THE UNIVERSITY OF MANITOBA

THE IN VIVO ACTIVATION OF ANTI-SUPPRESSOR PATHWAY SIX HOURS AFTER ALLOGENEIC STIMULATION OF MICE, WHICH FACILITATES THE GENERATION OF CYTOTOXIC-T-LYMPHOCYTES

by

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TO MY WIFE

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ABSTRACT

Six hours after allogeneic stimulation of C57Bl/6J ($H\text{-}2^b$) with P815 ($H\text{-}2^d$) mastocytoma cells or DBA/2 SC ($H\text{-}2^d$), the serum of the mice was found to contain Ig-Ag complexes. Allogeneic stimulation with D-region coded antigens using B10.AKM and B10.BR mice also resulted in the formation of this complex. Either Lyt.1^+ or Lyt.2^+ cells are necessary for the formation of the complex which is cytophilic for a subpopulation of T cells. The complex was found to specifically enhance the CTL response upon i.v. injection into the animals two hours before challenging with a low dose of the stimulating antigen. CTL was significantly augmented in the control groups, but not in the 6HS group if the assay was carried out after a 24 hour in vitro incubation (considered to eliminate suppressor cells). Treatment of mice with low doses of cyclophosphamide (CY) or with anti-I-J antisera enhanced the CTL response in normal mice as shown by others. However, 6HS did not produce further enhancement of the CTL response in CY or anti-I-J treated mice. Since both CY and anti-I-J treatments are considered to enhance the immune response by interfering with the T cell suppressor pathway, the question arises whether the enhancement observed with 6HS is also due to a similar mechanism. NWC-NAC cells injected in CY treated mice abolished the enhancement of CTL but had no effect on the 6HS induced enhancement.

Suppressor cells which were generated by high tolerogen dose (20×10^6 HT-TU) were tested by their ability to block CTL response in vivo upon transfer to normal C57Bl/6J mice. The 6HS blocked both the induction and the expression of Ts cells. However, with low tolerogen dose (7.5×10^6 HT-TU), 6HS did not block the induction of Ts cells although it still inhibited their function. The data suggest that the 6HS acts through an effector T cell which is Lyt.1^+. These 6HS activated T cells were called anti-suppressor (As) cells. In conclusion, the serum of mice collected 6 hrs after allogeneic stimulation contains an Ig-Ag

complex which acts as an immunoregulatory factor in a new pathway called an anti-suppressor pathway.

LIST OF ABBREVIATIONS

Ab	Antibody
AEF	Allogeneic Effect Factor
Ag	Antigen
Apc	Antigen presenting cells
As	Antisuppressors
B	Bone marrow - derived lymphocytes
B6	C57B1/6J mice
BSA	Bovine Serum Albumin
C	Complement
CDC	Complement-Dependent-Cytotoxicity
CGG	Chicken Gamma Globulin
Con-A	Concanavalin-A
^{51}Cr	Chromium 51
CS	Contact Sensitivity
Cs	Contrasuppressors
CTL	Cytotoxic-T-Lymphocytes
CTL-p	" " -precursors
CY	Cyclophosphamide
DC	Dendritic Cells
DPBS	Dulbecco's Phosphate Buffer Saline
DTH	Delayed Type Hypersensitivity
FCA	Freund's Complete Adjuvant
FcR	Receptor for the Fc fragment of Ig
FCS	Fetal Calf Serum
GvH	Graft versus Host
HBSS	Hank's Balanced Salt Solution
HT-TU	Heat Treated-Tumor cells (P815)
Ia antigen	Membrane bound antigen coded by I-A and I-E subregions of the MHC
IACF	Immunoglobulin and Antigen Complexing Factor
Ig	Immunoglobulin
id	idiotype
I-J antigen	Membrane bound antigen expressed mainly on the surface of Ts cells and coded by the I-J subregion of the MHC
IL-1	Interleukin-1
IL-2	Interleukin-2
i.p.	intraperitoneal
Ir	Immune response genes
ISC	Immune Spleen Cells
i.v.	intravenous
Lyt	phenotypic markers of T cells
Lyt.1$^+$ cells	T cells that carry the determinant of the Ly-1 locus of chromosome 19
Lyt.2,3(2/3)$^+$ cells	T cells that carry the determinant of the Ly-2,3 (2/3) locus of chromosome 6
MAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MLC	Mixed Lymphocyte Culture
MLR	Mixed Lymphocyte Reaction
Mls	Minor histocompatibility locus

m. wt.	Molecular weight
NMS	Normal Mouse Serum
NRG	Normal Rabbit Ig
NSC	Normal Spleen Cells
NWC-NAC	Nylon Wool Column - Non Adherent Cells
PBS	Phosphate Buffer Saline
PFC	Plaque Forming Cells
P815	Methylcholantherene-induced mastocytoma in DBA/2 mice
RICA	Reverse Immune Cytoadherence
SIg	Surface Immunoglobulin
SRBC	Sheep Red Blood Cells
SAC	Splenic Adherent Cells
s.c.	subcutaneous
SC	Spleen Cells
6HS	Six hour serum
T	Thumus-derived lymphocytes
TNP	Trinitrophenyl group
Ts	T-suppressors
Ts-P	T-suppressor -precursors
VH	Variable region of Ig or Ab heavy chain

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INTRODUCTION

Six hours after antigenic stimulation, an increase of Ig^+ cells are observed in the peripheral lymphoid organs of mice which was detected by a technique known as Reverse Immune Cytoadherence or RICA (1). The technique utilizes a 5S hybrid antibody with two combining sites; one site is composed of anti-Ig which interacts with the Ig molecules on the cell surface, while the other site is composed of an antibody to a protein (i.e. BSA) which interacts with SRBC coated with BSA, thus forming a rosette (2). This technique is considered more sensitive than other techniques which detect SIg, due to the lack of Fc fragment in the hybrid antibody which contributes to the non-specific binding of Fc portion of Ig molecule to the FcR (3). Using the RICA technique Paraskevas et al (4) detected an increase in Ig^+ cells in the spleen and lymph nodes of the immunized mice between 3-6 hrs after immunization with particulate antigens or soluble foreign proteins in Freund's Complete Adjuvant (FCA). Although several possibilities were considered to explain this phenomenon several independent lines of evidence indicated that a population of cells which bear no surface Ig before immunization becomes Ig^+ due to the acquisition of a "cytophilic" Ig. Thus the increase in Ig^+ cells could be reproduced in vitro when normal spleen cells were incubated with serum collected 6 hrs after antigenic stimulation (4). It was also shown the cell which became Ig^+ was a T cell which normally is Ig^-. The number of Ig^+ cells per 1000 cells in the spleen of Balb/c mice is between 300-350 and an increase of 7-8% in Ig^+ cells was observed when normal cells were incubated with the six hour serum (6HS) (4).

