosinic-Poly cytidylic acid. Actinomycin D treatment of MEF which inhibited mRNA synthesis by > 90% as determined by ^{3}H-uridine uptake, totally inhibited the increased MHC expression by WNV-infection. Thus, the increase in class I MHC antigen expression is dependent upon cellular RNA synthesis.

INTRODUCTION

Cytotoxic (Tc) cells are important in the cellular immune response of an organism to virus infections (Blanden, 1974). Virus - immune Tc cells mediate lysis of infected cells *in vitro* and presumably also *in vivo* before assembly of progeny virus, thereby preventing continued viral replication. Class I MHC surface antigens on virus - infected cells act as restriction elements in the recognition of virus - infected cells by virus - immune Tc cells (reviewed in Zinkernagel and Doherty, 1979). Thus lytic activity of Tc cells generated against virus - infected cells is restricted to infected target cells expressing the same class I MHC antigens as the sensitizing cells as well as being specific for the sensitizing virus (Zinkernagel and Doherty, 1974). Furthermore, quantitative variations in the class I MHC antigen expression correlate with the efficiency of lysis of virus - infected target cells by MHC restricted virus - immune Tc cells (O'Neill and Blanden, 1979; King *et al*, 1985).

We have previously detailed the parameters relating to the generation of flavivirus-immune Tc cells, in particular those for West Nile virus (WNV) (Kesson *et al.*, 1987, 1988). The standard ^{51}Cr - release assay described, commonly makes use of mouse embryo fibroblasts (MEF) as a target. In this report we show that WNV - infection of MEF induces a significant increase in the surface expression ~~of~~ individual class I MHC (H-2K and H-2D) antigens, which is independent of α/β-interferon (α/β-IFN). This unusual phenomenon may have important implications for the combat of flavivirus disease by the cellular immune system as well as for the ~~stratergy~~ strategy of survivial of these viruses.

MATERIALS AND METHODS

Mice.

Mice were bred under pathogen free conditions at the Animal Breeding Establishment of the John Curtin School of Medical Research. Female mice of the strains CBA/H ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), C3H.H-2^{02} ($H\text{-}2^{02}$), DBA/2 ($H\text{-}2^d$), B10.A ($H\text{-}2^a$), (A/J X

DBA/1) F_1 (H-$2^{a/q}$), (C3H. H-2^{02} X DBA/1) F_1 (H-$2^{02/q}$), (BIO.BYR X SJL) F_1 (H-$2^{by1/s}$), (BALB/c X C57Bl/6) F_1 (H-$2^{d/b}$) were used between the ages of 6 and 16 weeks.

Virus Stocks.

1) Vero Cell - Grown. Vero cells were grown in Dulbecco's Modified Eagles Medium (Cat. No.430 - 1600, GIBCO, Chargrin Falls, Ohio, U.S.A.), supplemented with 5% FCS, 200µg/ml Streptomycin, 200 U/ml Penicillin G and 125µg/ml Neomycin Sulphate (DMEM) in Nunclon Delta 175 cm^2 tissue culture flasks (No 56502) in a humidified atmosphere of 5% CO_2 in air at 37° C. When the Vero cell monolayer was confluent the medium was poured off and the cells infected with 10^8 p.f.u. of West Nile virus (WNV), Kunjin, Murray Valley encephalitis (MVE) ~~and~~ or Japanese B encephalitis (JBE) in 1 ml of DMEM (i.e. m.o.i. of 5) for 1 h at 37° C. The cells were then washed three times with 50 ml phosphate-buffered saline (PBS) and 20ml of DMEM was then added. After 48 h incubation in a humidified atmosphere of 5% CO_2 in air at 37° C the cells and medium were frozen at -70° C and then thawed to release any intracellular virus. The medium and cell debris were poured off and clarified by centrifugation at 1500g for 20 min at 4° C. Further virus was recovered after sonication and centrifugation of the remaining pellet in 5 ml of the supernatant. The supernatants were pooled and aliquots were stored at -70° C for future use as stock virus.

2) Purified Virus. Purified virus was prepared using a modification of the method described by Lobigs *et al.* (1986). Briefly, Vero cell monolayers cultures were grown in DMEM in 850 cm^2 roller bottles (Corning, New York. No. 25140). When confluent, the cell monolayer was infected with WNV at a m.o.i. of 10. Twelve hours post infection the culture medium was removed and infected cells were incubated with actinomycin D (Sigma Chemical Co. St Louis, Mo. U.S.A.) at 5µg/ml in 20 ml DMEM for 30 min. This medium was replaced with Eagles minimal essential medium Cat. No. 410-1500 GIBCO,

Chargrin Falls, Ohio, U.S.A., containing 2% dialysed fetal calf serum, 20mM hepes buffer (pH 8.0) and [5-^{3}H] uridine (5μCi/ml) (Amersham). Culture fluids were harvested at 36 and 48 h post infection, pooled and clarified by centrifugation. The virus was concentrated from this clarified culture fluid by addition of 40% polyethylene glycol 6000 to a final concentration of 8% with stirring at 4^o C and was banded by centrifugation on a 10% - 30% glycerol gradient in a SW28 rotor at 2000 rev./min for 3.5 h at 4^o C in a Beckman L5-50 ultracentrifuge. Virus containing fractions were collected on an Isco (Lincoln Nebraska U.S.A.) fraction collector and counted on a Beckman Scintillation Counter. Fractions with counts higher than backgound were aliquoted and stored at -70^o C for future use.

Titres of all virus stocks were determined by serial dilutions and titrations on Vero cell monolayers as described by Taylor and Marshall 1975.

Cell Culture.

Mouse embryo fibroblasts (MEF) were obtained by trypsinization of BALB/c, CBA/H, C3H.H-2^{02} or B10.A foetuses at 16 days of gestation. The cells, designated primary MEF, were seeded at $10^{6.3}$ cells / 80 cm^2 plastic flask (Nunclon) in 20ml of DMEM in an atmosphere of 5% CO_2 in air at 37^o C for 4 days after which time they were removed to room temperature and kept for use as required for up to 3 weeks prior to subculture. Four days prior to requirement primary MEF were subcultured by trypsinisation and reseeding at $10^{6.3}$ cells / 80 cm^2 flask, subjected to culture conditions identical to the first four days of primary MEF culture, and designated secondary MEF. Secondary MEF were used after 4 days as the source of tertiary MEF (Sinickas *et al.*, 1985).

Interferons and Anti-Interferons.

1) Beta-Interferon (β-IFN): Murine β-IFN (Batch No. 83001; Cytoimmune Reagents, Lee Biomolecular, San Diego. U.S.A.) was used at a concentration of 200 units / ml.

2) Affinity purified polyclonal rabbit anti-mouse α/β interferon globulin (α/β-IFN

antibody) (Batch No. 21031 Cytoimmune Reagents, Lee Biomolecular, San Diego. U.S.A.) was used at a concentration of 200 units / ml.

Both reagents were titrated prior to use on MEF using MHC labelling (see below). In the case of β-IFN, the concentration which caused the maximal increase in class I MHC antigen expression after 48h was used. The α/β-IFN antibody was found to abrogate the effect of β-IFN on a unit for unit basis. A total volume of 2 ml, comprising culture medium and reagents, was added to petri dish cultures; a total volume of 30µl was added to wells in microtitre plates.

Treatment of cells with polyinosinic acid : polycytidylic acid (poly I.C.).

MEF were grown in petri dishes (Kayline. Australia No. 420140) for 24 h, divided into three groups for treatment (see Fig. 4) and incubated in 5% CO_2 in air at 37° C for 48h. To approximate conditions of viral infection poly I.C. was left in the culture supernatant for the duration of the experiment.

Treatment of cells with Actinomycin D (AMD) and measurement of ^{3}H-uridine incorporation

MEF were grown either in petri dishes or 96-well flat - bottomed tissue culture plates (Nunc, Roskilde, Denmark) for 24 h. The cells were drained of medium and 10 p.f.u. / cell of WNV in a volume of 2ml of DMEM was added to petri dishes while a volume of 30µl containing 10 p.f.u. / cell of WNV was added to relevant wells of tissue culture plates. After incubation for 1 h at 37° C in an atmosphere of 5% CO_2 in air the cells were washed, AMD (Sigma) was added at a concentration of 5 µg / ml in DMEM (30µl total volume in tissue culture wells, 2 ml in petri dishes) and the cells incubated for a further 30 min under the same conditions. This concentration of AMD (determined by prior titration) reduced the incorporation of ^{3}H-uridine by more than 90%. The MEF were then washed twice with fresh DMEM at 37° C and DMEM added for 24 h at 37° C in an atmosphere of 5% CO_2 in air.

^{3}H-uridine incorporation by MEF in the 96-well tissue culture plate was measured after 25µl of ^{3}H-uridine (Amersham Int. Ltd., Amersham, U.K.) (25µCi / ml) was added for a 6 h interval 18 h after beginning the incubation. Labelled MEF were harvested onto glass fibre paper using a multiple sample harvester (Titertek 530 Flow Labs. U.K.). The dried samples of glass fibre paper were placed into 7 ml of of scintillation fluid and β emission was counted in a liquid scintillation counter (Beckman U.S.A. LS3801.). Twelve samples were assayed in each group and the mean counts per minute (c.p.m.) and standard errors of the mean (S.E.) calculated. Significance was determined by Student's t Test and P values of < 0.05 were considered significant.

Antisera.

Anti-H-2K^d/Ia^d and H-2D^d sera were raised in (A/J X DBA/1)F$_1$ and (C3H.OH X DBA/1) F$_1$ mice, respectively, injected intraperitoneally at weekly intervals with $10^{7.7}$ DBA/2 spleen cells for 4 weeks and bled 1 week after the final injection. Anti-K^k sera was raised in (B10.BYR X SJL) F$_1$ injected ~~intrapertoneally~~ intraperitoneally with $10^{7.7}$ B10.A spleen cells and anti-D^k sera in (BALB/c X C57BL/6) F$_1$ injected intraperitoneally with $10^{7.7}$ C3H.OH spleen cells using the above protocol. Pre-immune sera from relevant F$_1$ mice were used as a normal serum controls.

In addition, monoclonal antibodies against K^d/D^d (clone 34-1-2S; American Type Culture Collection [ATCC] No. HB-79; Ozato *et al.*, 1982), K^k (clone 11-4.1; Beckton Dickinson, California, USA), I-A^k (clone 11-5.2.1.9; ATCC No. TIB-94; Oi *et al.*, 1978) and I-A^d (clone MK-D6; ATCC No HB-3; Kappler *et al.*, 1981) were used to label class I MHC antigens. Supernatants from hybridoma cultures were concentrated 10x and used at dilutions giving saturation labelling of the relevant antigens. Sera and supernatants were stored at -20^o C until required, thawed, and stored thereafter at 4^o C. The same batch of each antiserum or supernatant was used throughout. Reagents were used for no more than 6 weeks after thawing.

Rabbit anti-mouse immunoglobulin-fluorescein-isothiocyanate (RAMIg- FITC) was

prepared by standard methods from antiserum raised in a New Zealand white rabbit against mouse immunoglobulin (Goding, 1976). RAMIg- FITC was never frozen, but stored at 4° C in lightproof vials.

Cell Labelling for Fluorescence-Activated Cell Sorting (FACS) Analysis.

Secondary MEF were trypsinised and seeded prior to treatment at 10^5 cells/ml into tissue culture plastic petri dishes for labelling and FACS analysis. MEF were then infected/treated with WNV/β-IFN/Poly I:C and/or anti-α/β-IFN antibody and incubated as described above. After this, MEF were trypsinized off the plastic with 0.25% trypsin in buffered isotonic saline, washed once in DMEM to inactivate the trypsin, washed once in Hank's balanced salt solution, and suspended for 30 min at 4° C in freshly prepared periodate-lysine-paraformaldehyde in distilled water (pH 7.3), 3 ml 0.1 M L-lysine in 0.1 M phosphate buffer (pH 7.3), and 9 mg paraperiodic acid.

Periodate-lysine-paraformaldehyde is a gentle fixative maintaining the serological detectability of surface H-2 (King and Parr, 1982). After fixing, the cells were washed first in Hank's solution and then in 50% normal rabbit serum in Hank's solution to reduce subsequent non-specific binding of antibody during specific labelling procedures. All steps of this and subsequent procedures were carried out at 4° C.

Aliquots of $10^{5.7}$ BALB/c MEF were incubated for 60 min in 100 µl anti-H-2 ~~serum~~ sera (anti-K^d/Ia^d or anti-D^d) at a dilution that gave maximum specific binding. Aliquots were then washed three times in DMEM and incubated for 60 min in 150 µl RAMIg-FITC at a dilution that gave maximum specific binding. After three washes, each aliquots was suspended in 0.5 ml DMEM for flow cytometry. Controls were BALB/c MEF incubated with anti-K^k, anti-D^k antisera or normal mouse serum (NMS) and RAMIg-FITC or with RAMIg-FITC alone. BALB/c MEF labelled with anti-K^d/Ia^d and/or anti-D^d and RAMIg-FITC were always significantly more fluorescent than controls. MEF are I-A^d negative, thus only K^d antigens were detected by the anti-K^d/Ia^d serum. King *et al.* (1985)

WNV-infected and mock-infected MEF from CBA/H (H-2^k), C3H.H-2^{02} (H-2^{02}) and B10.A (H-2^a) strains were also labelled for class I MHC antigens in a protocol identical to that used for BALB/c MEF using polyclonal antibodies as follows: anti-K^k and anti-D^k for CBA/H, anti-K^d/Ia^d and anti-D^k for C3H.H-2^{02}, and anti-K^k and anti-D^d for B10.A. Control MEF samples from the respective strains were labelled with an irrelevant polyclonal antibody and NMS from the above panel.

Labelling of WNV-infected and mock-infected MEF from the above strains was also carried out using monoclonal antibodies (MAb) against K^d/D^d, K^k, I-A^d and I-A^k (see Antisera). Class I MHC antigen labelling with MAb gave results identical to those obtained with polyclonal antisera.

FACS Analysis.

Fluorescence was measured using a FACS IV (Beckton - Dickinson) with an argon ion laser set at the standard excitation wavelength of 488 nm for FITC. The emitted fluorescence between 515 and 540 nm was measured. 10^5 cells were analysed from each labelled sample. We found no difference in any of the MEF populations detectable by low angle (cell size) or right angle (membrane configuration) scatter analysis on the FACS (see Melamed *et al.*, 1979). Thus, differences in the MHC-fluorescence distributions between any of the MEF groups cannot be explained by cell size differences between or within the sample.

RESULTS

Flavivirus Infection Induces Increased Class I MHC Antigen Expression in Mouse Embryo Fibroblasts.

BALB/c MEF (H-2^d) were infected with 40 p.f.u. / cell of purified WNV for 16 h and 40 h. The level of cell surface class I MHC antigen expression of both infected and uninfected control MEF was measured by FACS analysis. Fluorescence profiles of the

WNV-infected cells showed a significant increase in class I MHC antigens compared with uninfected control MEF (Fig. 1), and the level of fluorescence was higher at 40h after infection than at 16h. Class II MHC antigens were not detected before or after infection with WNV in BALB/c MEF (data not shown). After this first demonstration all the subsequent experiments used Vero cell-grown WNV seeded from brain stocks and harvested after the first passage to reduce possible variations in virulence.

Increases in class I MHC antigen expression were demonstrated after 48h infection with the four Vero grown flaviviruses tested, WNV, Kunjin, MVE and JBE. (Figure 2). Furthermore, similar increases in class I MHC antigen concentration were demonstrated in CBA/H (K^k and D^k), BALB/c (K^d and D^d), C3H.H-2^{O2} (K^d and D^k) and B10.A (K^k and D^d) MEF infected with these flaviviruses, indicating that mouse strain background or H-2 haplotype had no profound influence on the phenomenon (data not shown).

Flavivirus-Induced Increase in Class I MHC Antigen Expression in MEF Occurs by Mechanism(s) Partly Independent of β–Interferon (β–IFN) Secretion.

Beta-IFN can induce increased class I MHC antigen expresssion on cells (Wallach *et al.*,1982). Theoretically, WNV infection of MEF could result in β-IFN production by the infected MEF which could upregulate the MHC expression of the whole MEF population including the MEF that remained uninfected. Therefore, anti-α/β-IFN antibody was added to the MEF cultures at the time of infection with WNV in an attempt to inhibit the action of any endogeneously secreted β-IFN and to determine if the increase in class I MHC measured after WNV infection is due solely to the action of secreted ß-IFN or whether WNV infection can upregulate MHC expression by some other mechanism.

Figure 3 (upper panel) shows that the increase in class I MHC antigen expression caused by WNV infection (profile A) was partially inhibited by the presence of anti-α/β-IFN antibody (profile B); however, some 66% of the MEF population remained significantly more fluorescent than the control MEF.

To ascertain if all secreted β-IFN in Group B was neutralized by the anti-α/β-IFN

antibody and therefore if the increase in class I MHC antigen expression seen was due to residual IFN or an additional mechanism(s), a second experiment was performed. We have previously demonstrated that WNV does not affect MHC antigen expression after irradiation with $10^{6.7}$ rads from a ^{60}Co source but β-IFN remains active (data not shown). We therefore irradiated the supernatants of the MEF cultures from Groups A and B with $10^{6.7}$ rads to inactivate any remaining virus. These supernatants were then added to fresh MEF cultures for 48 h and the level of class I MHC antigen expression measured. β-IFN was also added to a control group of normal, irradiated MEF supernatants to determine whether the nutrients in the medium were still sufficient to support an increase in MHC expression in MEF in response to β-IFN.

The results from this experiment (Fig. 3 lower panel) demonstrated that the supernatant from 48 h WNV-infected MEF caused increased MHC expression on BALB/c MEF (profile G), those from WNV-infected MEF treated with anti-α/b-IFN antibody from the start of infection contained insufficient free β-IFN to cause a detectable increase in the surface class I MHC antigens (profile E) thus anti-α/β-IFN antibody was able to inhibit completely the MHC increasing effects of ~~endogeneously~~ endogenously produced and secreted β-IFN acting through MEF surface IFN receptors. This strongly suggests that increased class I MHC expression in WNV-infected MEF treated with anti-α/β-IFN antibody was due to mechanism(s) independent of secreted β-IFN. It is also clear from these experiments that secreted WNV-induced β-IFN contributed to the increased class I MHC antigen expression in infected MEF.

The Effect of Poly I:C on the Induction of Class I MHC Antigen Expression in MEF. Treatment of BALB/c MEF with poly I:C caused a significant increase in the level of expression of class I MHC antigens over and above the control MEF (Figure 4.); this was completely inhibited by the addition of 200 units of anti-α/β-IFN antibody. We assumed, therefore, that poly I:C-induced β-IFN was completely neutralized by the anti-α/β-IFN antibody particularly as previous experiments titrating the anti-α/β-IFN antibody on poly

I:C-treated MEF showed an inhibition of the class I MHC antigen increase proportional to the concentration of anti-α/β-IFN antibody used (data not shown).

Increased Class I MHC Antigen Expression by WNV - Infected MEF is Dependent upon Host Cell mRNA Synthesis.