It was also shown that reconstitution of irradiated mice with thymocytes but not with bone marrow cells resulted in an increase of Ig^+ cells six hours after immunization (5). This result again supported the notion that T but not B cells give rise to the sudden increase of Ig^+ cells 6 hours after immunization. Experiments were also designed to define the nature of the "cytophilic" Ig which was present in the serum which with class specific hybrid antibodies was shown to

belong to the IgG class. Using radioactively labelled antigens, it was found that the "cytophilic" Ig represented complexes of Ig with antigen. The complexes could not represent conventional antigen-antibody complex since no antibody is synthesized within six hours after antigenic stimulation; even DNA synthesis of Ig secreting cells does not start until 24 hrs after stimulation (7). It was also shown that the formation of these complexes depended on the presence of a factor. Since no complexes were detected upon stimulation of the animals with soluble proteins in the absence of FCA the role of FCA was investigated. Serum collected 6 hrs after injection of FCA (6HS-FCA) was shown to generate "cytophilic" Ig upon addition of a soluble protein (antigen). The "cytophilic" Ig was detected by incubating the serum and antigen with normal spleen cells.

Fractionation of the 6HS-FCA on Sephadex G-200 column showed that the 4S fraction (68,000 daltons) contained a factor which upon addition of an antigen and 7S Ig (collected from NMS) generated "cytophilic" Ig (?). This factor was found in vitro to be produced by thymocytes or T cells and was called Ig and Antigen Complexing Factor or IACF (8). The production of the IACF in vitro was studied in detail and it involves an interaction of macrophages and T cells (9). No physical contact was required since a supernate collected from macrophages could stimulate the release of IACF from T cells. The macrophage factor has not been fully characterized but it seems that free-SH group is necessary for its activity. For example, SH containing reagents, i.e. mercaptoethanol can bypass the requirement for the presence of macrophage (9). It was found that in vivo after stimulation by FCA the IACF is produced by the $Ly1^+$ cells. The complexes which are present in the 6HS are taken up by the $Ly2^+$ subpopulation (10).

The activity of IACF is removed by an anti-Ia column indicating that I region coded determinants is important for its function. Ia determinants are also involved in the uptake of the complex by T cells since only T cells which are FcR^+ and Ia^+ take up the complexes. Furthermore, the Ia antigens rather than the FcR primarily, if not exclusively, take up the 6hr complexes while the conventional immune antigen

antibody complexes are taken up by the FcR. This property strikingly differentiates the 6hr complexes from conventional immune complexes. T cells which lost their Ia antigen after 4 hrs in culture loose the ability to take up the complexes. However, this ability was reconstituted after the reconstitution of Ia antigen on the surfaces of T cells by macrophage supernatants (11).

The biological significance of these phenomena became apparent when it was shown that cells from the spleens of lethally irradiated mice reconstituted with thymocytes collected 6 hrs after antigenic stimulation enhanced both IgM and IgG plaque forming cells (PFC) seven days after adoptive transfer into sublethally irradiated recipients together with bone marrow cells (12). It was also found that this enhancement depended on the presence of two T cell subpopulations, one of which carries the Ig-Ag complexes and was found to be resistant to treatment with anti-Thy1+C. The second subpopulation of T cells were sensitive to anti-Thy1+C treatment and was Ig^-. The two subpopulations were found to act synergistically (13). Recently, Lee and Paraskevas (14) have found that serum collected 6H after antigenic stimulation with SRBC (or CGG or BSA in FCA) can enhance the Ig responses upon i.v. injection into naive mice, when given with subimmunogenic doses of the stimulating antigen. Best enhancement was obtained when the serum was injected two hours before the subimmunogenic challenge of the antigen. Such enhancement was found to be specific. When the 6HS was passed through either anti-Ig or anti-antigen columns, it was found that both the "cytophilic" complexes (as determined by RICA) and the enhancing activity were abrogated (15). The following conclusions can be drawn at this time: i) there is a factor formed 6 hrs after antigenic stimulation which is present in the serum; ii) the factor enhances antibody formation and iii) it is composed of complexes of Ig with Ag.

The "cytophilic" Ig or complexes are induced by a variety of antigens including particulate antigens or soluble proteins in FCA allogeneic cells and even syngeneic tumor cells (16). Synthetic polypeptides will also induce "cytophilic" Ig only

when they are composed of at least three amino acids (16). Thus GT copolymer as well as homopolypers do not induce "cytophilic" Ig.

The work presented here was undertaken in order to determine the biological significance of the complex formed after allogeneic stimulation in the generation of cytotoxic T cell responses.

LITERATURE REVIEW

1. THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

In the late thirties, it was found that molecules on cell surfaces in the mouse are responsible for allograft rejection upon transplanting of foreign tissues (17). Gorer et al (18) called these molecules histocompatibility antigens and the genes coding for them histocompatibility genes. Antigen II was the strongest in graft rejection and therefore the term H-2 antigens was coined to define these antigens. Interest in these molecules has been increased due to the fact that they are not only responsible for tissue rejection but are also involved in other aspects of the immune response. The Major Histocompatibility Complex which contains the H-2 genes is located on chromosome 17 and spans about 2 centrimorgans of DNA which corresponds to approximately 4000 kilobase pairs (Kbp) of DNA. The complex is divided into six regions called K,I,S,D,Qa and Tla (Fig. 1). The classical H-2 complex comprises the genes of the K through the D regions and encodes the class I transplantation antigens K, D and L, the class II and class III molecules. Qa and TL molecules, which are structurally closely related to the class I transplantation antigens are encoded by genes located to the right of the H-2 complex (20). Qa and TL differ from H-2D/K antigen in their expression on the surfaces of cells, in their polymorphism and in their homologies to each other, whereas Tla alleles appear to be much more homologous (21).

Class I polypeptide contains three external domains, each about 90 residues in length, a transmembrane region, and a cytoplasmic domain. The third external domain is noncovalently associated with B2-microglobulin, a small polypeptide that shows homology to the constant region domains of immunoglobulins and is not encoded in the MHC. Class II molecules are composed of two noncovalently associated polypeptide chains, denoted α and β, both of which are encoded in the MHC. Each of these polypeptides has two external domains that are of similar size as for class I molecules, a transmembrane region, and a small cytoplasmic domain (22).

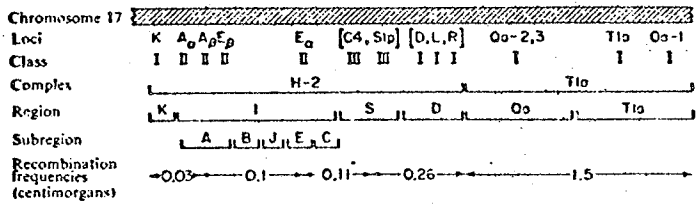


Fig. 1 Genetic map of the major histocompatibility complex of the mouse. The *K*, *D*, *L* and *R* genes encode the transplantation antigens which are found on all somatic cells, whereas the *Qa-2,3*, *Tla* and *Qa-1* genes encode class I molecules which are restricted in their expression to haematopoietic cells. The class III genes *C4* and *Slp* encode C4 components in the *S* region. Genes in brackets have not been mapped with respect to each other.

Taken from 19

In the mid-1960s, fundamental discoveries were made by Benacerraf and McDevitt and their co-workers which demonstrated for the first time a function of MHC genes in the control of immune responses to antigens other than those related to transplantation antigens or reactions or both. Responder strains and non responder strains were distinguished by their ability or inability to manifest cell-mediated (delayed hypersensitivity) and humoral (antibody production) immune responses to the relevant antigens. Studies utilizing congenic-resistant strains of mice established the linkage of responder or non responder status to genes in the MHC, and the MHC controlling such responses were termed immune response (Ir) genes (289,290). These genes were shown to be inherited in a Mendelian fashion as autosomal dominant traits in most instances, although the responder phenotype have been later shown in selected cases to result from gene complementation thus implicating the involvement of at least two Ir genes in the control of such responses (Reviewed in 291).

Analysis with intra-H-2 recombinant strains of mice established the fact that Ir genes were not located in the terminal K (left) or D (right) regions of the complex, but rather were located in a new region, adjacent and to the right of the K region, which was denoted as the I region (292). The Ir genes were thought to influence the function of Th cells. Studies utilizing congenic-resistant mouse strain established that the genetic restriction in cooperative T cell-B cell interaction is linked to the H-2 gene complex (293). Further studies documented that the critical genetic locus or loci involved in controlling such interaction map in the I region of the histocompatibility complex (294), a remarkable association with the same region containing Ir genes and most potent alloantigens in terms of stimulating one type of transplantation reaction, namely the mixed lymphocyte reaction (MLR).

The identification of Ir genes in the I region motivated an attempt to prepare antibodies against determinants coded for by the I region gene products and expressed on the surface of cells of the immune system. Mice which differ genetically only at the I region (A.TH and A.TL) were immunized reciprocally with each other's lymphoid cells (295,296). The alloantisera obtained detected a new class of antigens expressed on most B cells, on a large fraction of macrophages (297) and also on a small percentage of T cells (298). These antigens were given the generic name Ia antigens (Reviewed in 259). They belong to class II antigens.

There is a large body of evidence supporting the contention that the Ia molecules which are the restricting elements for Th cells activation and proliferation, are actually products of the murine Ir genes. One piece of data was provided by Lin et al (23) who used APC from the B6. C-H-2^{bm12} mutant (where mutation occurs in the A_β chain, thus it differs slightly from B6 mice from which the mutant is derived. Mutation appears to consist of a limited change in the A_β chain). These cells lost the capacity to present bovine insulin to insulin-specific B6 T cells and T cell lines, whereas B6 APC presented the antigen normally. The response to bovine insulin is controlled by an I-A encoded Ir gene. Thus, a mutational change in class II molecule extinguishes its capacity to act as a restriction element in Ir gene controlled immune responses as well as in "conventional" immune responses.

A major line of evidence implicating class II molecules as restriction elements in Ir gene controlled systems is drawn from studies of F1 specific restriction elements and class II molecules. T cell response to the terpolymer of L-glutamic acid, L-lysine and L-phenylalanine (GLØ) is controlled by Ir genes mapping to both the I-A and the I-E regions of the MHC. B10.A(5R) ($1\text{-}A^b$, $I\text{-}E^k$) T cells specific for GLØ recognize a restriction element that is contributed to by genes in the $I\text{-}A^b$ and the $I\text{-}E^{k,d \text{ or } r}$ regions. The construction of this restriction element by gene complementation is precisely paralleled by the construction of a class II molecule from the E^b_β polypeptide encoded by the E polypeptide (299). The latter is encoded by a gene in the I-E region. Noting that Eα gene is expressed in strains of mice carrying the

k,d or r alleles of the I-E subregion. Thus, there is a precise parallelism in the gene complementation that resulted in the construction of a restriction element capable of presenting GLØ to primed T cells and in the construction of a unique class II molecule. Furthermore, monoclonal antibodies to this class II molecule inhibits the response of T cells to GLØ and the E^{b}_{β} $E^{k \text{ or } d}_{\alpha}$ restriction element.

The cytotoxic T lymphocytes (CTL) recognize foreign antigens in the context of K and D antigens (29). These CTL include the ones generated to minor histocompatibility complex (30), virus-associated antigen (31) and chemically modified-self (32). This phenomenon has been termed H-2 restriction. MHC restriction was also observed between MØ and T cells. First in the guinea pigs by Rosenthal and Shevach (300) and later in the mouse, where the more sophisticated genetics allowed the mapping of the genes involved to the I-A subregion of the MHC for antigen such as DNP-OVA (301). The MHC also restricts the interaction between Th cells and B cells, at least those that do not express the Lyb5 surface marker and present in the CBA/N mice (302).

The effector cells induced in vitro were also found to belong to the T cell lineage (41,42). It is now known that CTL can be generated both in vivo and in vitro not only to the MHC antigens, but also against minor-histocompatibility antigens, virally-infected cells, chemically-modified cells and tumor-associated antigens. The cytolytic action of CTL is restricted to target cells sharing with the sensitizing cells some of the structures coded by the MHC. This phenomenon has been termed H-2 restriction (31, 32). The restricting determinants are coded for mostly by the H-2K and H-D genes.

2.1 In Vivo Generation of CTL

Mice grafted with allogeneic skin generate CTL several days before the graft is rejected. These cells were found in the draining lymph nodes, spleen,

blood and contralateral lymph nodes (43). Although Brunner et al (44) have generated strong CTL response by a single i.p injection of allogeneic tumor cells, Sabbadini (45) indicated that a single dose of allogeneic cells did not result in good CTL responses unless the mice were irradiated before immunization.

Another method for the induction of CTL directed against MHC antigens consists in the transfer of cells into lethally irradiated allogeneic or semiallogeneic recipients. The CTL-precursors (P) which are present in the transferred population proliferate and differentiate into CTL upon contact with the alloantigens of the recipient (reviewed in 46). Thus, relatively high CTL activity can be demonstrated in the lymphoid cell population recovered from the recipient spleen few days after transfer.

CTL in vivo were found to accumulate in the peritoneal cavity (site of allograft) after i.p. injection of allogeneic cells indicating that CTL differentiate at the site of the allograft (47). Brunner and Cerottini (48) also found that antigen-specific CTL are present in the peritoneal cavity of mice treated with repeated injection of X-irradiated syngeneic tumor cells. These CTL exhibited potent cytotoxic activity. However, the duration of the CTL activity was found to be short and decreased rapidly (44). Nevertheless, immunological memory at the level of CTL has been demonstrated (49).