WNV-infection could increase the expression of class I MHC antigen expression by one of several mechanisms. Theoretically, this could result from increasing cellular mRNA synthesis, increasing translation of mRNA already transcribed or increasing the transport of MHC antigens to the cell surface membrane. To investigate these possibilities further MEF were treated with AMD to block new mRNA transcription and the level of class I MHC antigen expression was determined by FACS analysis in parallel with the measurement of ^{3}H-uridine incorporation. The replication of flavivirus RNA is not inhibited by AMD (Leary and Blair,1983) and it is used in the method of purification of the virus (Lobigs *et al.*, 1986).

As shown in Figure 5, treatment with AMD totally inhibited the WNV-induced increase in MHC antigens. ^{3}H-uridine incorporation was reduced by more than 90% in the AMD-treated groups indicating that cellular RNA synthesis had been virtually abolished by the AMD treatment (Table 1), while more than 95% of the MEF remained alive, as assessed by trypan blue exclusion and low angle scatter parameters on the FACS (data not shown). Thus, we conclude that the WNV-induced increase in class I MHC antigen expression is dependent upon cellular mRNA synthesis. Whether this reflects a requirement for new mRNA synthesis, or replacement of short-lived mRNA is not known.

DISCUSSION

Tc cell lysis of cells infected with intracellular pathogens is crucial not only for controlling the spread of intracellular organisms and viruses, but also for their eventual eradication from the host (Kees and Blanden, 1976; Yap *et al.*, 1978; Oldstone *et al.*,

1986). In view of the central role played by class I MHC antigens in the recognition of virus-infected cells by virus-immune MHC-restricted Tc cells, the increase in class I MHC gene expression caused by flavivirus infection described here is an important immunological phenomenon.

WNV was used as the prototype model for detailed investigations, although infection for 48 h by all four of the flaviviruses tested so far, (WNV, Kunjin, MVE and JBE), produced the phenomenon. Analysis was usually performed on MEF infected for 24-48 h; nevertheless, class I MHC increases on BALB/c MEF were detectable by FACS analysis as early as 8 h after WNV infection and then increased up to at least 96 h after infection (data not shown). Inhibition of MHC increases in WNV-infected MEF by AMD treatment indicated that increased MHC expression requires transcription of mRNA from the host cell DNA, but detailed mechanism(s) by which MHC expression increases remain unclear.

Increases in class I MHC antigen concentration was only partially inhibited by treating the MEF with anti-α/β-IFN antibody at the time of infection. This treatment was shown to neutralize all MHC-increasing activity present in supernatants of infected MEF (probably due to β-IFN) or deliberately added as pure β-IFN, suggesting that increased class I MHC antigen expression on WNV-infected MEF was not caused solely by secretion of virus-induced interferons. The possibility that some β-IFN was bound to receptors on the MEF before being neutralized by the antibody, or that WNV-induced β-IFN was able to act internally, i.e. without being secreted by the cell cannot be excluded, although published evidence (Yarden *et al.*,1984; Leeuwenberg *et al.*, 1987), and the results of the poly I:C experiments reported here, make the latter unlikely. Furthermore, in other work (King *et al*, 1989), it is evident that WNV infection can increase class I MHC antigen expression independently of secretion of virus-induced IFNs. Primary trophoblast giant cell outgrowths from preimplantion blastocysts hatched *in vitro* may be induced to express class I MHC antigens *de novo* following infection with WNV for 16 h. These cells neither secrete virus-induced IFN's (Barlow *et al*, 1984), nor are they susceptible to the MHC increasing effects of extracellular IFNs (Ozato *et al*, 1984; King *et al*, 1987).

The heterogeneity in the MHC-fluorescence distribution of WNV-infected MEF in the presence of anti-α/β-IFN antibody revealed by profile B (figure 3) therefore presumably occurs because the MEF are influenced individually and unequally by WNV infection *per se* to increase MHC antigen expression, in contrast to MEF exposed to α, β or γ-IFN, or WNV plus WNV-induced β-IFN, which show an increase in MHC fluorescence as a whole population (i.e. in a log-normal distribution) identical to the fluorescence distribution of an untreated control MEF population (King *et al.*, 1985; N.J.C.King, unpublished observation).

Profile B in figure 3 further reveals two subpopulations of MEF. The first, some 66% of the total cell population, exhibited an increase in MHC antigen expression not abrogated by anti-α/β-IFN antibodies, and presumably caused by WNV infection via intracellular mechanism(s). The remainder showed an MHC antigen increase caused by secreted WNV-induced IFNs, and inhibited by an anti-α/β-IFN antibodies. This suggested that the second population (34%) was not infected. However, immunofluorescence studies on acetone-fixed, WNV-infected MEF using hyperimmune anti-WNV antiserum showed >80% of MEF nuclei to be brightly stained for WNV antigens 48 h after infection. Thus, while 15 to 20% of the population probably were not infected, 10-15% were WNV-infected, able respond to WNV-induced secreted IFN's, but refractory to the intracellular MHC-increasing effects of WNV.

The heterogeneous MHC-increasing response of the MEF to WNV infection may relate, among other factors, to time and m.o.i. of individual MEF. We have found, for example, the magnitude of WNV-induced MHC increase over 24 h is directly related to m.o.i. (data not shown). We are also currently investigating the possibility that cell cycle position at the time of infection may alter either the susceptibility or the MHC-increasing response of MEF to WNV-infection or both. Lastly, some fibroblasts, e.g. L929, readily infected by WNV (Kesson *et al.*, 1987) and sensitive to exogenously added IFNs, are refractory to the MHC-increasing effects of WNV (data not shown). It seems possible therefore, that polyclonal tertiary MEF populations may contain elements partially or

completely refractory to the MHC-increasing effects of WNV.

Previous studies have demonstrated that virus-immune, class I MHC- restricted Tc cells are capable of triggering clearance of established viral infections *in vivo* (Kees and Blanden, 1976; Yap *et al.*, 1978; Oldstone *et al.*, 1986). The mechanisms by which T cells mediate viral clearance are not fully elucidated but cytopathic activity is likely to be a contributor (Blanden, 1974). Flaviviruses infect a wide range of both vertebrate and arthropod hosts, and replicate in a wide variety of cultured cells (Brinton, 1986). *In vitro*, the latent phase of the flavivirus replication in Vero cells cycle lasts approximately 12 h, after which progeny viruses begin to be released, maximal viral titres occurring some 24 h after infection (Trent and Naeve, 1980). The relevance of these times to events *in vivo* is not fully understood but our findings suggest, that there could be a significant increase in MHC antigen concentration on infected cells before assembly of maximal numbers of progeny virions. It follows, therefore, that flavivirus- induced MHC increase could contribute to more efficient lysis by virus- immune Tc cells *in vivo* and thus to limitation of viral replication.

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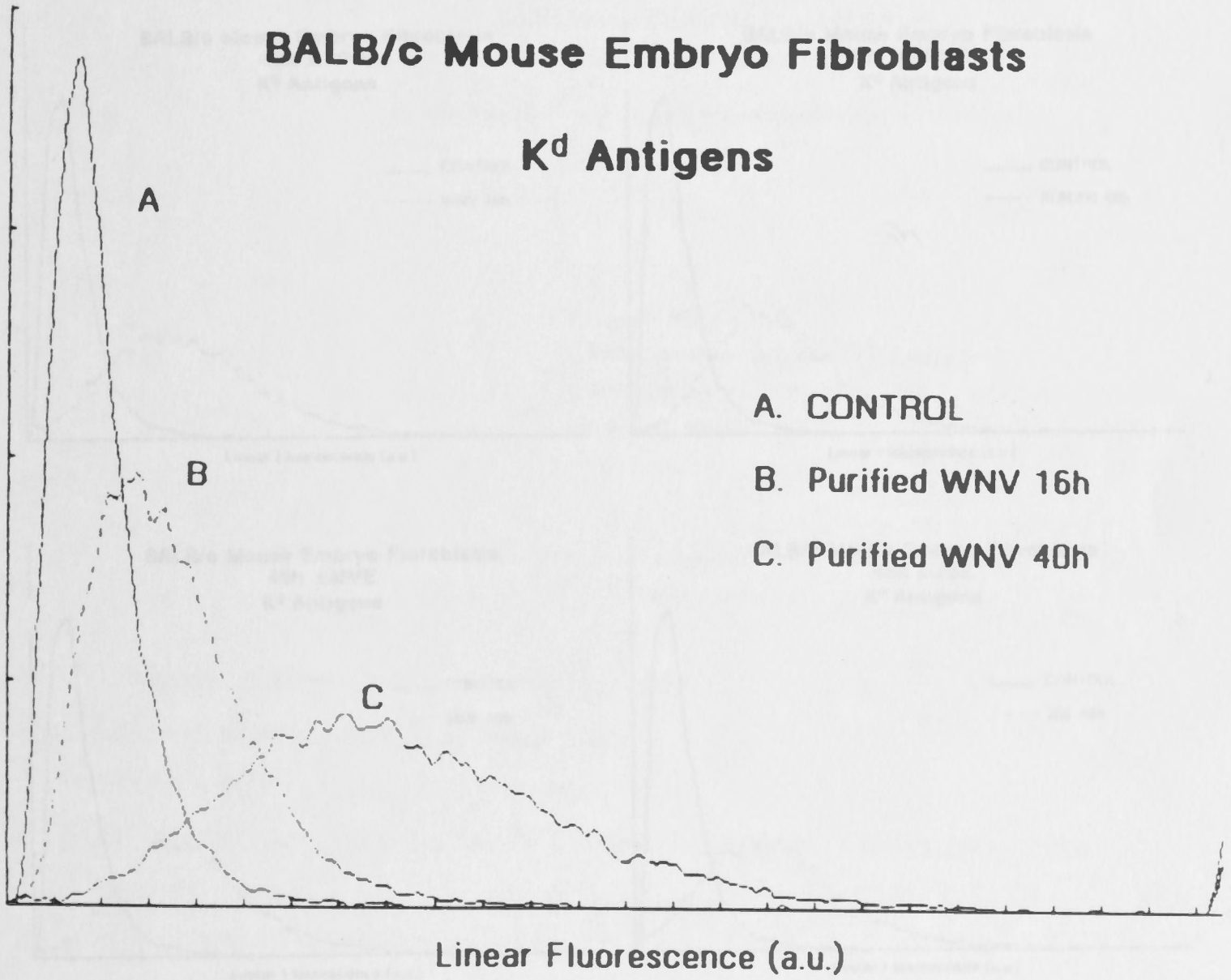
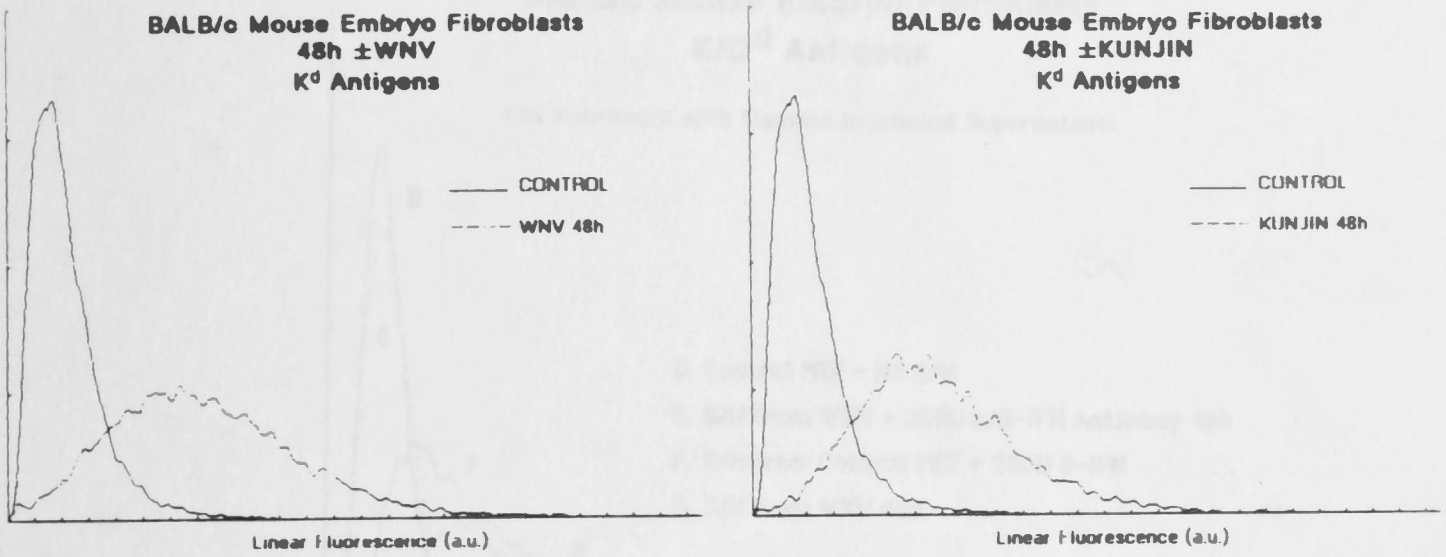
FIGURE 1.



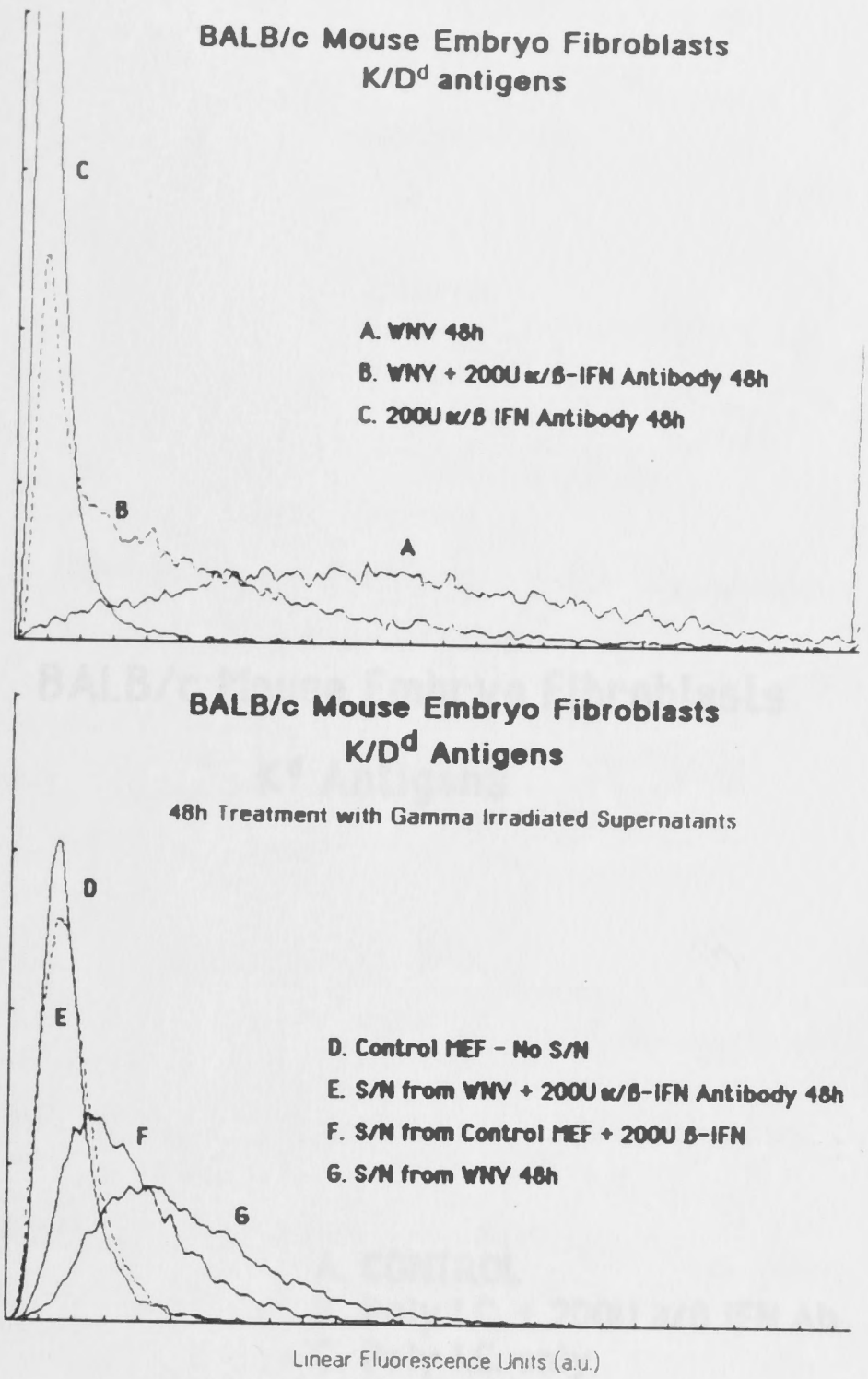
Induction of increased expression of H-2K^d on BALB/c MEF by WNV infection. Profiles A, B and C show control untreated MEF, MEF-infected with WNV for 16 h and MEF infected with WNV for 40 h respectively. The plots (from FACS analysis) shows fluorescence intensity divided into channels (abscissa), against cell number in each channel (ordinate).

FIGURE 2.



Induction of increased expression of H-2K^d on BALB/c MEF by flavivirus infection. The four panels show clockwise from the top left, WNV-, Kunjin-, MVE-, and JBE - infected MEF for 48 h compared with uninfected control MEF. The data presented as in figure 1.

FIGURE 3.



WNV-induced increase in expression of class I MHC antigens on BALB/c MEF is inhibited partially by anti-α/β-IFN antibody.

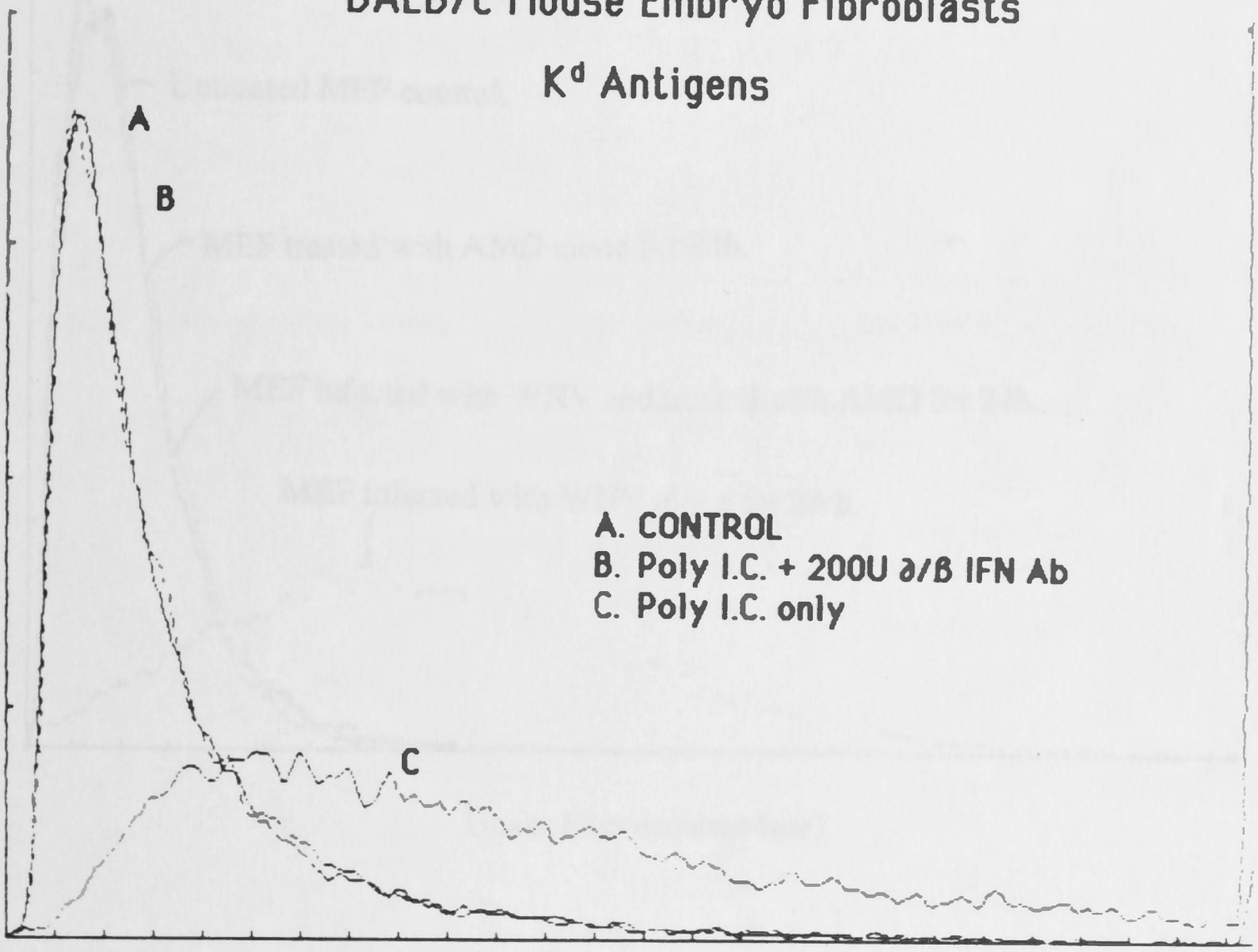
The top panel shows FACS analysis of:

A) MEF infected with 10 p.f.u .WNV for 48h; B) as A but MEF were treated simultaneously with 200 units of anti-α/β-IFN antibody for 48h; C) MEF treated with 200 units of anti-α/β-IFN antibody only for 48h.