2.2. In Vitro Generation of CTL

The most widely used in vitro system for the generation of CTL against MHC antigens is the mixed lymphocyte reaction (MLR) in which responder cells are mixed with allogeneic stimulator cells which are either irradiated or treated with mitomycin-C. Allogeneic stimulator cells are more efficient in inducing CTL formation in vitro. The reason for this may be due to the presence of antigen-specific T-suppressor cells as will be explained later.

CTL generation in vitro have also been achieved in various syngeneic model systems (32,50,51). Several other studies have confirmed that in vitro stimulation of in vivo immunized lymphoid cells is particularly useful way for obtaining CTL populations directed against non-MHC antigens (50,52,53). The usefulness of such procedure is best illustrated in studying the formation of CTL against minor-histocompatibility antigens (30) or the male-specific (H-Y) antigen in mice (54). In both instances no detectable CTL activity was obtained after primary in vivo or in vitro immunization, while active populations were obtained by combining the two procedures in sequence.

2.3. Lyt phenotype(s) of CTL

The discovery of Lyt antigen system was useful in classifying T cell subsets due to their restricted presence on T cells, thymocytes and leukemias of T cell origin. The Lyt.1 antigens are determined by alleles at the Ly-1 locus of chromosome 19 (55), whereas the Lyt-2,3 antigens are determined by alleles at the Lyt-2,3 locus of chromosome 6 (56). By analyzing the structures recognized by anti-Lyt-2 and anti-Lyt-3 antibodies, it was found that either Lyt-2 and Lyt-3 determinants exist on the same molecule or that Lyt-2 and Lyt-3 molecules are associated as a dimeric complex (57,58).

To select one T cell subclass using antibodies to the Lyt antigens it is necessary to eliminate the other subclasses. For example Lyt.1^+ cells are selected by treating the T cell population with either anti-Lyt.2 antisera plus complement (C) or anti-Lyt-3 antisera+C. This treatment removes the Lyt.23^+ and the Lyt.123^+ cells (both being Lyt.2^+ and Lyt.3^+) leaving the Lyt.1^+ cells intact. If on the other hand, Lyt.23^+ cells are selected, then the cells can be treated with anti-Lyt-1^+ + C, which removes Lyt.1^+ and Lyt.123^+ (both being Lyt.1^+) leaving the Lyt.23^+ cells intact. It was also found that Lyt.1^+ cells cannot give rise to Lyt.123^+ or to Lyt.23^+ cells, neither can Lyt.23^+ give rise to Lyt.123^+ or Lyt.1^+ cells. Therefore, it was suggested that Lyt.1^+ and Lyt.23^+

subclasses belong to different lines of differentiation and are not sequential stages of a single progression (59).

Using this technique, it was originally proposed that T-helper (Th) cells are Lyt.1^+2^- which proliferate in response to class II MHC alloantigens. These cells interact with Lyt.1^+2^+ CTL-P that differentiate into Lyt.1^-2^+ CTL-effectors. The latter are directed against class I MHC-antigens (60,61). The Lyt.2^+3^+ cells , however contain both CTL (60,62) as well as suppressor cells for both humoral and cellular immunity (60,62,63).

Since no cells expressed the Lyt.2^+3^- or Lyt.2^-3^+ phenotype, Huber et al (59) classified T cells into three subpopulations; the Lyt.1^+, the Lyt.23^+ and the Lyt.123^+. The latter subpopulation represents about 50% of the total peripheral T cell pool. These cells have been considered as precursors for both Lyt.1^+ and Lyt.23^+ subclasses (60,62), as suppressors in some systems (64) and as precursors of cytotoxic effectors to TNP-modified self (65,66). In contrast to Benacerraf et al (66), who indicated that Lyt.23^+ cells form the precursors for allogeneically-reactive Lyt.23^+ CTL, Simon and Abenhardt (67) have found that Lyt.123^+ T cells contain the precursors for all umprimed cell population. These precursors then mature into Lyt.1^-23^+ cytotoxic effectors after sensitization with the antigen in vitro. Once these cells are generated, they will either act as killer cells or as memory for CTL in the secondary response. In this case CTL in secondary responses derive from Lyt.1^-23^+ memory cells.

The idea that different subpopulations of T cells respond to one or the other MHC alloantigens may not be an absolute one. It was previously suggested that Lyt.1^-2^+ respond to class I alloantigens, whereas Lyt.1^+2^- respond to class II alloantigens (68). However, CTL were generated across the I-A barrier and these cells were able to lyse target cells that share I-A region with the stimulating cells (69). These clones of CTL were found to be fewer than those generated to class I alloantigen (70). Consistent with this idea are the findings of Panfili and Dutton (71) that Th cells were generated against K/D difference.

Also Melief et al (72) using H-2K mutants have found that CTL generated to the mutant allo-antigen have the phenotype Lyt.2^+3^+, with the participation of Lyt.1^+ Th cells (the same as in the classical pathway of CTL generation). In other words, Lyt.23^+ as well as Lyt.1^+ recognize the H-2K alloantigen (class I) and respond to it. In addition CTL generated toward H-2K+I differences were of Lyt.23^+ phenotype. These cells did not need the participation of Lyt.1^+ Th cells. He has therefore concluded that in this situation, the Lyt.23^+ CTL-P respond to H-2K alloantigen and in addition another CTL-P subpopulation, also of the Lyt.23^+ phenotype responds to the I-A alloantigens, making up for the two subpopulations of CTL responding to different MHC-complex alloantigens. These results indicate that the Lyt antigens expressed on the surface of T cells may not be associated with the functional activities of these cells but rather serve other functions.

A different pathway for CTL generation was suggested by Bach and Alter (73) who have found that CTL generated to H-2K/D differences have the Lyt.1^+2^+ phenotype and were derived from Lyt.1^+2^+ CTL-P. At the same time it was found that proliferation in primary MLC specific for H-2K/D mutant alloantigens is mediated by Lyt.1^+2^+ T lymphocytes (74). Using a different system Swain et al (75) found that helper cells for B cells responding to K/D differences have the Lyt.123^+ phenotype, whereas helper cells induced to whole MHC differences have the Lyt.1^+ phenotype.

In accordance with Bach and Alter, CTL generated to mutant K/D express the Lyt.1^+2^+ phenotype (76). These results were confirmed by other workers (77) who found that CTL-P in vitro have the Lyt.1^+2^+ phenotype which upon alloantigenic stimulation give rise to alloreactive CTL having the Lyt.1^+2^+ phenotype. This is true whether the stimulating antigens are whole-MHC difference or only H-2K different from the responding population of cells. The same conclusion was also reached using TNP-modified syngeneic cells for stimulation, indicating that CTL generated to both the alloantigens and to the TNP-modified self antigens are derived from the same pool of CTL-P which have the Lyt.1^+2^+ phenotype (78).

However, Nakayama et al (79) have shown that T cells involved in primary allo-antigen recognition can be divided into two subpopulations, cells which proliferate express the Lyt.1^+23^- phenotype while the cytotoxic cells express the Lyt.123^+ phenotype. Lyt.123^+ CTL recognize the K/D antigens and are derived from the Lyt.123^+ precursors, whereas the Lyt.1^+23^- recognize the I and the Mls differences and can give rise to Lyt.1^+23^- cytotoxic cells upon blocking the normal CTL (Lyt.123^+) by anti-Lyt antisera. Cerottini and McDonald (80) drew the same conclusions: there are two CTL populations, one derived from Lyt.123^+ CTL-P express the Lyt.1^+2^+ phenotype and can kill target cells specifically, whereas the second population is derived from Lyt.1^+2^- CTL-P, expresses the Lyt.1^+2^- phenotype and can kill target cells non-specifically in lectin-mediated cytotoxicity.

It should be noted here that these differences may be due to the system used and do not reflect the qualitative expression of Lyt antigens on the surface of T cells. Ledbetter et al (81) used Mab to Lyt.1, Lyt.2 and Lyt.3 antigens and found that all T cells express the Lyt.1 antigen. However, some cells express higher density of this antigen than others. The first population is sensitive to killing in complement-dependent-cytotoxicity, whereas the second population resists such treatment. These results were confirmed by others (80) who used in addition to anti-Lyt antisera, flow microfluorometry. It is therefore likely that differences in Lyt antigens present on the surface of CTL are quantitative (high vs low) rather than qualitative (present vs. absent) (82).

In accordance with this concept Vidovic et al (83) have found that CTL generated against the I-A differences have the Lyt.1^+2^- phenotype (Lyt.1 high, Lyt.2 low), i.e. the same phenotype as the classical Th cells which recognize the I-A gene products. On the contrary, CTL generated against the I-E subregion antigens express the Lyt.1^+2^+ (Lyt.1 high, Lyt.2 high) phenotype. These results indicate that the two major glycoproteins encoded by the I-region genes (the I-A

and the I-E products (84)) are recognized by different subsets of T cells. These results and the fact that CTL generated to K/D differences express the Lyt.123$^+$, while helper cells express the Lyt.1$^-$2$^+$ phenotype (85) indicate that Lyt antigens expressed on the surfaces of T cells are not associated with the functional activities of these cells. These activities are influenced by the stimulating allo-MHC antigens, i.e. the MHC-gene products determine the phenotype of functional T lymphocytes. As it will be apparent later, Lyt antigens may be involved in recognizing the different MHC alloantigens and depending on the kind of the stimulating antigen, triggering of different subsets of T cells occurs. For example, K and D alloantigen differ from I alloantigen in many biochemical characteristics and these differences lead to the triggering of different T cells depending on Lyt antigen which recognize these alloantigens.

3. NATURE OF THE T-CELL RECEPTORS

The specific immune response is initiated via the recognition of antigenic determinants by immunocompetent lymphocytes. Such recognition is mediated by antigen-specific receptors located on the surface of the lymphocytes. Conventional immunoglobulins (IgM, IgD...etc.) serve as antigenic receptors for B lymphocytes. However, one major difference between B cells and helper or cytotoxic T cells is that these T cells cannot directly bind soluble, or particulate antigens that have not been processed by macrophages. T lymphocytes require simultaneous recognition of foreign antigen along with self-histocompatibility antigen at the surface of the Antigen Presenting Cells (APC). Such differences in antigen recognition by T and B cells suggest a major difference in their receptors but do not rule out the possibility that T cell receptors utilize at least a portion of the antibody structure.

Humphrey was the first to suggest that T cell receptors might consist of heavy chain without light chain (86). It was later found that anti-immunoglobulin antibody raised between species widely separated in phylogeny can give heavy

labelling of thymocytes or T lymphocytes (270-272). However, T cell antigen receptors were found to bear determinants that are cross-reactive with antibody idiotypes or VH (287,288). For example, anti-idiotypic antibodies raised against T cell receptors show complete cross reaction with the relevant idiotypic B cell receptors (273). In contrast, anti-idiotypic antisera produced against B cell receptors or idiotypes located on the corresponding alloantibodies could be shown to react with additional idiotypes, which are present on B cell receptors but are absent from T cell receptors that are directed against the same determinants (273). Additional experiments revealed that anti-idiotypic antisera raised against B cell receptors frequently contained antibodies against either heavy-or light-chain determinants, whereas anti-T cell receptor sera contained antibodies only to heavy-chain idiotypes (273-289).

Furthermore, it was found that isolated heavy chains, but not light chains derived from idiotypic alloantibodies could inhibit anti-idiotypic sera produced against T-cell receptors of the corresponding specificity (273). Similar results were reported by Rubin et al (274). However, Kramer and Eichman have found in the same system that idiotypes on alloreactive T lymphocytes are coded by two sets of genes, one linked to the genes coding for heavy chain immunoglobulin and the other to genes within the MHC (275), a result that has been proven later by other investigators (276, 277).

Association between idiotypic receptors and specific immune function was demonstrated in several T dependent assays, using anti-idiotypic antibodies in affinity chromatography to enrich or deplete idiotype-positive cells, or using the antibodies in conjunction with complement to eliminate selectively the idiotype-bearing lymphocytes (90,278,279). These procedures have thus demonstrated that T lymphocyte responsible for reactivity in GVH reaction, MLC and cell mediated lymphocytoxicity at precursor or effector cell levels bear idiotypic receptors (91,279). In addition, anti-idiotype sera added to lymphocyte culture in the absence of complement, at low concentrations can induce enhanced

responses. It was also found that anti-idiotypic sera can restimulate MLC-primed T lymphocytes specifically to a similar magnitude as the corresponding allogeneic lymphocytes. In addition, the same anti-idiotypic sera were able to induce proliferative responses in normal T lymphocyte populations, as well as specific, highly efficient CTL (280). The intensity of these reactions is exemplified by the fact that anti-idiotypic sera could induce CTL in the absence of the corresponding Th cells. In summary, anti-idiotypic antibodies raised against T lymphocyte receptors specific for alloantibodies can suppress T-cell function in a highly specific way in the presence of complement, while at the same time in the absence of complement they have selective enhancing effect leading to specific immune reactions (281-283).

Recently, a number of monoclonal antibodies raised against cloned antigen-specific, MHC-restricted or alloreactive T cells of both mice (284-286) and human (287, 288) have been described. Antibodies with these activities have been proven to be clone-specific in their activity, suggesting that they recognize the idiotypic portion of the Ag/MHC receptors on target T cells.