The lower panel shows FACS analysis of:

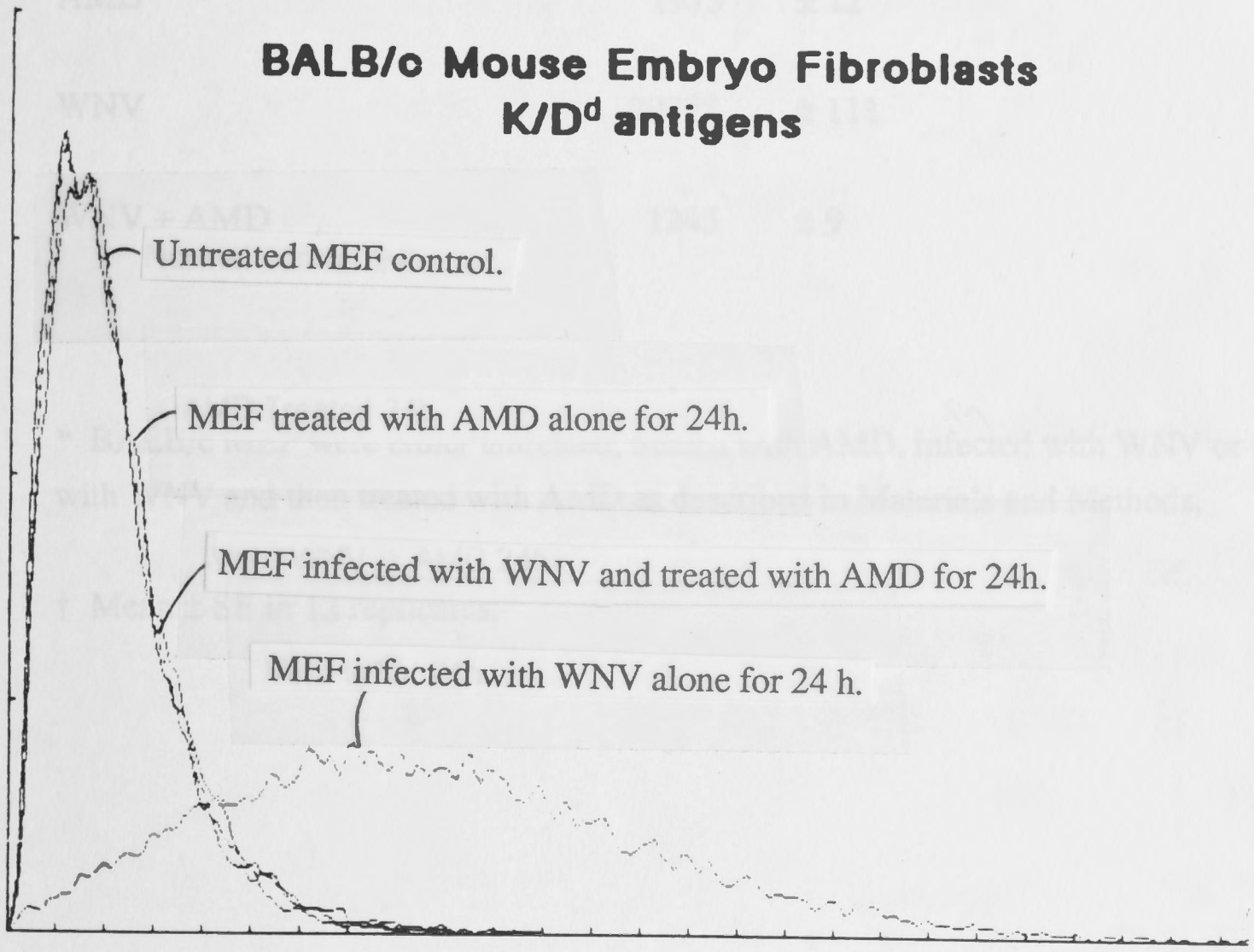
D) Control MEF without supernatant; E) MEF treated for 48h with γ-irradiated supernatant from Group B, i.e. WNV-infected MEF with 200 units of anti-å/ß-IFN antibody; F) MEF treated for 48h with γ-irradiated supernatant from control MEF with 200 units of β-IFN added; G) MEF treated for 48h with γ-irradiated supernatant from Group A, i.e. WNV-infected MEF. The data presented as in figure 1.

FIGURE 4.



Induction of increased expression of H-2K^d on BALB/c MEF by treatment with poly I:C. Profiles as follows: A) 200 units of α/β-IFN antibody; B) Poly I:C. at 25μg/ml (Sigma) and 200 units of α/β-IFN antibody;C) Poly I.C. at 25μg/ml only. The data presented as in figure 1.

FIGURE 5.



Inhibition of WNV-induced induction of class I MHC expression by AMD treatment of BALB/c MEF. The four groups show

Untreated MEF control.

MEF treated with AMD alone for 24h.

MEF infected with WNV and treated with AMD for 24h.

MEF infected with WNV alone for 24 h.

The data presented as in figure 1.

TABLE I

Effect of AMD on ^{3}H-Uridine Uptake in WNV - Infected MEF.

Treatment*	^{3}H-Uridine (c.p.m.)†	
Untreated	25107	± 98
AMD	1975	± 12
WNV	20988	± 111
WNV + AMD	1245	± 9

* BALB/c MEF were either untreated, treated with AMD, infected with WNV or infected with WNV and then treated with AMD as described in Materials and Methods.

† Mean ± SE in 12 replicates.

CHAPTER 5.

WNV-INDUCED MODULATION OF CLASS I MAJOR HISTOCOMPATIBILITY ANTIGENS: IMPLICATIONS FOR Tc CELL / TARGET CELL INTERACTIONS.

SUMMARY.

West Nile virus (WNV)-infection of tertiary mouse embryo fibroblasts (MEF) resulted in increased levels of expression of serologically detected class I MHC antigens which was reflected functionally by increased susceptibility to lysis by both secondary WNV- immune and allo-immune Tc cells. This increase is first detectable 8 h after infection and continues for at least 88 h causing increases in individual K and D antigens of 6-fold or more. However, increasing the level of class I MHC antigen expression of MEF as a result of treatment with γ-interferon or the supernatant from concanavlin A treated splenocytes leads not only to increased lysis by allo-immune Tc cells but also by secondary WNV-immune Tc cells. This increased lysis by secondary WNV-immune Tc cells is independent of the expression of viral antigens, nevertheless, a virus-specific subset of Tc cells can be demonstrated within the secondary WNV-immune Tc cell population by cold target competition experiments. The avidity of interaction between secondary WNV-immune Tc cells and WNV-infected or recombinant γ-interferon treated targets is greater than the avidity of interaction with uninfected targets as determined by blocking of lysis with anti-CD8 monoclonal antibody.

INTRODUCTION.

Murine class I MHC antigens (H-2K, D and L) act as restriction elements for antigen-specific cytotoxic T (Tc) cell responses (reviewed in Zinkernagel and Doherty, 1979). Previous studies have demonstrated that quantitiative variations in the level of class I MHC antigen expression correlate with the efficiency of lysis of virus-infected target cells by MHC-restricted virus-immune Tc cells (O'Neill and Blanden, 1979).

We have recently reported that infection of tertiary mouse embryo fibroblasts (MEF) with the flavivirus, West Nile (WNV) resulted in an increase in the expression of class I MHC antigens (King and Kesson, 1988). This increase in serologically detected class I MHC antigen expression was reflected functionally by increased susceptibility to lysis by both allo-immune and secondary WNV-immune Tc cells. However, these results do not allow definitive identification of the ligand necessary to trigger secondary WNV-immune Tc cell lysis of WNV-infected targets. The increased susceptibility to lysis of WNV-infected MEF by secondary WNV-immune Tc cells may be due to increased class I MHC ~~anitgen~~ antigen expression *per se*, or it may be due to expression of a determinant dependent upon both class I MHC antigen and WNV antigen together.

In this report we have explored the effects of increasing the level of class I MHC antigen expression alone by using γ–interferon treatment of MEF, and investigated the parameters affecting their susceptibility to lysis by secondary WNV-immune Tc cells. We show that increase in class I MHC antigen expression, without the expression of viral antigens, leads to increased lysis by both secondary WNV-immune Tc cells and allo-immune Tc cells. This observation is under dispute. However, a WNV-specific subset of T cells can be demonstrated in the secondary WNV-immune Tc cell population. These findings illustrate that the level of class I MHC antigen expression significantly affects the interaction between target cells and both allo-immune and secondary WNV-immune Tc cells.

MATERIALS and METHODS.

Mice:

Mice were bred under pathogen free conditions at the Animal Breeding Establishment of the John Curtin School of Medical Research. Female mice of the strains CBA/H ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), C3H.H-2^{02} ($H\text{-}2^{02}$), DBA/2 ($H\text{-}2^d$), B10.A ($H\text{-}2^a$), F1(A/J X DBA/1) ($H\text{-}2^{a/q}$), F1(C3H.OH X DBA/1) ($H\text{-}2^{02/q}$), F1(BIO.BYR X SJL) ($H\text{-}2^{by1/s}$), F1(BALB/c X C57Bl/6) ($H\text{-}2^{d/b}$) were used between the ages of 6 and 16 weeks.

Virus Stocks:

Vero cell grown WNV was prepared as described in King and Kesson (1988).

Cell Culture.

BALB/c MEF were grown as described in King and Kesson, (1988).

Supernatants from Concanavlin A Stimulated Splenocytes (Cs) and Interferons

1) Preparation of Cs. Cs was prepared by a method described by Sinickas *et al.*, (1985). ~~Breifly~~ Briefly splenocytes were cultured at a concentration of 10^7 nucleated cells/ml in 60ml of serum free Eagle's Minimal Essential Medium (Cat. No.430-1500, GIBCO, Chargrin Falls, Ohio, U.S.A.), supplemented with 10^{-4} M 2-mercaptoethanol (EMEM) and concanavlin A (Pharmacia) at a concentration of 5µg/ml for 1 h at 37° C in 175^2 cm tissue culture flasks (Nunclon). The cell layer was then washed three times with Hanks' balanced salt solution and 60ml of EMEM was added for a 16 h incubation at 37° C in an atmosphere of 5% CO_2 in air. The supernatant was harvested, centrifuged to remove cellular debris and concentrated 10-fold on a Amicon PM-10 membrane. The concentrated Cs preparation was sterilized by filtration and stored at -20° C. The preparation was added to MEF cultures at a final concentration of 3% (v/v).

2) Gamma-Interferon (γ–rIFN): Purified recombinant murine γ–rIFN was kindly provided by Boehringer Ingelheim and was used at a concentration of 500 units /ml. 2 mls of γ–rIFN was added to petri dish cultures and 30µl to the wells in microtitre plates.

Cs and γ–rIFN were ~~titred~~ titrated prior to use to the give maximal effect on MHC expression as measured by FACS analysis.

Cell Treatment for Cytotoxic Assays and FACS Analysis.

BALB/c MEF from the same population were seeded at 10^5 cells/ml into tissue culture plastic petri dishes (Kayline) for later labelling and FACS analysis or into 96-well microtitre trays (Nunclon) for cytotoxic assays. The petri dishes were seeded with a 7 ml volume containing the MEF, while the 96-well microtitre plates were seeded with 100µl volume. This was to give approximately equal numbers of cells for the respective cell-adhesive surface areas.

The cellswere drained prior to treatment and were either:

1) Infected for 1 h at 37° C in 5% CO_2 in air with WNV at a m.o.i. of 10 diluted in Dulbecco's Modified Eagles Medium (Cat. No.430-1600, GIBCO, Chargrin Falls, Ohio, U.S.A.), supplemented with 5% FCS, 200µg/ml Streptomycin, 200 U/ml Penicillin G and 125µg/ml Neomycin Sulphate (DMEM) being 30µl per microtitre well and 2ml per petri dish. DMEM was then added for the remainder of the culture period (up to 96 h).

2) cultured in DMEM containing Cs at 3% v/v.

3) cultured in DMEM containing γ–rIFN at 500 units / ml.

4) infected with WNV at a m.o.i. of 10 prior to culture with Cs or γ–rIFN.

5) left untreated.

The final volume of microtitre wells was 100µl and petri dishes 7 ml of DMEM.

Generation of Secondary WNV-Immune Tc cells

Secondary WNV-immune Tc cells were generated *in vitro* as described in Kesson *et al.*, (1988).

Generation of Allo-Immune Tc cells.

Spleens were collected in Puck's saline and teased apart with sterile needles to produce a single cell suspension. Viable cells were then counted using trypan blue exclusion and cell concentrations were adjusted as required in culture medium (Eagle's Minimal Essential Medium Cat. No. 410-1500 GIBCO, Chargrin Falls, Ohio, U.S.A., supplemented with 5% foetal calf serum (FCS), 200µg /ml Streptomycin, 200 U/ml Penicillin G, 125µg/ml Neomycin Sulphate and 10^{-4} M 2-mercaptoethanol). Allo-immune Tc cells were generated *in vitro* by culturing $10^{7.9}$ "responder" cells with $10^{7.3}$ allogeneic "stimulator" cells which had been irradiated with $10^{3.3}$ rads from a ^{60}Co source for 5 days in 50mls ($10^{6.3}$ cells per ml) of culture medium in Nunclon Delta 80^{2} cm tissue culture flasks (No 53732) in a humidified atmosphere of 5% CO_2 in air at 37^{o} C. The cells were then washed once with culture medium, viable cell numbers determined using trypan blue exclusion and cell concentrations adjusted as required.

CD8 Blocking of Secondary WNV- Immune Tc Cells.

Before addition to target cells, Tc cells were preincubated for 30 minutes at 37^{0} C with 2-fold dilutions of the supernatant fluids from rat hybridoma cell line 53.6.7 which produce monoclonal anitbodies against CD8. The Tc cells were suspended at a concentration of $10^{6.8}$ cells / ml in the monoclonal antibody dilutions. Following incubation, 100µL of the cell suspension (i.e. $10^{5.8}$ cells) was then added to targets in microtitre plates for cytotoxic assays. (MacDonald *et al.*,1981).

Cytotoxic Assays:

96-well flat bottomed microtitre trays were seeded with $10^{4.3}$ MEF per well in100µl of DMEM. Plates were left for 6 h at 37° C and 5% CO_2 in air to allow the cells to adhere. The wells were drained and the cells were treated as described above in "cell treatment". 100µL of DMEM was then added for variable times up to 80 h. Finally, 3µCi of $Na_2(^{51}Cr)0_4$ (Amersham Int. Ltd., Amersham, U.K.) in 100µl DMEM per well was then added to give a total volume of 200 µl, for 16 hours at 37° C and 5% CO_2 in air. After this the medium was flicked off and the cells washed twice with DMEM at 37° C. Secondary WNV- immune Tc cells or allo-immune Tc cells were incubated with the target cells for 6 h at 37° C in a humidified atmosphere containing 5% CO_2 in air.

Triplicate assays were set up for each dilution of WNV-immune or allo-immune Tc cells at the required effector to target cell ratio (E:T). Calculations of E:T were based on the estimated number of target cells being $10^{4.3}$ per well at the time of assay. For assays requiring greater than 16 h infection MEF were originally seeded at a cell concentration which would yield the above number of cells assuming two mean population doublings every 4 days (King *et al.*, 1986). Supernatants were harvested and the % specific lysis calculated as described in Kesson *et al.* (1988).

Data given are means of triplicates. Standard errors of the means were always less than 5%. Significance was determined by Student's t Test and P values of < 0.05 were considered significant.

Cold Target Competition.

Tertiary MEF were grown in 175^2 cm tissue culture flasks (Nunc). The media was drained and the MEF were infected with 25 p.f.u. per cell of WNV, treated with 25,000 units of γ–rIFN or left untreated for 1 h at 37° C. After this the cells were cultured in 50ml DMEM for 48 h at 37° C in 5% CO_2 in air. The cells were then trypsinized off the plastic with 0.25% trypsin in buffered isotonic saline, washed once in DMEM, and viable cell

numbers determined using trypan blue exclusion. $10^{6.5}$ cells from each of the three groups were labelled with ^{51}Cr for 1 h at 37° C, washed twice in DMEM and $10^{4.3}$ cells in 50µl added to each well of a 96-well tissue culture plate (Nunc). Unlabelled cells from the three groups were used as cold targets and 50µl aliquots added at ratios 8, 4, 2 and 1 times the number of labelled targets. Tc cells ($10^{5.8}$) were then added in 100µl per well. The assay was run for 6 h.

Preparation of Antisera and cell labelling.

Antisera were prepared and cells labelled as described in King and Kesson (1988).

FACS Analysis and Cell Size.

Fluorescence and cell size was measured as described in King and Kesson (1988).

RESULTS.

WNV- Infection of BALB/c MEF Increases their Sensitivity to Lysis by Allo-Immune and WNV-Immune Tc Cells.

In a previous paper we reported that WNV infection induced an increase in the expression of class I (H-2K and H-2D) MHC antigens (King and Kesson, 1988). The effect of WNV-infection of MEF on their susceptibility to lysis by secondary WNV-immune and allo-immune Tc cells was tested in a ^{51}Cr- release assay. BALB/c MEF were infected with WNV for 16, 48, or 96 h or left uninfected. These four MEF populations were then divided into two samples. One sample was used as targets for lysis by secondary WNV- immune or allo-immune Tc cells, while on the other quantitive changes in class I MHC antigen expression were determined by FACS. Figure 1 shows increased lysis of the 48 h and 96 h WNV-infected MEF compared with the 16 h and uninfected MEF by both secondary WNV-immune and allo-immune Tc cells. This

increased susceptibility to Tc cell mediated lysis correlated with increased fluorescence measured by FACS analysis as shown in figure 2.

Cs and γ–rIFN Induced Enhancement of Class I MHC Antigen Expression of MEF Increases their Susceptibility to Lysis by Secondary WNV-Immune Tc Cells.

The difference between uninfected and 16 h WNV-infected versus 48 h and 96 h WNV-infected MEF in susceptibility to lysis by allo-immune and secondary WNV-immune Tc cells could be attributed to the differences in the level of class I MHC antigen expression. From the above data it is impossible to determine whether the increase in Tc cell mediated lysis of MEF was due to increased MHC antigen expression *per se*, or if the MHC antigen increase facilitated the association of MHC antigen with WNV antigen which was then the determinant seen by the secondary WNV-immune Tc cells. If the former were true, viz secondary WNV-immune Tc cells ~~repond~~ respond to MHC antigens alone, then increasing the level of class I MHC antigen expression of MEF with IFN should also increase their susceptibility to lysis by secondary WNV-immune Tc cells. Therefore, tertiary BALB/c MEF were divided into four groups: A) untreated, B) WNV-infected, C) treated with Cs or D) both WNV-infected and then treated with Cs for 48 h. These four MEF populations were divided into two samples. One sample was used as targets for lysis by secondary WNV-immune or allo-immune Tc cells while on the other quantitative changes in the level of expression of class I MHC antigens was determined by FACS.

Figure 3 shows increased lysis of the WNV-infected, Cs-treated and WNV-infected and Cs-treated MEF (groups B, C and D respectively) compared with the uninfected and untreated MEF (group A) by both secondary WNV-immune and allo-immune Tc cells. The increased fluorescence measured by the FACS on the MEF which were infected with WNV and treated with Cs (group D) was greater than either of the the WNV-infected (group B) or Cs-treated (group C) groups (figure 4). However, this increase in fluoresence of the MEF treated with Cs and infected with WNV (group D) was not reflected by an increase in

susceptibility to lysis by either the allo-immune or secondary WNV-immune Tc cells.

Cs contains γ–interferon which is normally released by activated T cells and has been demonstrated to increase the level of class I MHC antigens on cells which are exposed to it (Wong *et al.*, 1984; Pasternack *et al.*,1984). γ–interferon, unlike α- and β–interferon, increases the MHC antigen expression of cells at doses approximately 100-fold lower than those required for antiviral effects (Wallach *et al.*,1982). Therefore, the experiment described above was repeated using murine recombinant γ–interferon (γ–rIFN) instead of Cs. The increased fluoresence detected by the FACS was very similar to that shown in figure 4 (data not shown). Furthermore, increased lysis of the three treated groups, WNV-infected, γ–rIFN-treated and WNV-infected and γ–rIFN-treated MEF was mediated by both secondary WNV-immune and allo-immune Tc cells (figure 5). However, the lysis by allo-immune Tc cells of the WNV-infected MEF was greater than that of the other treated groups and cannot be solely explained by the level of MHC antigen expression.

Cold Target Competition.

The previous results suggested that the secondary WNV-immune Tc cells were largely cross-reactive. That is, the BALB/c WNV-immune Tc cells lyse both BALB/c WNV-infected and BALB/c γ–rIFN-treated MEF. Therefore, cold target competition was used to determine if any WNV-specific Tc cell subset was detectable. The rationale for cold target competition is thus: if competition is more efficient using WNV-infected cold targets than γ–rIFN- treated cold targets when secondary WNV-immune Tc cells are used to lyse hot WNV-infected targets then the existence of a WNV-specific Tc cell population would be demonstrated. Therefore a cold target competition experiment was performed.

Secondary WNV-immune and allo-immune Tc cells were assayed on ^{51}Cr -labelled MEF which had been WNV-infected, γ–rIFN-treated or left uninfected, and unlabelled MEF competitors which were either WNV-infected, γ–rIFN-treated or left uninfected were added at various ratios to the labelled targets. The results in figure 6a demonstrate the

ability of WNV-infected cold targets to compete to a much greater extent than either the γ–rIFN or untreated MEF when secondary WNV-immune Tc cells were used as effector cells to lyse WNV-infected ^{51}Cr-labelled MEF. There was no significant competition by any of the cold targets when secondary WNV-immune Tc cells were used as effector cells to lyse ^{51}Cr-labelled γ–rIFN-treated MEF. The results in figure 6b demonstrate that all three of the cold targets competed when allo-immune Tc cells were used as effector cells to lyse uninfected MEF, however the γ–rIFN treated and WNV-infected MEF competed to a greater extent than uninfected MEF when either the γ–rIFN treated or WNV-infected MEF were hot targets. Taken together these results support the existence of two subsets of Tc cells in the population of secondary WNV-immune Tc cells i.e. those which lyse cells expressing class I MHC antigens alone and those which are specific for WNV antigen(s) in association with class I MHC antigens. Furthermore, allo-immune Tc cells also seem to contain at least two subsets of Tc cells. Those of low affinity which preferentially lyse the γ–rIFN treated or WNV-infected MEF and those of relatively high affinity which lyse both the uninfected MEF expressing lower levels of class I MHC antigens and γ–rIFN treated or WNV-infected MEF expressing higher levels of class I MHC antigens. These apparent subsets are defined by the target cell chosen for lysis. Within a population of allo-immune Tc cells it is presumed that there will be a range of affinities from high to low. Therefore a given population of target cells may or may not exceed the threshold of antigen concentration needed to trigger a functional Tc cell response (Shimonkewitz *et al.*, 1985; Blanden *et al.*, 1987).

Blocking with Monoclonal anti-CD8 Antibody of Target Cell Lysis Mediated by Secondary WNV-Immune Tc Cells.

The cold target experiment confirmed that a subset of Tc cells in the secondary WNV-immune Tc cell population may recognize class I MHC antigens alone. To determine the avidity of interacion between secondary WNV-immune Tc cells and the target cells an

experiment using CD8-blocking of effector cells was performed. In theory, CD8 antibody blocking of secondary WNV-immune Tc cells is more efficient for low than high avidity interactions with target cells.

After treatment with anti-CD8 antibody, secondary WNV-immune Tc cells were added to MEF which had been either infected with WNV, treated with γ–rIFN or left untreated for 48 h prior to assay. Anti-CD8 antibody blocked the lysis of uninfected MEF targets to a greater extent than it blocked the lysis of either the WNV-infected or γ–rIFN treated MEF targets, thus the interaction of the secondary WNV-immune Tc cells with the WNV-infected or γ–rIFN treated MEF targets is of greater avidity than the interaction of secondary WNV-immune Tc cells with uninfected targets (Figure 7).

DISCUSSION.

The increased susceptibility of Cs or γ–rIFN treated MEF to lysis by secondary WNV-immune Tc cells generally paralleled their increased susceptibility to lysis by allo-immune Tc cells, suggesting a similar quantitative requirement for cell surface class I MHC antigen expression on target cells for both syngeneic secondary WNV-immune and allogeneic allo- immune Tc cells. Both Cs or γ–rIFN treatment of WNV-infected MEF resulted in a further increase in serologically detectable class I MHC antigens, over and above that already induced by WNV-infection. However, this increase in K and D antigen expression was not reflected by an increase in lysis by either secondary WNV-immune or allo-immune Tc cells. This suggests that either optimal levels of both MHC and viral antigens were achieved or that the level of viral antigen expression is limiting, thus preventing any further increase in lysis by secondary WNV-immune Tc cells. However, the failure to detect increased lysis of these targets by allo-immune Tc cells suggests that optimal lysis of targets can occur when lower levels of MHC antigens are expressed. Thus, the efficiency of both secondary WNV-immune and allo-immune Tc cells mediated

lysis may be at the plateau of maximal killing, and the increased expression of class I MHC antigens does not increase the target cell lysis (King *et al.*, 1986).

Both Bukowski and Welsh (1985) and King *et al.* (1986) demonstrated that treatment of MEF with interferon or Cs greatly enhanced the susceptibility of virus-infected MEF to lysis by virus-immune Tc cells. Both groups attributed their results to the increased level of class I MHC antigen expression making the targets more susceptible to lysis by virus-immune Tc cells and suggested that these results supported the view that interferons enhance Tc cell recognition of virus-infected cells by enhancing class I MHC antigen expression. However, in the case of flavivirus infections it appears that the increase in class I MHC antigen expression by either IFN or WNV- infection *per se* resulted in optimal lysis by both secondary WNV-immune and allo-immune Tc cells. It is impossible to determine from these experiments whether there is a role for viral antigens in the recognition of WNV-infected MEF by secondary WNV-immune Tc cells or whether the class I MHC antigens alone are the target for the secondary WNV-immune Tc cells. An alternative explanation i.e. that BALB/c WNV-immune Tc cells are cross-reactive and lyse both BALB/c WNV-infected and γ–rIFN-treated MEF was tested by cold target experiments. These experiments excluded the possibility that "H-2^d-WNV" cross-reacts with "H-2^d" and thus demonstrated the existence of a WNV-immune Tc cell subset in the secondary WNV-immune Tc cell population generated *in vitro*.

Blanden *et al.* (1987) hypothesized that the signal ~~recieved~~ received by a T cell depends upon the receptor-ligand interaction as well as the concentration of both the ligand on the target cell and the receptors on the T cell. Furthermore, the imposition of self-tolerance on the T cell repertoire requires the deletion of self-reactive Tc cells from the mature T cell population. (This makes no comment on the mechanism(s) of generating the T cell repetoire). That is, T cells which express high affinity T cell receptors for self would be deleted and low affinity self-reactive T cells would be triggered only when the concentration of the ligand on the target cell was sufficient to generate an activation signal.

Therefore, Blanden *et al.*, (1987) hypothesized that if among a population of virus-immune Tc cells there were clones which, in addition to being virus-specific were also self-reactive then these clones would most probably have low affinity for self antigens. Therefore, anti-CD8 antibodies would block lysis by such clones of uninfected syngeneic targets to a greater extent than that of infected syngeneic targets. The data in figure 7 demonstrate that CD8 blocking of secondary WNV-immune Tc cells had a significantly greater effect on the lysis of uninfected targets than on the lysis of either γ–rIFN-treated or WNV-infected targets. Furthermore, the lysis of γ–rIFN-treated targets by secondary WNV-immune Tc cells was not blocked by anti-CD8 antibodies. Thus, the secondary WNV-immune Tc cells had increased avidity of binding to these targets.

Both WNV-infected and γ–rIFN-treated MEF expressed higher levels of class I MHC antigens than uninfected MEF and this increased level of class I MHC antigen expression may be sufficient to allow them to act as targets for low affinity self-reactive Tc cell clones with the secondary WNV-immune Tc cells. Generation of WNV-immune Tc cell clones will allow for a definitive answer to this question by splitting the clone and testing lysis on WNV-infected γ-IFN-treated and uninfected / untreated target cells.

Use of recombinant vaccinia viruses containing cDNA encoding Kunjin polypeptides to infect target cells has clearly shown that such targets are lysed by either WNV-immune or Kunjin-immune MHC-restricted Tc cells (A.Hill *et al.*, 1989 personal communication). There is ~~definate~~ definite evidence for the existence of WNV-immune Tc cells that recognize flavivirus derived antigen plus MHC.

ADDENDUM.

Data provided by Dr A Mullbacher is in conflict with the data presented in this chapter.

Secondary B10.A(2R) (K^kD^b) MEF were divided into three groups: A) untreated, B) treated with 500 units of γ-rIFN or C) infected with WNV for 48 h.

These three MEF populations were divided into two samples. One sample was used as targets for lysis by secondary CBA (K^kD^k) WNV-immune or BALB/c (K^dD^d) anti-CBA allo-immune Tc cells while on the other sample, quantitative changes in the level of expression of class I MHC antigens was determined by FACS.

Compared with the untreated MEF, γ-rIFN-treated MEF showed enhancement of K^k expression by 83.3% while WNV-infected MEF showed enhancement of K^k expression by 40.2% (data not shown).

The data in table 1 shows increased lysis of the WNV-infected and γ-rIFN-treated MEF by the allo-immune Tc cells. In contrast, lysis of the WNV-infected MEF is increased compared with the γ-rIFN-treated and untreated MEF by WNV-immune Tc cells. There is decreased lysis of the γ-rIFN-treated MEF compared with the untreated MEF which is unexplained, but clearly there is no increase in lysis of γ-rIFN-treated MEF compared with uninfected MEF by secondary WNV-immune Tc cells.

On this occasion elevated levels of MHC antigen expression did not result in increased lysis by secondary WNV-immune Tc cells ~~Thus,~~ thus suggesting that secondary WNV-immune Tc cells consist of a population which recognize a determinant consisting of WNV antigen and MHC. A population of Tc cells with low-affinity to self antigens which could be triggered by a higher level of MHC antigen expression were not demonstrated within the secondary WNV-immune Tc cells. The discrepancy between this data and my own data presented earlier is not easily explained and further experiments are needed to clarify these observations. However, I have demonstrated increased lysis of γ-IFN- or Cs-treated MEF by WNV-immune Tc cells on five occasions. Dr Mullbacher was unable

to repeat these experiments on one attempt. Unfortunately, I had left the John Curtin School when this discrepancy arose and I have been unable to repeat my work and confirm my findings.

TABLE 1.

Lysis of Uninfected, γ-rIFN-Treated and WNV-Infected MEF by Secondary WNV-immune and Allo-immune Tc cells.

		% Specific Lysis of MEF Target Cells.*		
Cytotoxic Cells	E/T†	WNV-Infected+	γ-rIFN-Treated§	Uninfected
WNV-Immune¶	160	86.2	16.2	34.5
	53	75.0	10.8	26.3
	18	61.4	3.5	10.5
Allo-Immune•	100	70.5	67.7	28.8
	33	49.9	53.0	22.3
	11	28.7	26.2	12.7

*) Means of triplicate cultures with standard errors of the means less than 3.5%.

†) Effector to target cell ratio.

+) Infected with 10 p.f.u WNV per target.

§) Treated with 500 units γ-rIFN.

¶) Primed with 10^6 p.f.u WNV i.v. and spleen cells boosted *in vitro* 7 days later.

•) BALB/c spleen cells boosted *in vitro* with CBA spleen cells.

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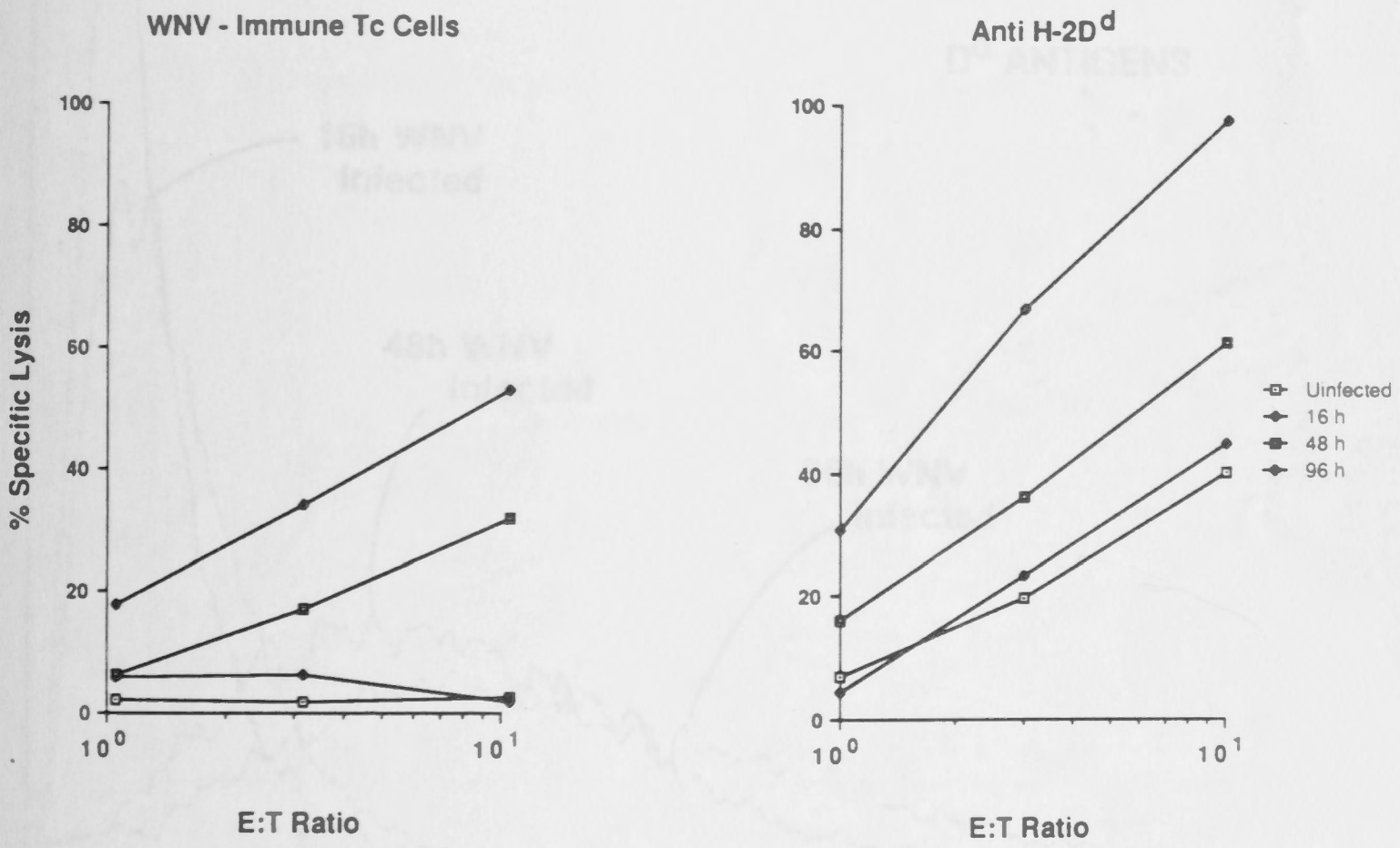
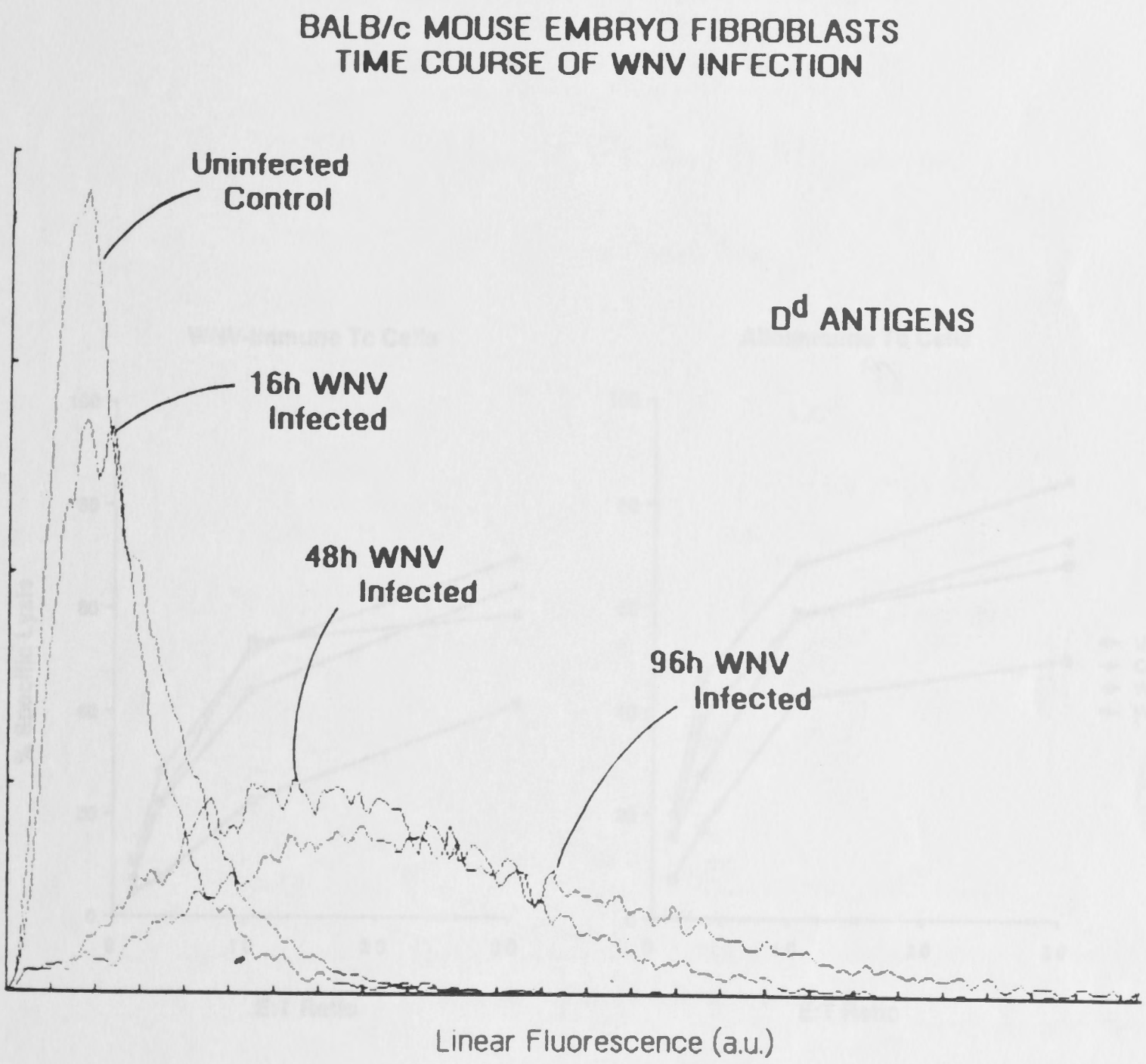
FIGURE 1.