Although all this work indicated the presence of idiotypic determinants on the surface of T lymphocytes and that these determinants are involved in recognition of the antigen, hybridization experiments using mRNA for T cell hybridomas and VH DNA probes argue against VH genes being used to code for T cell idiotypes (for review, see 266 and 267). However, it is possible to prepare various models by which the serelogical data, pointing so strongly to shared gene pools for B and T cell receptors, can be reconciled with molecular genetic data suggesting that distinct gene pools are used. The first model would say that T cells do indeed use Igh-V genes, but that the rearrangement involves sister chromated exchange rather than deletion, leaving

the T cell with genes in the Igh gene complex that appear to be in the genomic configuration. One might also propose that distinct joining genes and perhaps D gene segments are used by T cell-receptor gene formation, leading to differences in the behaviour of the molecules. For instance, a postulated JT gene could have a 12 base pair (bp) spacer between its haptomer-nonamer joining sequences allowing joining with Igh-V genes without need for a D segment gene. Another possibility is that the IgT gen family (for review on IgT gene family, see 303) has its own V genes that arose by gene duplication or inversion from an ancestral Igh-V gene pool. If the genes arose from a common ancestor, then they could be very similar although differences would accumulate during the separate evolution of the two gene families. Gene conversion mechanisms operating on the portions of the IgT-V genes that confer idiotypy and antigen-binding specificity could maintain idiotypic similarity, while allowing the genes to diverge sufficiently in other coding and flanking regions so that they fail to hybridize strongly with Igh-V gene probes.

4. THE GENERATION OF CTL

The T cell mediated cytotoxicity can be divided into three stages, a RECOGNITION PHASE in which T cells recognize the alloantigens resulting in clonal proliferation of specific antigen activated cells. This stage needs the participation of Th cells which provide the second signal for CTL-P. When such interactions occur, the second phase of T cell mediated cytotoxicity occurs which is known as the REGULATION PHASE. The third phase is characterized by the killing of the target cells bearing the sensitizing antigens. This stage is known as the EFFECTOR PHASE.

4.1. The Recognition Phase (Antigen Recognition)

As indicated earlier, T lymphocytes recognize foreign antigen in the context of MHC antigens. This was first established when the lytic activity of CTL by LCMV-sensitized T cells against infected target cells was detected only when LCMV infected target cells were compatible at the H-2 gene complex (31). Lymphocytes from H-2 incompatible donors did not cause any specific lysis, and the unrelated targets were not lysed at all (92, 93). Also, Ectromelia virus-specific lysis was blocked by anti-H-2 antisera better than with hyper-immune anti-virus antisera (94). This activity was due to the ability of anti-H-2 antisera to block the H-2 determinants of target cells (95). Similar results were found with CTL specific for TNP-modified self antigens (reviewed in 96) and also for minor histocompatibility antigens (30). These results indicate that CTL must have receptors for foreign (nominal) antigens as well as for MHC antigens and that both antigens must interact with these receptors.

The interaction of two entities may result in a complex in which both components fully reserve their integrity and properties (95) or they may impart conformational changes on each other (253) or they may merge with each other to form a new entity (95, 97). The latter would still preserve certain features of both components and it is referred to as neoantigen or altered self.

T cell must recognize the complex of self-molecule plus antigen. Whether there is a single receptor or two different receptors for these antigens, has been and still is a matter of controversy (97-100, 265, 268). Whereas the MHC-restricted responses can be explained by both one or two receptor hypotheses, the strong alloreactive response to foreign H-2K and D antigens which are recognized

independently of self-antigen, i.e. unrestricted, poses a problem for the two-receptors model (97). The one receptor model of T cell may also apply to the alloreactive responses if one considers that the receptor will recognize an alloantigen plus self complex.

Janeway et al (101) proposed a model in which T cells have two receptors; R1 recognizes the nominal antigen (X), while R2 recognizes and interacts with the MHC-gene products regardless of their origin (self MHC or allo MHC). However, the allo-MHC antigen has higher affinity for R2 and therefore can activate T cells by just interacting with this receptor, whereas self-MHC has lower affinity for R2. Hence, self-MHC need another signal provided by the interaction of the nominal antigen with the R1 receptor. According to this hypothesis, nominal antigens also have lower affinity to R1 and that only the combination of self MHC-R2 + nominal antigen-R1 interactions can activate the T cells. Binz et al (102) further isolated T cell receptor material specific for allogeneic MHC antigens using anti-idiotypic antibodies and the isolated receptor was coupled to Sepharose column. Radiolabelled allogeneic products were then passed through this column and found to react with the receptors with high affinity, whereas it bound with low affinity to self-MHC products and not at all with unrelated third-party molecules.

On the other hand, Langman (254) predicted that alloreactive cells are the same as anti-X (where X is a nominal antigen) reactive cells and that the anti-self receptors are selected in the thymus while the anti-X receptors on T cells are not influenced very much by self-H-2 but the separate selected anti-self receptor is responsible for giving alloreactive cells a self-preference in restriction. Furthermore, Bevan (255) indicated that the CTL receptor which binds H-2 alloantigens in an alloreactive response is influenced by the self-H-2 antigens present in the thymus. In other words the repertoire of alloreactive receptors is dependent on the allele of self-H-2. Bevan also indicated that T cells have one receptor which is used in conventional and in

allo-responses and that the repertoire is skewed during differentiation to react preferentially with self-plus-X or altered self.

The receptors, regardless of their nature, were believed to be generated and diversified during ontogenesis by means of somatic mutation from a limited gene pool (256, 257). However, Wagner et al (258) indicated that the repertoire of peripheral CTL-P is unlimited in that it includes self-MHC as well as allo-MHC restricted antigen specific CTL-P. The diversity of T cell receptors has been recently shown by Siliciano et al (105) using Ag specific MHC-restricted clones of T cells.

Whatever the explanation is, it should be mentioned that these receptors (Whether to nominal antigen or to MHC antigen) are clonally distributed on the surface of T cells (103). In other words, an individual CTL is generally restricted for only one of the K and D gene products present in a given individual (103, 104).

4.1.1. Role of Lyt antigens in antigen recognition

As indicated before Lyt antigens present on the surfaces of T lymphocytes are not related to the functional activities of these cells but rather act as receptors for them. Cytolytic activity of CTL was inhibited by monoclonal antibodies directed toward Lyt.2,3 antigens (82,106). These MAb have been also found to inhibit both cell proliferation and the generation of CTL in bulk allogeneic MLC (107,108). This inhibition of proliferation may be due to the ability of anti-Lyt.2,3 MAb to inhibit the specific recognition of antigen by CTL-P which result in the inability of these cells to express the IL-2 receptors on their surfaces (109). In addition to recognizing specific antigens, Lyt.2 antigens may be involved in recognizing lectins (e.g. Con A) which leads to the activation of CTL, i.e. lectins interacts with the clonally distributed receptors (e.g. Lyt.2 antigen) on the surfaces of T cells the same way that specific antigens do, rendering these cells susceptible to the proliferation

events mediated by IL-2. Anti-Lyt.2 MAb inhibited such interaction, therefore, it inhibited mitogen-dependent cytotoxicity of Lyt.2^+ CTL (110).