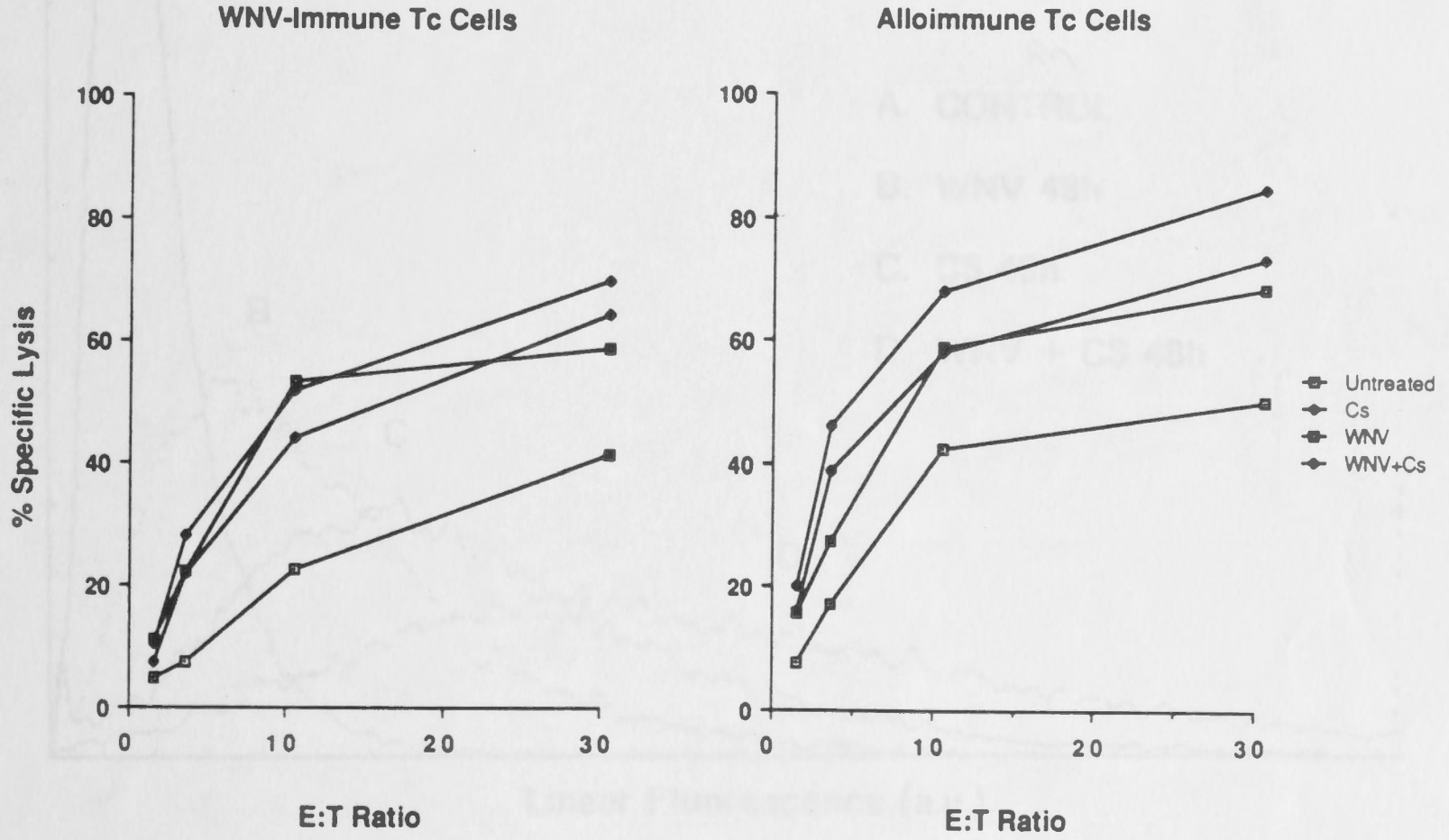
Cytotoxic activity of BALB/c secondary WNV-immune or anti-H-2D^d Tc cells on 16, 48 or 96 h WNV-infected or uninfected BALB/c MEF. Each point represents the mean of triplicate assays. The standard error of the mean is always less than 4.8% and is omitted for clarity.

FIGURE 2.



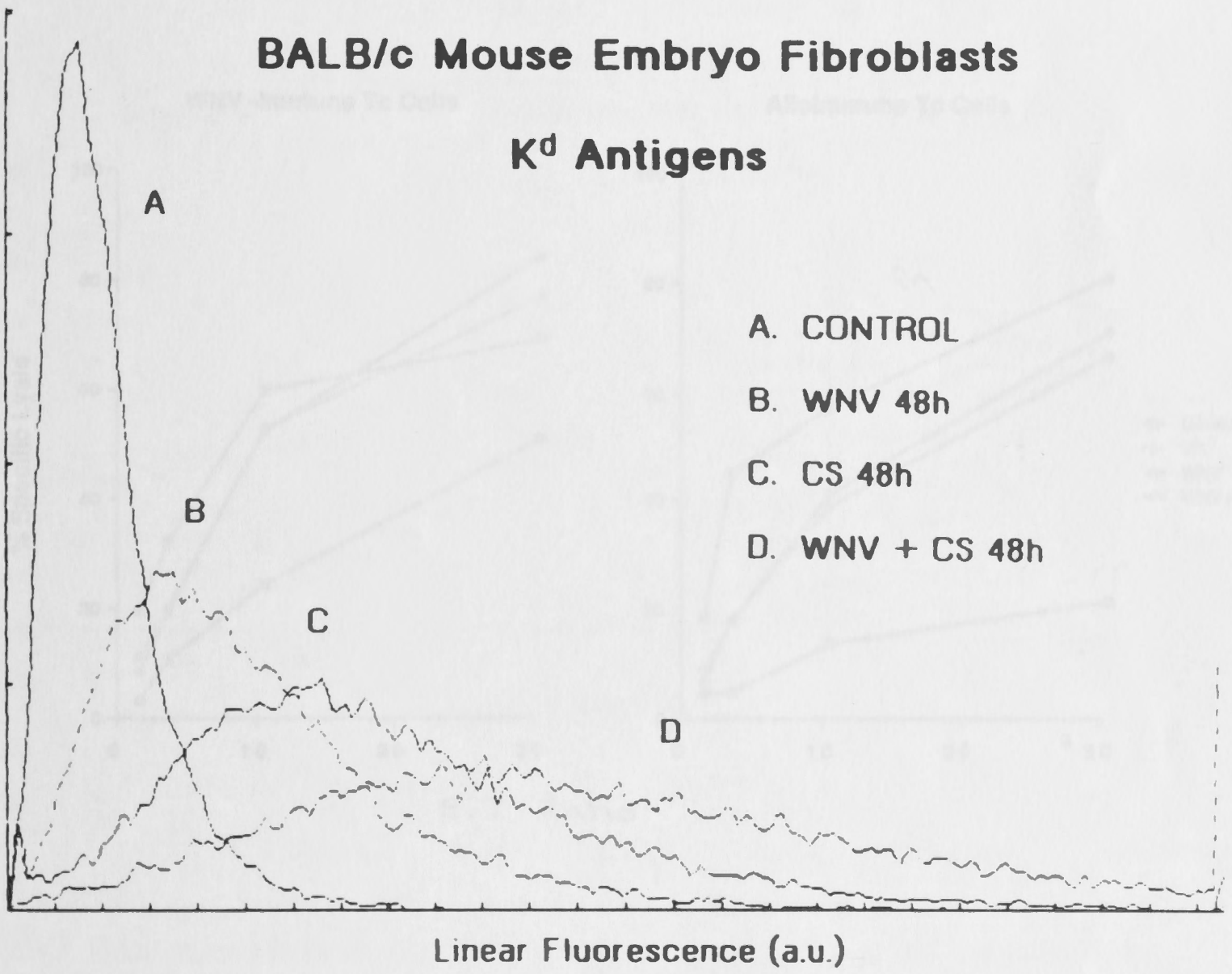
Induction of increased expression of H-2D^d on BALB/c MEF. The MEF were infected with WNV for 96, 48, 16 h or left uninfected. The plot from the FACS analysis shows fluorescence intensity divided into channels (abscissa), against relative cell number in each channel (ordinate).

FIGURE 3.



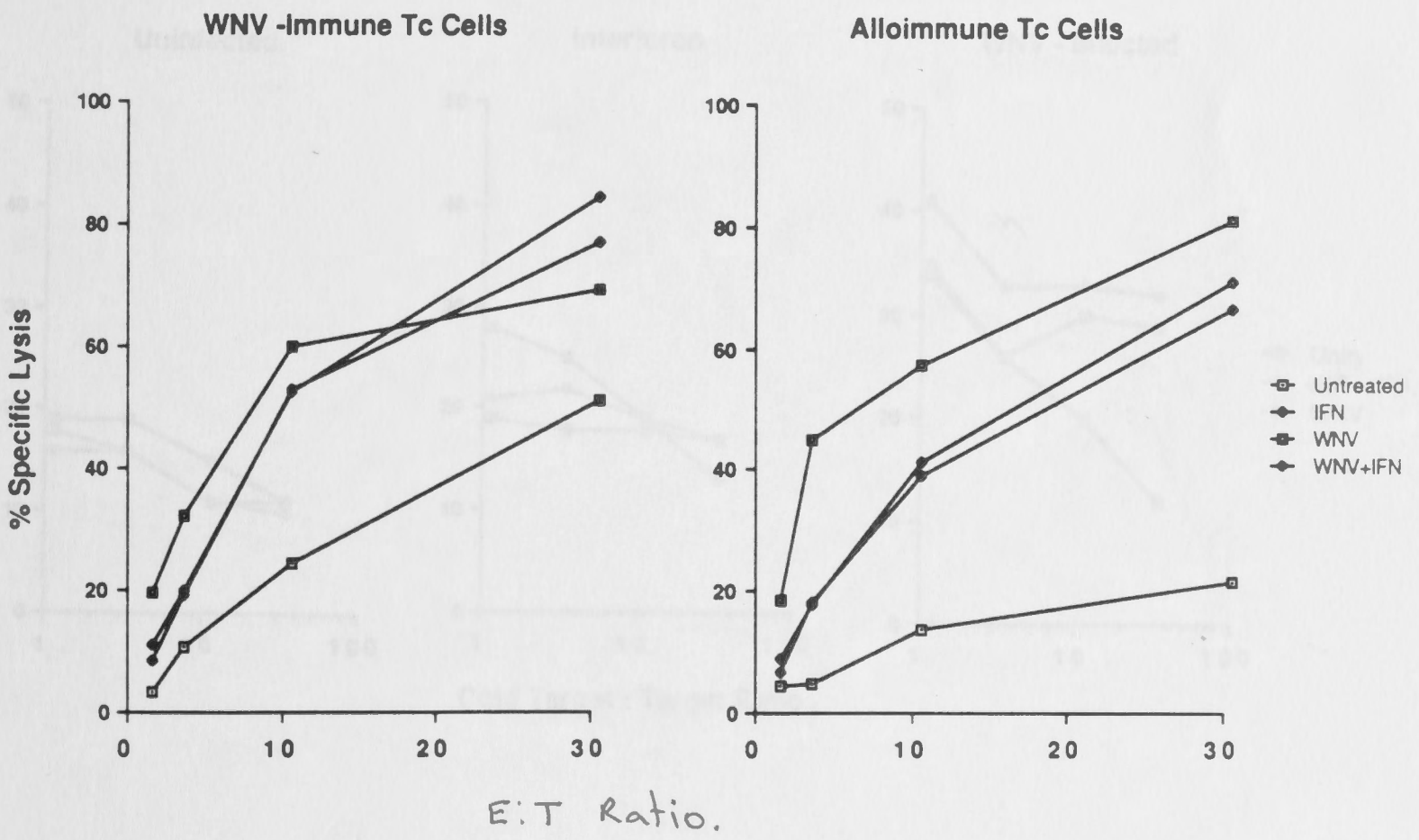
Cytotoxic activity of BALB/c secondary WNV-immune or anti-H-2K^d Tc cells on BALB/c MEF treated with Cs, infected with WNV, infected with WNV and treated with Cs or left uninfected. Each point represents the mean of triplicate assays. The standard error of the mean is always less than 4.2% and is omitted for clarity.

FIGURE 4.



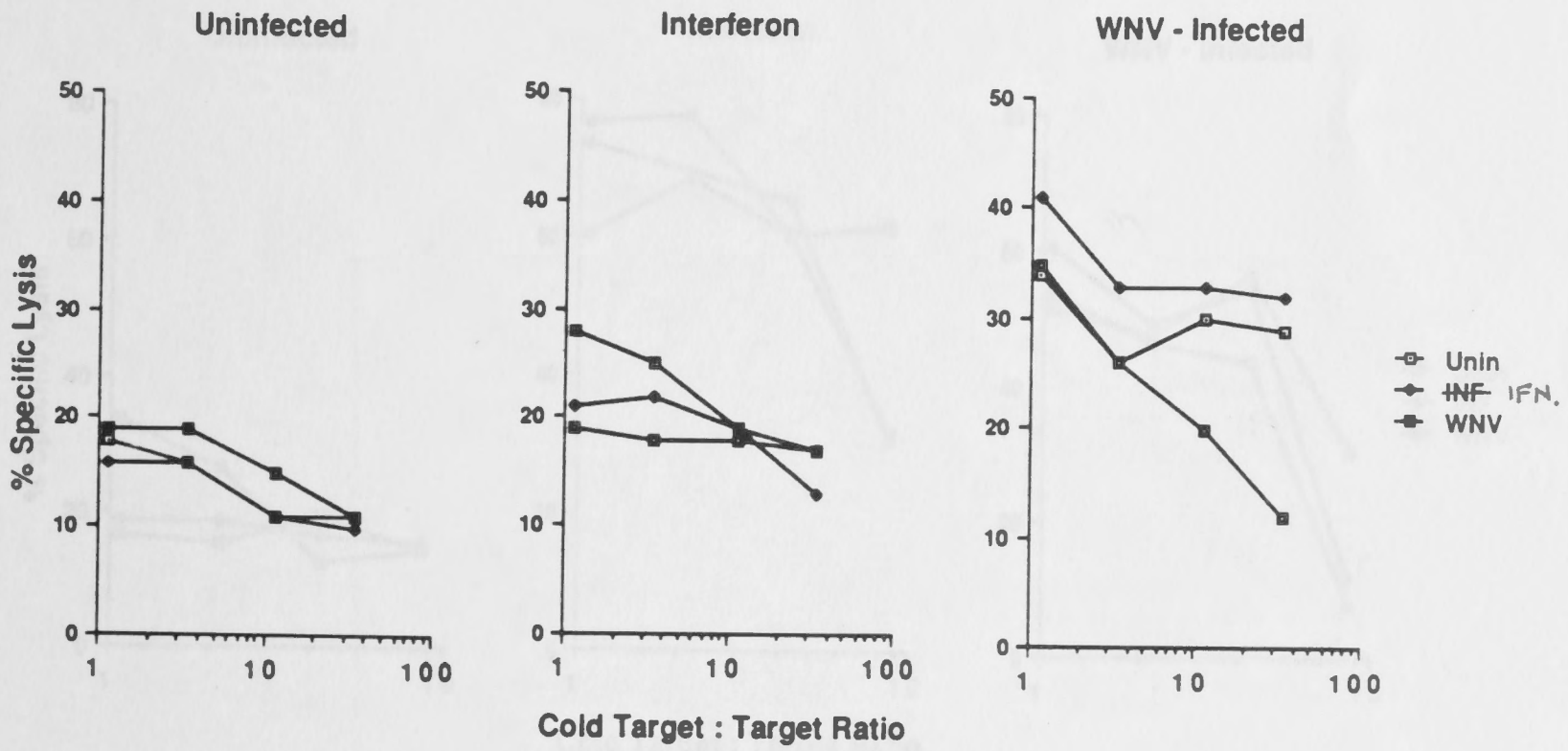
Induction of increased expression of H-2K^d on BALB/c MEF. The MEF were A, untreated: B, infected with WNV for 48 h; C, treated with Cs for 48 h or D, infected with WNV and treated with Cs for 48 h. The data presented as in figure 2.

FIGURE 5.



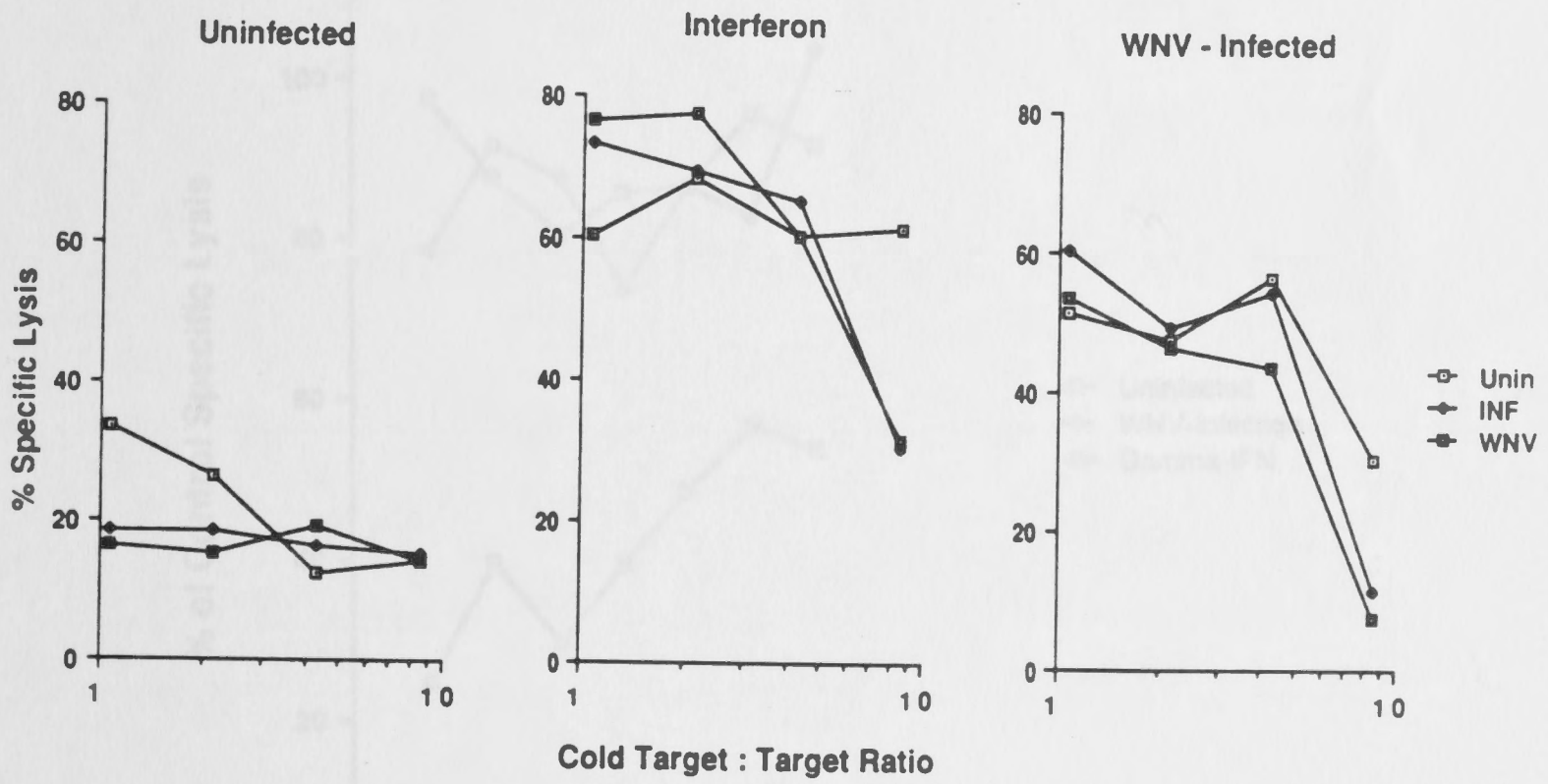
Cytotoxic activity of BALB/c secondary WNV-immune or anti-H-2K^d Tc cells on BALB/c MEF treated with γ–rIFN, infected with WNV, infected with WNV and treated with γ–rIFN or left uninfected. Each point represents the mean of triplicate assays. The standard error of the mean is always less than 3.9% and is omitted for clarity.

FIGURE 6a.



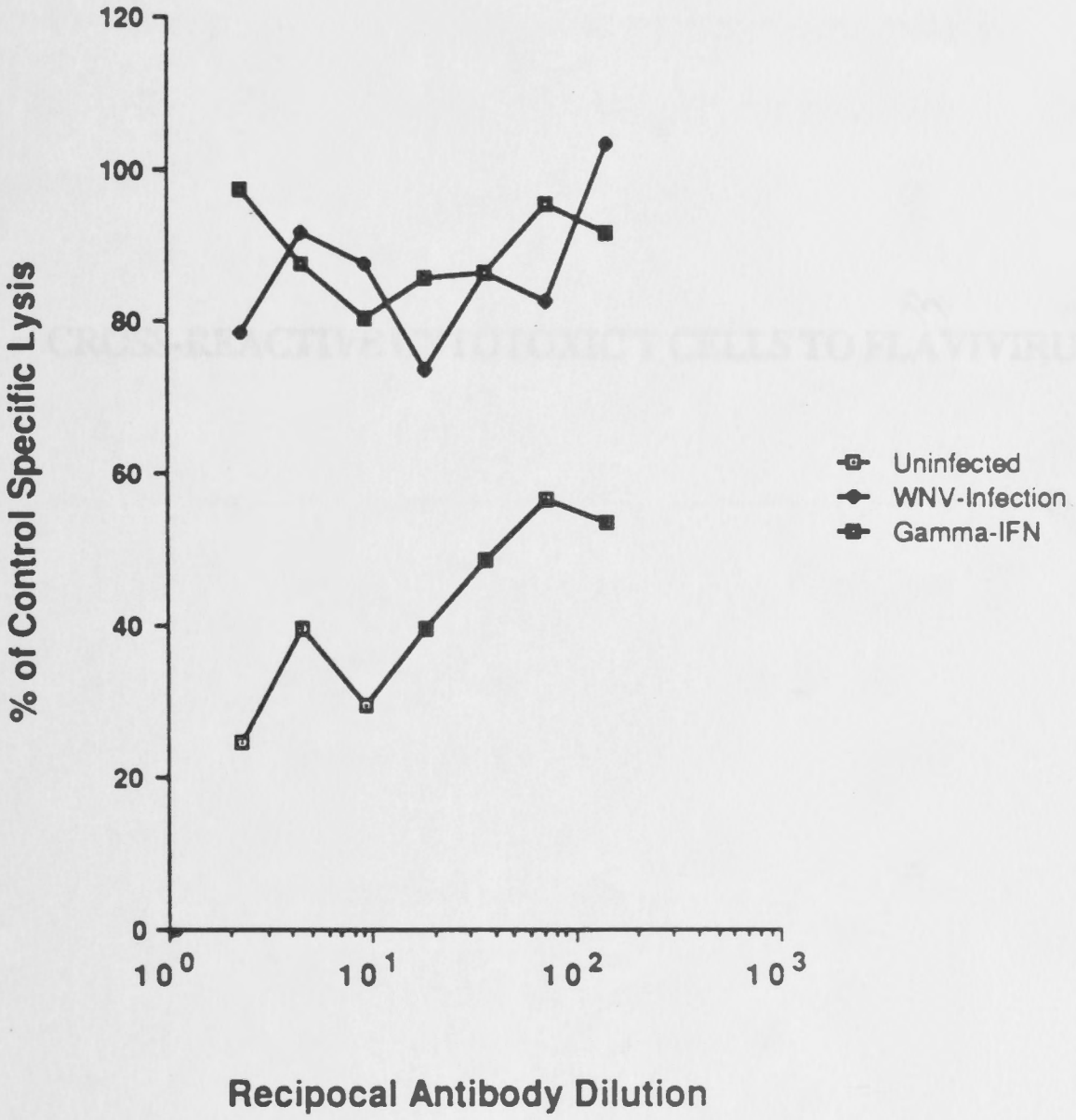
Inhibition of Tc cell mediated cytotoxicity by cold target competitors infected with WNV (WNV) or treated with γ–rIFN (IFN) or left uninfected (Unin). Secondary WNV-immune Tc cells were treated for cytotoxicity on ^{51}Cr-labelled BALB/c MEF targets which were either WNV-infected , treated with γ–rIFN or left uninfected in the presence of cold competitiors as shown in the figure. The ratio of Tc cells to ^{51}Cr-labelled BALB/c MEF was 30:1. Each point represents the mean of triplicate assays. The standard error of the mean is always less than 5.7% and is omitted for clarity. Control specific lysis without cold competitors was 17.1%, 31.0% and 47.1% for uninfected, γ–rIFN-treated and WNV-infected MEF respectively.

FIGURE 6b.



Inhibition of Tc cell mediated cytotoxicity by cold target competitors infected with WNV (WNV) or treated with γ–rIFN (IFN) or left uninfected (Unin). Allo-immune Tc cells were ~~treated~~ tested for cytotoxicity on ^{51}Cr-labelled BALB/c MEF targets which were either WNV-infected, treated with γ–rIFN or left uninfected in the presence of cold competitiors as shown in the figure. The ratio of Tc cells to ^{51}Cr-labelled BALB/c MEF was 30:1. Each point represents the mean of triplicate assays. The standard error of the mean is always less than 6.1% and is omitted for clarity. Control specific lysis without cold competitors was 46.3%, 86.9% and 66.9% for uninfected, γ–rIFN-treated and WNV-infected MEF respectively.

FIGURE 7.



Blocking by addition of anti-CD8 antibody prior to assay of cytotoxicity mediated by secondary WNV-immune Tc cells on ^{51}Cr -labelled BALB/c MEF which were either WNV- infected, treated with γ–rIFN or left untreated as shown in the figure legend. Results are expressed as percent ~~reduction below~~ of control ~~levels of~~ specific lysis in the absence of antibody.

CHAPTER 6.

CROSS-REACTIVE CYTOTOXIC T CELLS TO FLAVIVIRUS INFECTION.

SUMMARY

Secondary flavivirus-immune Tc cells reactive to West Nile virus (WNV), Kunjin, Murray Valley encephalitis (MVE) or Japanese B encephalitis (JBE) were generated *in vitro* following priming *in vivo*. These four flavivirus- immune Tc cell populations were cross-reactive at the level of target cell lysis as they lysed target cells infected with WNV, Kunjin, MVE or JBE significantly more than they lysed uninfected targets. Furthermore, cross-reactivity was also detected at the level of induction of the secondary flavivirus-immune Tc cells. Cold target competition experiments confirmed the cross-reactivity of flavivirus-immune Tc cells and no virus-specific subset was demonstrated.

INTRODUCTION.

Members of the Flaviviridae may cause infections in humans with significant morbidity and mortality (Sanford, 1987). Protection from lethal flavivirus infection has been demonstrated using both humoral and cellular immune responses. Adoptive transfer of specific antibody (Chaturverdi *et al.*, 1978; Mather *et al.*, 1983) and immune spleen cells (Jacoby *et al.*, 1980) have been shown to be protective in experimental murine flavivirus infections however, the immune response may be deleterious in some situations. In dengue haemorrhagic shock syndrome the presence of antibodies to one serotype of dengue virus is thought to mediate significant immunopathology when secondary infection with another serotype of dengue virus occurs (Halsted, 1982).

T cells have beeen demonstrated to be important in recovery from viral infections (Blanden, 1974; Ada *et al.*, 1981) and furthermore cross-protection by T cells has been demonstrated to occur in influenza infections (Yap and Ada, 1978). Acute and chronic lymphocytic choriomeningitis virus (LCMV) infection can be cleared by T cells (Mims and Blanden, 1972) even from the central nervous system (Oldstone *et al.*,1986). However, T cells have been demonstrated to mediate significant immunopathology in some viral infections. Studies of mice with LCMV encephalitis have provided evidence that T cell mediated injury of the brain may be central to the immunopathological process (Cole *et al.*, 1972; Zinkernagel and Doherty, 1973). The role if any of T cells in mediating immunopathology in flavivirus infections is not clear. However there is evidence that cellular immune responses in flavivirus encephalitis may enhance the pathological response (Camenga and Nathanson, 1975; Semenov *et al.*, 1975).

Influenza-immune Tc cell populations contain two subsets. A cross-reactive subset which responds to heterologous virus of a different subtype and a second Tc cell subset specific for the immunizing strain of virus. Different influenza virus antigens are recognized by the different subsets (Braciale, 1977). In contrast, alphavirus-immune Tc cells comprise only a single cross-reactive population (Mullbacher *et al.*, 1979).

Uren *et al.* (1987) demonstrated asymmetrical cross-reactivity with $CD4^+$ flavivirus-specific T cell clones within a subgroup of flaviviruses. WNV-specific clones proliferated strongly when stimulated with WNV but to a lesser extent when stimulated with MVE virus however, MVE specific clones proliferated strongly when stimulated with both WNV and MVE. Gajdosova *et al.* (1980, 1981) demonstrated cross-reactive flavivirus-immune cytotoxic spleen cells with flaviviruses from different subgroups.

In this report we demonstrate that secondary flavivirus-immune Tc cell populations generated against viruses from within one subgroup are cross-reactive both for target cell lysis and induction of the secondary response.

MATERIALS and METHODS.

Mice:

Mice were bred under pathogen free conditions at the Animal Breeding Establishment of the John Curtin School of Medical Research. Female mice of the strain CBA/H ($H\text{-}2^k$) were used between the ages of 6 and 16 weeks and were age matched in all experiments.

Virus:

West Nile, Sarafend strain (WNV), Kunjin (KUN MRM16 prototype strain), Murray Valley encephalitis virus (MVE 96961/53 prototype strain) (MVE) and Japanese B encephalitis (Nakayama strain) (JBE) were obtained from Dr I. D. Marshall, and grown in suckling mouse brains (Taylor and Marshall, 1975). Virus was recovered after sonication of a 10% w/v of brain homogenate in gelatin saline (pH 7.2, 0.5% gelatine in borate buffered CaMg saline) for 15 s using a Branson B12 Sonifier (Branson Sonic Power Co., Danbury, Conn., U.S.A.) at 50 W and centrifugation at 1500g for 20 min at 4^o C. Aliquots of supernatant were stored at -70^o C for future use as stock virus. Titres of virus were determined by serial dilutions and titrations on Vero cell monolayers as described by Taylor and Marshall. (1975).

Target Cells:

The C_3H (H-2^k) fibroblast cell line, L929, was grown as described in Gardner *et al.*, (1974).

Generation of Secondary Flavivirus-Immune Effector Cells In Vitro:

Secondary flavivius-immune Tc cells were generated using the method described in Kesson *et al.* (1988) Briefly, $10^{7.9}$ "responder" cells from mice primed with 10^6 p.f.u. of WNV, Kunjin, MVE or JBE were cultured *in vitro* for 5 days with $10^{7.3}$ non-immune syngeneic "stimulator" spleen cells which were infected with either the homologous or a heterologous virus and then irradiated with $10^{3.3}$ rads from a ^{60}Co source.

Cytotoxic Assay:

The method has been described in Kesson *et al.* (1988). The target cells were infected with an m.o.i of 10. The cells were left for 48 h at 37° C prior to assay. Data given are means of triplicates and standard errors of the means were usually less than 5%. Significance was determined by Student's t Test and P values of < 0.05 were considered significant.

Cold Target Competition.

L929 target cells (10^7 cells per ml) were infected with 25 p.f.u. per cell of WNV, MVE or left uninfected for 1 h at 37° C. After this the cells were cultured in 50ml Dulbecco's Modified Eagles Medium (Cat. No.430-1600, GIBCO, Chargrin Falls, Ohio, U.S.A.), supplemented with 5% FCS, 200µg/ml Streptomycin, 200 U/ml Penicillin G and 125µg/ml Neomycin Sulphate (DMEM) at a cell concentration of $10^{5.3}$ per ml, in a 50ml plastic centrifuge tube (Corning, New York). The tube was ~~gased~~ gassed with a mixture of 10% CO_2, 7% N_2 and 83% O_2 sealed and incubated for 48 h while being rotated at 20 revolution per minute to prevent cell adherence to the wall of the culture vessel. Following incubation the cells were then washed once in DMEM, and viable cell numbers determined using trypan

blue exclusion. $10^{6.5}$ cells from each of the three groups were labelled with ^{51}Cr for 1 h at 37° C, washed twice in DMEM and $10^{4.3}$ cells in 50µl added to each well of a 96-well tissue culture plate (Nunclon Roskilde, Denmark). Unlabelled cells from the three groups were used as cold targets and 50µl aliquots added at ratios 8, 4, 2 and 1 times the number of labelled targets. Secondary Tc cells ($10^{5.8}$) were then added in 100µl per well. The assay was run for 6 h.

RESULTS.

Cross-Reactivity of Secondary Flavivirus-Immune Tc Cells in Vitro.

Spleen cells from CBA/H mice primed 7 days earlier with a flavivirus were restimulated *in vitro* with syngeneic stimulator spleen cells infected with the same virus and tested on L929 targets, uninfected or infected with the homologous or heterologous virus. Previous studies showed that lysis by a population of secondary WNV-immune cells was inhibited when they were treated with anti-Thy and complement or anti-CD8 and complement; the cells thus had the phenotype of cytotoxic T (Tc) cells (Kesson *et al.*, 1988). As can be seen in table 1, secondary flavivirus-immune Tc cell poulations lysed all infected L929 target cells significantly more than uninfected cells. The lysis of the JBE-infected targets was generally lower than the lysis of the other infected targets. In contrast, WNV-infected targets were lysed more efficiently than all other infected targets, Kunjin- and MVE-infected target cells were intermediate. Presumably, this variation between the infected target cells reflected the fact that varying proportions of the target cells were successfully infected. Despite these limitations, it is apparent that there was substantial cross-reactivity between WNV and MVE and between WNV and Kunjin, but rather less cross-reactivity between Kunjin and MVE. Thus, cytotoxic activity of secondary Kunjin-immune Tc cells was 3-fold less on MVE infected targets than on Kunjin infected targets. Similarly, MVE-immune effector cells lysed MVE and WNV-infected targets more efficiently than Kunjin infected targets. Finally, JBE-immune Tc cells lysed

Kunjin-infected targets less than other infected targets, possibly indicating less cross-reactivity between JBE and Kunjin than between JBE and WNV or MVE.

Stimulation of Secondary Flavivirus-Immune Tc Cells with Homologous or Heterologous Virus.

WNV-primed CBA spleen cells were restimulated *in vitro* with syngeneic stimulator spleen cells infected with WNV, Kunjin, MVE, JBE or left uninfected. Cytotoxicity of these five populations of secondary flavivirus-immune Tc cells was then tested on uninfected and WNV-infected L929 target cells (Table 2). Lysis of infected targets over and above uninfected targets was highest in the homologous situation but was also detectable when stimulation was done by Kunjin, MVE or JBE-infected cells. Lysis of uninfected targets was highest when stimulation was done by uninfected spleen cells.

To further investigate if induction of secondary flavivirus-immune Tc cells is more efficient when homologous rather than heterologous virus is used to infect the stimulator cells a second experiment was performed using only WNV and MVE (Table 3). The lysis of infected targets was always greater than the lysis of uninfected targets but the latter was substantial, and was greater in the case of MVE than WNV-immune Tc cells. Little difference was observed when the flavivirus-primed spleen cells where boosted *in vitro* with stimulator cells infected with homologous or heterologous virus.

Cold Target Competition.

The results in Tables 1, 2 and 3 suggested that the secondary flavivirus-immune Tc cells were largely cross-reactive. Therefore, cold target competition was used to determine if any flavivirus -specific Tc cell subset was detectable. The rationale for a cold target competition experiment is thus: if competition is more efficient using cold targets which are infected with the same virus used for hot target infection than with cold targets infected with a heterologous virus, then the presence of virus-specific Tc cells would be demonstrated.