More direct results about the involvement of Lyt.2/3 in antigen recognition came from the work of Dialynas et al (111) who showed that Lyt.$2^-/3^-$ variants derived from CTL clone, have lost the expression of specific cytolytic activity but retained the ability to lyse target cells non-specifically in the presence of lectins. In addition Fan and Bonavida (112) showed that the recovery of Lyt.2,3 antigens on CTL after trypsinization closely paralleled the recovery of cytotoxicity. Inhibition of killer activity by anti-Lyt.2 or anti-Lyt.3 MAb may indicate that these antigens are integral parts of antigen binding receptors. Alternately, their presence may be in close proximity to the antigen-binding receptors on T cells and the anti-Lyt.2 or anti-Lyt.3 MAb may cause steric hindrance resulting in blocking the actual antigen receptors (82). It is known that Lyt.2 antigen is coded for by Lyt.2 locus which is present on chromosome 6 which contains the closely linked genes (Kappa genes) (113). Consequently, Lyt.2 antigen may represent the antigen binding site or is very close to it, therefore, blocking of Lyt.2 antigen resulted in the inhibition of T cell activation. Recently, Glasebrook et al (114) indicated that anti Lyt. 2,3 MAb inhibited both cytotoxicity to specific antigens as well as proliferation and lymphokine secretion of cloned CTL which proliferate to specific antigens without added IL-2. Although these results suggested that anti-Lyt MAb inhibit specific antigen recognition (presumably, Lyt.2/3 antigens), it was later found that these clones of CTL are heterogenous in respect to the inhibition by anti-Lyt.2,3 MAb, i.e. some clones were inhibited, whereas others were not, in spite of the fact that both clones express similar amounts of Lyt.2 antigen on their surfaces as determined by flow cytofluorimetry (115). Therefore it is suggested that Lyt.2,3 molecules function to stabilize the interaction between putative CTL receptors and the corresponding stimulating antigens. Such stabilization may be required by CTL with few or low affinity receptors and as the number and/or

the affinity of the receptors increase, the requirement for stabilization decrease (114). This could happen at the CTL-P level, where high affinity CTL-P react with stimulating antigen, hence expressing IL-2 receptors independent of Lyt.2/3, whereas low affinity CTL-P require Lyt.2/3 molecules to stabilize the interaction between CTL-P and the sensitizing antigen (116).

Other investigators produced anti-H-2D CTL hybridomas which express specific killing activity but do not express Lyt.2/3 (304). Also, Giorgi et al (305) noted spontaneous Lyt.2/3 variants of a Balb/c syngeneic plasmacytoma CTL line. This variant which is Lyt.2/3$^-$ had undiminished killing activity compared to the Lyt.2/3$^+$ parent line. Hence, the role of Lyt.2/3 antigens as a recognition unit for CTL should be elucidated more since there is no allelic exclusion of Lyt.2/3 expression in F1 cells (306). Also their molecules do not appear to possess the variability required to discriminate between distinct antigens.

Similar results to those with anti Lyt.2 MAb were found with anti-Lyt.1.1 MAb which inhibited CTL responding to TNP-self antigen (117). The activity of this MAb was directed toward Th cells rather than CTL-P. The authors suggested that Th cells recognizing self-modified antigen have low affinity for this antigen hence, they need Lyt.1 molecules to stabilize the reaction, whereas Th cells recognizing allogeneic antigen have high affinity for these antigens. Therefore, they do not need Lyt molecules to stabilize the interaction (117).

4.2 The Regulation Phase

The generation of cytotoxic responses to antigens, whether these are allo or self-modified, is not a simple interaction between CTL-P and the sensitizing antigens, but rather require a complex interaction between the various subsets of T cells including Th, CTL-P and reactive CTL, in addition to cells of non-lymphocytes lineage like the macrophages and the dendritic cells (DC). The latter are collectively known as accessory cells.

4.2.1. The role of T-helper (Th) cells

In the last eight years, it was established that there is a cascade of interactions between the different subsets of T cells including Th cells and cytotoxic cells. Th cells upon activation release soluble mediators which are needed for the proliferation of various T cells. These mediators are known as cytokines. They are produced by a variety of cells. The two most important ones which regulate the immune responses are derived from lymphocytes and accessory cells. Interleukin-1 (IL-1) is derived from monocytes and was previously known as lymphocyte-activating-factor (LAF) (118), whereas interleukin-2 (IL-2) which was called by many names (see for review Immunol. Reviews, Vol. 51. 1980) is released from lymphocytes. Both of these factors augment the proliferation of thymocytes in lectin-induced mitogenicity. However, these cytokines differ in their source of production, biochemical and biological properties (see for review 119). IL-1 induces the production of IL-2 (120,122) which is then released from a mature Lyt.1^+ subpopulation. IL-2 induces the proliferation of CTL-P, namely the Lyt.2^+ cells.

A.1. Accessory cells-Th cells interaction

Degiovanni et al (123) showed the need for accessory cells in secondary CTL responses to particulate or tumor alloantigens. Weinberger et al (124) showed that spleen adherent cells (SAC) are involved in presenting the sensitizing antigen to Th cells in the context of their Ia antigen, even when the antigens involved are membrane proteins (for example purified MHC molecules like H-$2K^k$). When such presentation occurs, Th cells release a soluble factor (IL-2) which sustains the proliferation of CTL-P which need another signal (alloantigen) in order to differentiate into alloreactive CTL. A direct evidence for the involvement of Ia^+ accessory cells in activating Th cells needed for the generation of TNP-specific CTL is recently provided by Kruisbeck et al (178).

CTL per se do not need SAC for the presentation of antigens, but rather they recognize the antigen on the surface of stimulator cells, hence they differentiate into reactive cells as long as the helper signal is provided by IL-2 (125). Antigen presentation by accessory cells to Th cells is known as signal-1, whereas IL-1 provides signal-2. Accessory cells provide signal-1 to responding cells present in bulk cultures or in cortisone resistant population of thymocytes but they do not provide any signals to CTL present in cortisone resistant populations or to CTL-P present in limiting dilution assays (126). Recently, it was found that Th cells required two sets of macrophages for activation. Macrophage 1 provides signal 1 in the form of antigen-MHC gene products (Ia antigen), whereas macrophage 2 provides signal 2 in the form of IL-1. Such interaction leads to the expression of IL-2 receptors on Th cells which proliferate upon interaction with IL-2. If these cells do not interact with IL-2, they will return to the resting stage again (127) (Fig. 2).