Therefore, WNV-immune Tc cells were assayed on ^{51}Cr-labelled L929 target cells which were uninfected or infected with WNV or MVE and unlabelled L929 competitors either uninfected or infected with WNV or MVE were added at various ratios to labelled targets. Figure 1 shows one such experiment. All three of the cold targets, WNV-, MVE-infected and uninfected, competed equally with the lysis of uninfected hot targets by the WNV-immune Tc cells. Lysis of WNV-infected targets by WNV-immune Tc cells was reduced equally by WNV-infected or MVE-infected cold competitor L929 cells but was not influenced by uninfected competitiors. Similarly, MVE- infected target cell lysis was reduced by both MVE- and WNV- infected but not by uninfected competitors.

DISCUSSION.

The secondary flavivirus-immune Tc cells generated *in vitro* against WNV, Kunjin, MVE and JBE viruses showed cross-reactivity at the level of target cell lysis when tested on a panel of flavivirus-infected targets infected with these four viruses. However, these results do not allow definitive interpretation of the cross-reactive pattern because the variation between infected targets may reflect variations in infection of target cells. Determination of the percentage of cells infected could be measured by immunofluoresence microscopy of fixed cells labelled with hyperimmune serum. While this technique does not demonstrate that the infected cells are presenting viral antigens on their surface associated with MHC molecules to form a determinant recognized by the Tc cells, it does provide presumptive evidence of the same. However, the low level of lysis of Kunjin-infected targets by MVE-immune Tc cells and MVE-infected targets by Kunjin-immune Tc cells may reflect a lesser degree of cross-reactivity. Furthermore, the lower lysis of Kunjin-infected targets compared with WNV-, MVE- and JBE-infected targets by JBE-immune Tc cells possibly also reflects a lesser degree of cross-reactivity. However, optimization of target cell infection and repeat experiments are required to confirm this.

The data in table 2 showed that when WNV-primed spleen cells were boosted *in vitro*

with either uninfected stimulator spleen cells or with stimulator spleen cells infected with Kunjin, MVE or JBE higher lysis of uninfected L929 target cells occurred than when boosting was done with homologous virus. That is, the detectable anti-viral activity (lysis of infected targets over and above uninfected targets) was higher in the homologous system. However, this pattern was not reproducible because in a cross-stimulation experiment between WNV and MVE, WNV-primed spleen cells were stimulated equally by stimulator cells infected with either MVE or WNV (table 3) and cross-reacted completely on MVE-infected target cells. These data confirmed that cross-reactivity occurs at the level of stimulation. Variation from one experiment to another probably reflects variations in infection of stimulator and target cells. This explanation is compatible with the importance of m.o.i. of L929 target cells presented in chapter 3.

Studies by Uren *et al.* (1987) using $CD4^+$ T cell clones demonstrated that $CD4^+$ T cell activity was cross-reactive between related flaviviruses from the same subgroup. They demonstrated that there was strong cross-reactivity between WNV and Kunjin. However, while 6 WNV- immune clones were stimulated by both WNV and MVE cross-reactivity was unidirectional i.e. 6 MVE-immune clones were stimulated by MVE but not by WNV.

In cold target competition experiments uninfected, WNV- and MVE- infected cold targets competed equally when secondary WNV-immune Tc cells lysed hot WNV- or MVE-infected targets. The competitor inhibition by WNV- or MVE-infected targets was similar. Thus, virus-specific Tc cells were not detected raising the possibility that only cross-reactive Tc cell populations were generated. DeMadrid and Porterfield (1974) demonstrated that WNV, Kunjin, MVE and JBE are all members of the same serological subgroup. We have not tested for cross-reactive Tc cells across different serological subgroups. However, Gajdosova *et al.* (1980, 1981) demonstrated cross-reactive flavivirus-immune cytotoxic spleen cells using flaviviruses from different subgroups. This pattern of cross-reactivity across subgroups is in contrast to influenza-immune Tc cells which comprise two distinct populations, one specific for the priming virus and a second cross-reactive subset (Braciale, 1977).

The high lysis of uninfected targets suggested the presence of two subsets of Tc cells within the secondary WNV-immune Tc cell population, those that lyse both uninfected and infected targets and those which lyse only infected targets (Kesson *et al.*, 1988). The results of the cold target experiments support this proposition. Lysis of the ^{51}Cr-labelled uninfected targets was inhibited to an equal exent by uninfected, WNV- and MVE-infected targets, while the lysis of the ^{51}Cr-labelled infected targets was inhibited to a greater extent by the infected than the uninfected targets. Thus, two subsets of Tc cells are present in the secondary WNV-immune Tc cell populations. One subset lysed uninfected cells expressing self antigens and a second subset lysed infected cells expressing flavivirus-induced antigenic changes but does not discriminate between WNV and MVE.

King and Kesson (1988) demonstrated that these four flaviviruses, WNV, Kunjin, MVE and JBE induced an increase in the level of class I MHC antigen expression on the surface of cells which they infect. Furthermore, if increasing the level of class I MHC antigen expression with γ-rIFN treatment of cells leads to increased susceptibility to lysis by secondary WNV-immune Tc cells as suggested in chapter 5 then it may be that the cross-reactivity demonstrated is due to increased susceptibility to lysis by flavivirus-immune Tc cells when class I MHC antigen expression is increased by flavivirus-infection of target cells and that viral antigen expression is not a requirement. Further studies using the limit dilution approach and splitting of the T cell clones are required to absolutely resolve the specificities of the Tc cell subsets at the clonal level and in particular, the question of clones specific for individual viruses in the panel of the four studied here.

A second experimental approach could be used to determine whether the cross-reactivity demonstrated with flavivirus-immune Tc cells depends on the level of class I MHC antigen expression or if it depends upon viral antigen expression. Using a vaccinia virus construct with flavivirus genes inserted into the genome it is probable that flavivirus antigens would be expressed on the cell surface without a concomitant increase in the level of class I MHC antigen expression. Vaccinia virus is known to inhibit host cell DNA,

RNA and protein synthesis (Moss, 1968). Therefore, if the above hypothesis is correct and cross-reactivity is demonstrated it is presumptive evidence of a population of flavivirus-immune Tc cells which recognize viral antigens.

A. Hill *et al.* (1989 personal communication) has demonstrated that Kunjin-immune and WNV-immune Tc cells both lyse targets infected with recombinant vaccinia virus containing cDNA encoding Kunjin polypeptides. Thus, cross-reactivity dependent upon viral antigen expression has been demonstrated.

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TABLE 1.

Cross-Reactive Secondary Flavivirus-Immune Tc Cells.*

		% Specific Lysis of L929 Target Cells.†				
Secondary Tc Cells.	E:T+	Uninfected	WNV	Kunjin	MVE	JBE
WNV-immune	30	11.2§	68.5	58.0	67.7	30.2
	10	13.7	62.9	54.9	49.2	25.1
	3	4.9	55.5	27.3	24.2	11.1
Kunjin-immune	30	15.2	58.9	56.1	42.8	31.0
	10	13.6	46.8	48.6	33.1	19.7
	3	2.9	42.0	25.3	9.7	5.5
MVE-immune	30	15.6	63.4	48.4	62.6	36.8
	10	9.0	50.8	23.0	51.3	25.4
	3	9.4	36.9	10.9	28.0	5.6
JBE-immune	30	13.1	56.4	32.5	46.4	42.3
	10	14.4	38.0	24.7	28.0	29.5
	3	5.7	37.2	8.1	18.0	9.7

*) Female mice greater than 6 weeks old were primed with 10^6 p.f.u. of WNV, Kunjin, MVE or JBE i.v. and their spleen cells used as responder cells 7 days after priming.

†) Infected with 10 p.f.u. / target of WNV, Kunjin, MVE, JBE or left uninfected for 48 h prior to assay.

+) Effector to target cell ratio.

§) Means of triplicates given with standard errors of the means less than 6.2%. The spontaneous release was 17.2%, 34.4%, 24.7%, 29.3% and 22.8% for uninfected, WNV-, Kunjin-, MVE- and JBE- infected targets respectively.

TABLE 2.

Cross-Stimulation of Flavivrus-immune Tc cells.*

CBA/H WNV-Immune Tc cells. Secondary Stimulatio	E:T+	% Specific Lysis of L929 Target Cells.† Uninfected.	WNV.
WNV	30	28.1§	60.2
	10	17.8	31.3
	3	5.4	11.7
Kunjin	30	38.7	58.9
	10	24.8	38.6
	3	8.8	16.3
MVE	30	42.3	53.0
	10	23.8	37.5
	3	10.1	13.3
JBE	30	46.2	56.3
	10	23.8	35.5
	3	6.2	13.3
NIL	30	60.6	63.2
	10	34.3	40.1
	3	9.8	12.2

*) Female mice greater than 6 weeks of age were primed with 10^6 p.f.u. WNV or MVE i.v. 7 days prior to secondary stimulation of their spleen cells. Secondary stimulation *in vitro* was as described in Materials and Methods.

†) Infected with 10 p.f.u. / target of WNV, MVE or left uninfected for 48 h prior to assay.

+) Effector to target cell ratio.

§) Means of triplicates given with standard errors of the means less than 6.4%. The spontaneous release ranged from 10.4% to 29.9%.

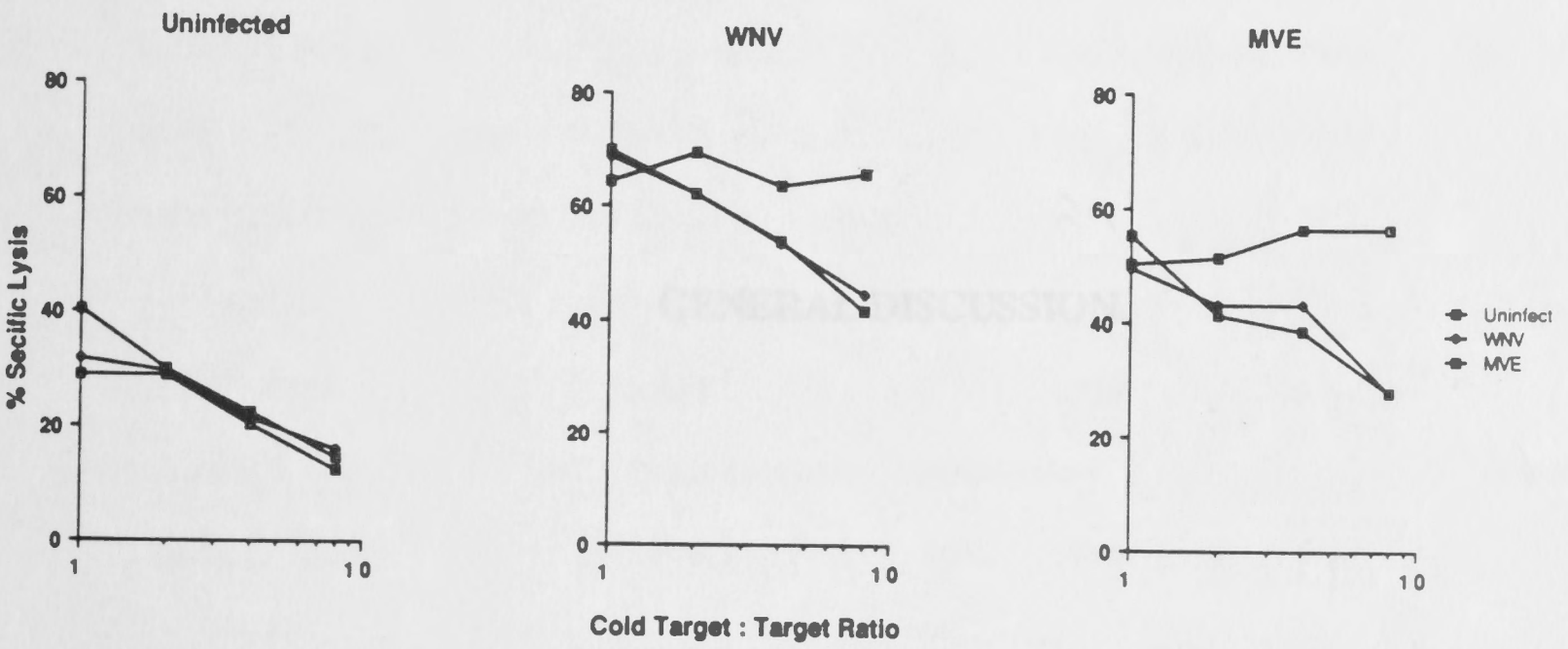
TABLE 3.

Cross-Stimulation of Flavivrus-immune Tc cells.*

CBA/H Tc cells.			% Specific Lysis of L929 Target Cells.†		
Primary Stimulation.	Secondary Stimulation.	E:T+	Uninfected.	WNV.	MVE.
WNV	WNV	30	49.6§	68.2	73.8
		10	34.0	70.1	57.7
		3	24.0	32.2	34.1
WNV	MVE	30	43.4	64.9	71.0
		10	28.0	54.0	58.4
		3	17.9	32.8	38.2
MVE	MVE	30	60.6	68.1	74.3
		10	50.6	67.0	74.9
		3	29.1	38.8	55.3
MVE	WNV	30	60.6	64.3	75.2
		10	49.4	64.1	65.8
		3	29.9	39.3	50.9

Footnotes as for Table 2.

FIGURE 1.



Similar inhibition of Tc cell mediated cytotoxicity by cold ~~competitors~~ competitors infected with either homologous or heterologous flavivirus. Secondary WNV-immune Tc cells were tested for cytotoxicity on ^{51}Cr-labelled L929 targets infected with either WNV, MVE or left uninfected (Unin) in the presence of cold competitors as shown in the figure. The ratio of Tc cells to ^{51}Cr-labelled L929 targets was 30:1. Standard error of the mean was always less than 4.8% and is omitted for clarity. Control specific lysis without cold competitors was 45.8%, 72.5% and 59.1% for uninfected WNV- and MVE-infected targets respectively. The spontaneous release ranged from 23.3% to 26.6%. For details see Materials and Methods.

CHAPTER 7.

GENERAL DISCUSSION.

Flavivirus-Immune Tc Cells.

Cytotoxic T (Tc) cell responses have been studied for many virus infections however, little work has been done to date to study the Tc cell response to flavivirus infections. Both Sheets *et al.* (1979) and ~~Gajosova~~ Gadjosova *et al.* (1981) reported flavivirus-immune Tc cell population in the spleens of mice which were immunized with flaviviruses. The work presented in this thesis describes the method required for optimal generation and detection of cytotoxic flavivirus-immune spleen cells. This work was extended by the *in vitro* generation of a secondary flavivirus-immune Tc cell population. These secondary flavivirus-immune Tc cells are more potent than the cytotoxic flavivirus-immune spleen cells thus allowing the investigation of MHC-restriction and *Ir* gene effects. Furthermore, careful investigation was undertaken of the interactions between T cell subsets necessary for the generation of secondary flavivirus-immune Tc cells.

Flavivirus-immune Tc cells express the phenotype common to other Tc cells. Primary WNV-immune Tc cell activity resided in the $CD8^+$ spleen cell population of mice immunized with WNV 4 to 7 days previously. Furthermore, a secondary WNV-immune Tc cell population could be generated *in vitro* from these responder spleen cells which were depleted of $CD8^+$ cells by antibody and complement mediated lysis. However, there was a 3-fold reduction in cytotoxic activity in the resultant Tc cell population. The lytic activity of a secondary WNV-immune Tc cell population resides in a $CD8^+$ population. Therefore, a $CD8^+$ WNV-immune Tc cell population could be generated *in vitro* from $CD8^-$ WNV-immune responder spleen cells. Russell *et al.* (1987) demonstrated that the cell surface expression of CD8 increases up to 3-fold on activated allo-immune Tc cell clones as determined by flow cytometry. Furthermore, studies using anti-CD8 antibody blocking of lytic function indicate that the increase in CD8 expression on the activated T cells is reflected by an increase in the lytic potential of the cells by an amount equal to the relative increase in its expression. No concomitant increase in T cell receptor expression of the cells was detected. Thus, it appears that the majority of responding flavivirus-

immune spleen Tc cells are $CD8^-$ as defined by complement mediated lysis and give rise to flavivirus-immune Tc cells which express higher levels of CD8 as they become activated. However, detection of CD8 antigen by anti-CD8 antibody and complement may not be as sensitive as detection by FACS analysis. It is probable that the level of expression of CD8 antigen on a population of Tc cells ranges from low to high and a significant proportion of responder cells express CD8 antigen at a level below the threshold of detection by anti-CD8 antibody and complement. FACS analysis may detect a subset of low CD8 antigen expressing responder cells which escape lysis by anti-CD8 antibody and complement but which respond to stimulation *in vitro* and generate a Tc cell population expressing higher levels of CD8.

A $CD4^+$ T cell subset was necessary for the *in vitro* generation of secondary flavivirus-immune Tc cells. The requirement for the $CD4^+$ subset was replaced by the addition of exogenous IL-2. Thus the requirement for a $CD4^+$ helper T cell subset was demonstrated. Previous studies have challenged the prevailing view, that help provided by $CD4^+$ cells is necessary for the induction of most functions of $CD8^+$ cells. Recent evidence suggests that under curtain circumstances Tc cells can differentiate without the requirement for exogenous help. Widmer and Bach (1981); vonBoehmer *et al.* (1984) and Sprent and Schaefer (1985) have demonstrated the existence of Tc cells which proliferate in response to antigen *in vitro* in the absence of exogenous IL-2 secreted from $CD8^-$ cells. Guimezanes and Schmitt-Verhulst (1985) showed that IL-2 production by T cells stimulated with class I MHC antigens was totally inhibited by anti-CD8 monoclonal antibody. Sinickas *et al.* (1985) demonstrated using murine cytomegalovirus-immune lymph node cells that both lymphokine release (IL-3) and cytotoxicity were dependent on $CD8^+$ cells. These studies all demonstrate that help can be provided by a $CD8^+$ population when the cells are responding to class I allo-antigens or when the response to the foreign antigen is class I restricted. Recently, a study by Buller *et al.* (1987) demonstrated *in vivo* induction of an ectromelia-immune Tc cell population in the spleens of mice which were

made deficient in $CD4^+$ T cells by innoculating them with monoclonal anti-CD4 antibody. Furthermore, these mice deleted of the $CD4^+$ cells showed significantly less morbidity and mortality when injected with ectromelia virus than their nude (nu/nu), $CD4^-$, $CD8^-$ littermates and thus the $CD4^-$, $CD8^+$ mice can mount an immune response *in vivo* and clear the virus.

Data presented in chapter 3 suggested that help could be provided by a $CD4^-$ T cell population in the generation of secondary flavivirus-immune Tc cells when the $CD8^+$ cells were cultured *in vitro* at an optimal cell concentration, but the effects of a residual contaminating population of $CD4^+$ T cells could not be absolutely excluded. Further studies could be undertaken to confirm the release of IL-2 from $CD8^+$ flavivirus-immune Tc cells by measuring proliferation of an IL-2 dependent cell line. If IL-2 release was demonstrated, further investigation of flavivirus-immune Tc cell clones could be undertaken to determine if $CD8^+$ Tc cells are capable of performing both cytolysis and lymphokine secretion. Thus, a $CD4^+$ T cell population is not an absolute requirement for the generation of flavivirus-immune Tc cell populations *in vitro* and help may be provided by $CD4^-$ T cells as was demonstrated in chapter 3. Further studies analogous to those performed by Buller *et al.* (1987) could be undertaken in the murine flavivirus model by inoculating the nice with monoclonal anti-CD4 antibody to determine the *in vivo* requirement for $CD4^+$ T cells in the generation of flavivirus-immune $CD8^+$ Tc cells.

Modulation of MHC Antigens.

The major histocompatibility antigens play a crucial role in the stimulation of the immune response by T cells (reviewed in Zinkernagel and Doherty, 1979). A T cell response to a foreign antigen is stimulated by a determinant which depends upon both the foreign antigen and an appropriate MHC antigen. In the mouse, products from the *H-2K* and *H-2D* regions (class I) are involved in cytotoxic T cell responses (Doherty *et al.*,1976) and *H-2I* region products (class II) are involved in helper (Erb and Feldman,1975) and

delayed hypersensitivity responses (Miller *et al.*,1976).

Studies have shown that virus-immune, class I MHC-restricted Tc cells are capable of clearing established viral infections *in vivo* (Kees and Blanden, 1976; Yap *et al.*, 1978; Oldstone *et al.*, 1986). This immune response is important to control the spread of these infectious organisms and also for their eventual eradication from the host. The mechanism(s) by which T cells mediate viral clearance are poorly understood and has been a matter of much research for many years. However it is known that class I MHC antigens play a central role in the recognition of virus infected cells by virus-immune Tc cells. Therefore, modulation of class I MHC antigen expression by flaviviruses as described in this thesis is a phenomenon with significant immunological implications.

Other virus infections have previously been demonstrated to modulate the expression of MHC antigens. Murine hepatitis virus (Suzumura *et al.*,1986), Epstein Barr virus (McCune *et al.*,1975) and the retrovirus Moloney murine leukaemia virus (Flyer *et al.*, 1985) all induce an increase in the level of MHC antigen expression. On the other hand, ectromelia (Gardner *et al.*, 1975), adenovirus (Eager *et al.*, 1985; Burgert *et al.*, 1987), Herpes Simplex virus type 2 (Jennings *et al.*, 1985) and the retrovirus Moloney sarcoma virus (Flyer *et al.*, 1985) down-regulate the expression of MHC antigens on the cells they infect.

Flavivirus induced increase in MHC antigen expression may be of advantage to the host. Tc cells lyse infected target cells and this arm of the immune response is thought to be a mechanism which helps control the spread of infectious organisms within a host by lysing infected cells before progeny virus can be assembled and released. The susceptibility to lysis of flavivirus infected cells by flavivirus-immune Tc cells correlates closely with the level of MHC antigen expression (chapter 5). The first detectable increase in MHC expression occurred 8 h after the cells were infected with the flavivirus (data not given). Studies of the growth kinetics of flaviviruses in Vero cells (Trent and Naeve, 1980) have demonstrated a latent phase which may last up to 12 hours after initial infection and

during this latent phase there is little production of infectious virus. Virions are then assembled and released reaching a maximum titre about 24 hours after infection. Thus, the early increase in the level of MHC antigen expression may occur before progeny virions are formed and the ~~increaed~~ increased susceptibility to lysis by flavivirus-immune Tc cells could occur therefore before the progeny virions are assembled and released , thus limiting the spread of infection within the host.

T Cell Tolerance.

A T cell must come into contact with an appropriate antigen presenting cell or target cell for it to receive an antigen specific signal. Furthermore, the concentration of antigen on the antigen presenting cell or target cell influences the extent of activation of the responding T lymphocyte (O'Neill and Blanden, 1979; Goldstein and Mescher, 1987). This indicates that there is a requirement for multivalent interactions between the T cell receptor (TCR) and the respective ligands on the cell surface (Hermann and Mescher, 1986). A T cell responds to a signal received via the TCR in two ways. The cell becomes "activated" and/or it will secrete lymphokines e.g. IL-2. For a T cell to receive an antigen specific signal, the TCR must interact with an appropriate ligand. When a sufficient number of receptor-ligand interactions have occurred a signal will be generated, the T cell will be triggered and a response as described above will ensue. Blanden *et al.* (1987) postulated that the number of receptor-ligand interactions or signal generating complexes formed in any cell-cell interaction depends upon both the affinity between the receptor and ligand as well as the number of receptors and ligands available for interaction on the respective cell surfaces.

A polyclonal T cell population contains T cells expressing TCRs with a range of affinities for a given ligand. The simplest model for triggering of a T cell proposes a direct relationship between the total signal strength generated and the number of receptor-ligand interactions. The number of receptor-ligand interactions will depend upon the number of

ligands and receptors available on the interacting cell surfaces and also on the affinity of interaction between the receptor and the ligand. Thus, each T cell clone will have a range of affinities for differing ligands. A T cell is triggered when it receives a signal above its "threshold" level and this results in a functional action by the responding T cell (Blanden *et al.*, 1987).

These two requirements for T cell activation namely the affinity of interaction between the receptor and the ligand as well as the number of receptor-ligand interactions are also mandatory when triggering self-reactive T cell clones. Imposition of self-tolerance in the mature T cell pool of an individual should theoretically result in the deletion or suppression of T cells which exceed the activation threshold when exposed to self-antigens. Self-reactive T cell clones are deleted or suppressed from the repertoire of the mature T cell population either during T cell ontogeny in the thymus or in secondary lymphoid tissue. The resulting population of mature T cells would have few self-reactive T cell clones, with the high affinity self-reactive clones deleted and any residual self-reactive clones having low affinity receptors for self-antigens. However, by increasing the level of expression of self antigens and therefore increasing the number of possible receptor-ligand interactions some low affinity self-reactive clones may be triggered.

The level of class I MHC antigen expression is critical to the interaction between Tc cell and target cell. Furthermore, it is critical for the ~~survivial~~ survival of the organism that Tc cell activity is not directed towards self epitopes. Therefore, an increase in the level of class I MHC antigen expression due to flavivirus infection may increase the number of receptor-ligand interactions sufficiently to increase the signal threshold and trigger low affinity self-reactive T cell clones which would have otherwise not have been triggered. Blanden *et al.* (1987) postulated that thymic dendritic cells which express very high levels of both class I and class II MHC antigens may be important in setting the limits for signal threshold above which self-reactive T cells are triggered. They argued that evidence suggests that thymic dendritics are involved in the imposition of self-tolerance to non MHC

antigens (vonBoehmer and Hafen, 1986) as they are accessible to circulating antigens (Kyewski *et al.*, 1986). These data suggest that thymic dendritics could be involved in deleting self-reactive T cell clones during ontogeny of T cells resulting in a mature T cell repetoire with a high signal threshold for triggering self-reactive clones. Blanden *et al.* (1987) postulated that increasing the antigen density on the cell surface would trigger some low affinity self-reactive T cell clones. Thus "T cell tolerance would be a quantitive phenomemon determined in part by self ligand concentrations on cells concerned with self tolerance imposition, and not solely related to affinity between receptor and antigen." This hypothesis is further supported by the finding that increasing the level of class I MHC antigen expression by γ–rIFN-treatment of uninfected MEF resulted in an increased susceptibility to lysis by secondary WNV-immune Tc cells and thus it is postulated that low affinity self-reactive T clones generated in the secondary WNV-immune Tc cell population were triggered because of the increased availability of ligands for receptor binding. This latter observation is under dispute as A. Mullbacher (chapter 5) was unable to demonstrate increased lysis of γ–rIFN-treated targets compared with untreated targets by secondary WNV-immune Tc cells. However the, lysis of untreated targets was blocked to a greater extent by anti-CD8 antibody than lysis of γ–rIFN treated targets suggesting that the increased class I MHC antigen concentration on the γ–rIFN treated targets resulted in a Tc cell / target cell interaction of higher avidity than when the Tc cells interacted with untreated targets (Chapter 5).

These data support the hypothesis that tolerance to self antigens is at least partly a quantitative phenomenon determined by receptor-ligand interactions. Further studies are in progress at present to determine if the increased level of class I MHC antigen expression by WNV-infection is greater than that expressed on thymic dendritics. If this is the case then WNV-infection may result in a level of class I MHC antigen expression which can activate self-reactive T cell clones and provide a mechanism for virus-induced autoimmune phenomenon.

Lysis of Uninfected Target Cells.

If WNV-infection causes an increase in the level of class I and class II MHC antigen expression on antigen presenting cells, and there is some evidence that it does (Lui *et al.*, 1989), then the modulations in the level of expression of MHC antigens may have a significant effect on the avidity of secondary WNV-immune Tc cell populations for self MHC antigens. Using synthetic beads coated with membrane (~~psuedocytes~~ pseudocytes) Goldstein and Mescher (1987) demonstrated that the antigen density on the surface of the psuedocytes had a significant effect on precursor Tc cell triggering. Precursor Tc cells responding to psuedocytes with a low antigen density resulted in effector Tc cells which were of higher affinity, as determined by CD8 blocking of Tc cells, than when Tc cells were generated from precursors responding to psuedocytes with high antigen density. This finding could go part way to explaining the high lysis of uninfected targets seen with secondary WNV-immune Tc cells. Theoretically, WNV-infection of antigen presenting cells could increase the level of MHC antigen expression and these antigen presenting cells with increased levels of MHC antigen could stimulate low-affinity self-reactive clones. The WNV-immune Tc cells generated could then theoretically contain a population of T cells which express TCRs with low affinity for self MHC antigens. These WNV-immune Tc cells with low affinity for MHC antigens could then cross-react and lyse uninfected targets expressing MHC antigens without viral antigens. Thus, WNV-infection of antigen presenting cells leading to high levels of MHC antigen expression may generate a WNV-immune Tc cell population which contains a population of Tc cells with low affinity for self MHC antigens.

Komatsu *et al.* (1978) demonstrated self reactivity with influenza-immune Tc cell clones. Using limiting dilution cultures they were able to split the T cell clones and assay for cytolysis on both uninfected and influenza-infected L929 targets. Clones were regarded as L929-specific if they lysed both infected and uninfected targets or as virus-specific if they lysed only infected targets. The data show that approximately 75% of the clones

isolated on day 2 lysed uninfected targets while by day 5 of culture most clones were influenza-specific. They argued that virus-infected cells are recognized by different clones with a range of affinities. Therefore, the subset of clones which lysed the uninfected targets represents those with low avidity for influenza-infected targets but with sufficient avidity to recognize MHC antigens. These findings are consistent with the data presented in chapter 3 but it is difficult to explain why self-reactive clones are generated early after antigenic stimulation, while virus-immune clones require 5 days to reach maximal numbers. Rammansee *et al.* (1984) postulated that veto cells along with suppressor cells and anti-idiotypic T cells may form part of a mechanism which is active *in vivo* in removing self-reactive clones. It is therefore possible that veto cells are somehow involved in removing self-reactive clones generated *in vitro* . It may be that the cytotoxic activity generated by 5 days *in vitro* culture is directed against a specific antigen while the self-reactive clones which are present early in the culture period are deleted by veto cell activity.

A similar phenomenon was reported by Andrew (personal communication) using influenza specific Tc clones. She demonstrated that influenza-immune Tc cell clones transiently underwent a profound change in functional specificity after antigenic stimulation. This change was associated with specific activation of the clones and was manifest as lysis of uninfected targets *in vitro* . The level of lysis of influenza-infected targets did not change when the clones cycled from their quiescent to active state. However, the lysis of uninfected targets was greatest early after antigenic stimulation and declined ~~with~~ with increasing time after activation. The lysis of influenza-infected targets must be the resultant lysis of clones directed at determinants shared by both ~~the~~ the infected and uninfected targets. Thus, for lytic activity of the influenza-infected targets to remain constant while the lytic activity of the uninfected targets declines needs to be explained. Clearly, the specificity of the TCR of a clone cannot change and the affinity of interaction between the TCR and the respective ligand will therefore be constant. However, a

mechanism for altering the lytic activity of a Tc clone would be to alter the overall avidity between the clone and the target. This alteration in avidity could be achieved in one of two ways.

Firstly, decreasing the number of TCR expressed on the cell surface would result in a decreased avidity of interaction with the target cell. Meuer *et al.* (1984) demonstrated that T cell activation was associated with a decrease in the number of TCR expressed on the cell surface and a concomitant rise in the expression of IL-2 receptors. Thus, for a T cell clone with decreasd expression of TCR after activation to receive a signal above threshold and be triggered to lyse a target cell requires a high affinity interaction between the receptor and the ligand with a resultant high level of specificity for the antigenic determinant (Blanden *et al.*, 1987). Thus, if influenza-immune Tc clones express lower levels of their TCR after activation a greater affinity for the influenza antigenic determinant expressed on the influenza-infected target is required for triggering of cytolysis by the clone.

A second method by which an influenza-immune Tc clone could increase the avidity of interaction with the target cell is to increase the number of molecules responsible for binding activity. CD8 molecules bind to class I MHC antigens (Swain, 1983) and aparently amplify the signal from the TCR binding to the same MHC molecule with or without expression of a foreign antigen (Governman *et al.*, 1986). Increased CD8 expression is associated with activation of T cell clones and the increase in CD8 expression on activated cells has been demonstrated to approximate the increase in lytic potential of the cell (Russell *et al.*, 1986).

Self-reactive Tc cells were demonstrated in lymphocytic choriomeningitis virus (LCM) infected mice (Pfizenmaier *et al.*,1975). The spleen cells of mice infected with LCM showed high lysis of uninfected targets with maximal activity peaking 4-6 days after immunization. This occurred earlier than the peak response for LCM-infected targets which was maximal on days 7-8. At this time there was a limited amount of residual lytic activity against self targets.

In contrast, WNV-immune spleen cells showed consistently low levels of lysis of uninfected targets (Chapter 2). However, WNV-immune spleen cells boosted *in vitro* for 5 days with WNV-infected stimulator cells exhibited a high level of lysis of uninfected targets when the priming interval was less than 4 days (Chapter 3). These data, along with the observation that a $CD8^+$ Tc cell population can be generated *in vitro* from a $CD8^-$ responder population support the hypothesis that the avidity of self-reactive Tc clones is enhanced during activation *in vitro* by the generation of increased numbers of CD8 molecules (Russell *et al.*, 1986). However, further studies using limiting dilution analysis and splitting of flavivirus-immune T cell clones is required to determine if the same clone is responsible for lysis of infected and uninfected targets. Once flavivirus-immune Tc clones are established, FACS analysis of the surface expression of the TCR and CD8 could be studies along with functional assays to determine alterations in avidity of interaction of the Tc cell with its target cell.

It seems unlikely that the lytic activity against uninfected self targets by secondary WNV-immune Tc cell populations (anti-self) is mediated by natural killer (NK) cells. NK cells spontaneously lyse syngeneic and allogeneic target cells in a manner unrestricted by the MHC and the level of expression of MHC antigens has a significant effect on the target cell lysis by NK-cells. Target cells exhibiting lower levels of MHC antigens are reported to be more susceptible to lysis by NK-cells than when they are induced to express higher levels (Harel-Bellan *et al.*,1986). Lui and Mullbacher (1988) were unable to demonstrate NK cell lysis of WNV-infected astrocytes and provided evidence which argued against the absolute concentration of MHC antigen on the cell surface being a factor in determining the targets susceptibility to lysis by NK cells. Furthermore, the "anti-self" lysis is inhibited when the effector population is treated with anti-Thy or anti-Lyt 2 and complement (chapter 3) and thus must be mediated by Tc cells.

Flavivirus Infection of the CNS.

The central nervous system (CNS) of mammals is a special environment with regard to immunological responses. Early experiments demonstrated that allogeneic skin grafts were not rejected from the brains of rabbits unless they were presensitized (Medawar, 1948). The level of MHC antigen expression in the normal mammalian brain is low and there is very little lymphocyte traffic. These factors along with the blood-brain barrier partially shield the brain from immunological responses. Because of this relative isolation of the CNS from immune responses, the CNS is described as being an "immunologically privileged site". The evolutionary reason for this immunologically privileged situation of the CNS is not known, however, most immune reactions involve a degree of tissue necrosis. It is therefore probably advantageous if such reactions are minimized in the CNS as the regenerative capacity of neurones is very limited.

However, immune responses can develop in the brain as evidenced by:

1) the production of immunoglobulins in the cerebrospinal fluid of patients with multiple sclerosis (Tourtelotte and Ma, 1978),

2) the lymphocyte infiltrations during viral encephalitis (Zinkernagel and Doherty, 1973),

3) prolonged ~~survivial~~ survival of mice given immunosuppressive agents in certain syndromes involving CNS immunopathology (Zinkernagel and Doherty, 1973) and

4) the transfer of experimental allergic encephalitis by myelin basic protein-immune cells (reviewed in Werekle and Fierz, 1985).

It has been demonstrated under appropriate circumstances that astrocytes, oligodendrocytes, microglial cells and endothelial cells can all be induced to express MHC molecules and to present antigen (Wong *et al.*, 1984; Fierz *et al.*, 1985; Traugott and Raine, 1985). Fierz and Fontana (1986) postulated that within the CNS, astrocytes are effective antigen presenting cells owing to their ubiquity and ability to phagocytose and process antigens. The CNS is both anatomically and physiologically separated from other

regions of the body. It seems unlikely that viruses can reach the CNS without first being accessible to the immune system, stimulating an immune-response and generating virus-immune T cells. Such virus-immune T cells entering the CNS could initiate an immune response provided the necessary MHC and viral antigens are expressed on cell surfaces. MHC antigen expression on CNS cells could be enhanced either by lymphokines secreted by the activated T cells notably γ–interferon and/or directly by virus infection. In the case of flavivirus infection, astrocytes may play a significant role in the immune response aimed at clearing the infection. If under normal circumstances astrocytes had high constitutive levels of class II MHC antigen expression there may be a risk of inapproapriate presentation of self antigens e.g. myelin basic protein. This may have special relevance to the development of immune tolerance to brain antigens as the normally low level of MHC expression in the CNS combined with the low level of lymphocyte traffic imposed by the blood-brain barrier may mean that lymphocytes which could otherwise respond to brain antigens are not normally triggered. Flaviviruses have been demonstrated to infect murine astrocytes both *in vivo* and *in vitro* , resulting in increased levels of class I and class II MHC antigen expression (Lui *et al*., 1989). Thus as a consequence of flavivirus encephalitis MHC antigen expression on astrocytes may be enhanced sufficiently to allow for an immune ~~respons~~response to other antigens e.g. myelin basic protein and thus provide a mechanism for an immunopathological response in the CNS and potentiate tissue damage. Studies of mortality in mice infected intracerebrally with a lethal dose of a flavivirus compared with the mortality in a matched group of immunosuppressed mice similarly infected may help resolve this issue. If the immunosuppressed mice survived for a longer period it would suggest an immunopathological ~~componant~~component occurs during flavivirus encephalitis. Furthermore, T cells from mice with flavivirus encephalitis adoptively transferred into naive animals may induce an encephalitic syndrome.

Evidence to support this hypothesis is found in a report by Massa *et al*. (1986) who demonstrated that murine hepatitis virus particles can induce Ia antigen expression on

astrocytes. Furthermore, lymphocytes from rats infected with murine hepatitis virus, restimulated *in vitro* with myelin basic protein and adoptively transferred into naive animals leads to lesions resembling experimental allergic encephalomyelitis in the recipient animals. Thus, viral infection of the CNS is capable of initiating an autoimmune response leading to chronic demyelinating disease (Watanabe *et al.*, 1983).

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