Recently Hunig et al (128) indicated that accessory cells are important for both the release of IL-2 from Th cells as well as in rendering T cells susceptible to stimulation by IL-2. Whether the producer and the consumer cells for IL-2 are the same is not known. According to the above authors, IL-1 is not enough by itself to render T cells responsive to IL-2, but physical contact between macrophages-T cells is necessary for that. Therefore, it seems that both signal 1 and 2 are important for the release of IL-2 but not for the susceptibility of Th cells to IL-2. In addition to macrophages, DC were found to be important for both the proliferation of T cells in MLR as well as the generation of CTL. This is due to the release of IL-1 from DC in these cultures (129). Finally the T cells which proliferate in oxidative mitogenesis reactions induced by sodium periodate, require the presence of DC which induce the release of IL-2 and the expression of IL-2 receptors on T lymphocytes. Once these events occur, T lymphocytes respond to IL-2 without the need of the presence of DC (130).

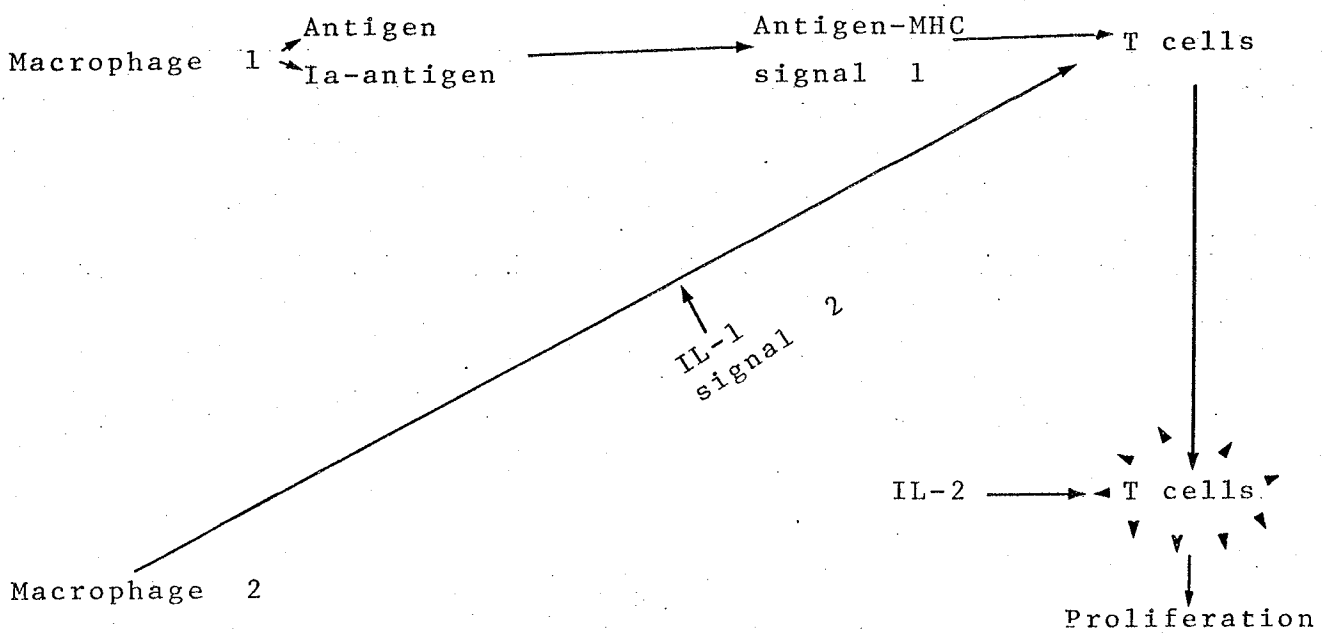


Fig. 2. Two subsets of Macrophage are needed for the activation of Th cells.
Both signal 1 and signal 2 lead to cell activation

Represent the IL-2 receptors

A.2. Th-CTL-P interaction

Plate was the first to describe that soluble factors derived from T cells can replace the requirement for Th cells in the generation of alloreactive CTL in the MLR (131). A similar factor has been described by Fink et al (132) who showed that thymic cells can generate CTL if assisting factor derived from T cells is added to the culture. Wagner and Rollinghoff (133) described a factor in Con A or in MLR supernatants after activation of Lyt.1$^+$ T cells with Con A or allogeneic antigens respectively which triggers alloantigen-primed Lyt.23$^+$ cells to proliferate and differentiate into alloreactive CTL. This action could only be induced in alloantigen primed T cells but not resting primary T cells. It is therefore, suggested that CTL are generated after receiving two signals: Signal 1 provided by the sensitizing antigen and signal 2 provided by IL-2 (134). Amplifying factors are also released in secondary MLC from Lyt.1$^+$-Th cells. These factors stimulate CTL to UV and heat-treated alloantigens (135).

The optimal production of IL-2 depends on the presence of two Th cells in MLR. These cells differ in their Qa-1 alloantigens; one being Qa-1$^+$ (Qa-1 high) and the other Qa-1$^-$ (Qa-1 low). Either one of these cells produce low amounts of IL-2, whereas both of them are required for optimal production (136).

IL-2 was found to sustain the growth of CTL when added in 4-day intervals to limited numbers of alloantigen-primed purified Lyt.23$^+$ cells which can grow autonomously without added antigen as long as IL-2 is provided in the culture medium. These cells have specificity for the sensitizing antigen. Thus it appears that IL-2 controls the clonal expansion of alloantigen-primed T cells (137,138). It is therefore, apparent that although Th cells are involved in the generation of CTL to alloantigens and mitogens (139,140), the main action of these cells is the release of soluble factor (IL-2) which can substitute for the requirement for the presence of Th cells in the generation of CTL in the MLR (141).

Recently, challenging data for the two signals hypothesis came from two laboratories. First, Von Boehmer and Hass (142,143) have found that K/D antigen restricted CTL clones can proliferate to cell bound antigens in the absence of exogenous IL-2. These results were confirmed recently by the same authors who indicated that the capacity of these clones to respond to cell bound antigens is lost upon growth in IL-2 media for several weeks (144). These results indicate that CTL (or even CTL-P) have the capacity to proliferate autonomously in response to cell bound antigens at certain stages of differentiation, a result that has been previously observed with CTL clones specific for alloantigen (145). Secondly, and contrary to the above data, Ballas and Ahman (146) indicated that CTL-P can generate CTL that kill preferably modified syngeneic target cells, in the presence of only IL-2.

Reddehase et al (147) have found that IL-2 is efficient only in providing help to alloreactive CTL in the presence of whole-MHC. When IL-2 is added to K/D stimulus, no CTL activity is observed unless an I-region incompatible stimulus is added to the culture indicating that I-region enhances Th cells which provide another signal to CTL-P. Previously, Lafferty et al (148) indicated that control supernatant which enhances CTL responses carry two factors, one that acts as early as three days in culture and the second is a late acting maintenance factor (IL-2) which is required for cell proliferation. From these data it seems that IL-2 causes only the proliferation (clonal expansion) of CTL-P that are already activated by antigens. Recently, two reports were published at the same time which showed exactly the same results and which clear up some of the confusion. Wagner et al (149) showed that IL-2 caused only the proliferation of CTL-P. However, there is another factor known as cytotoxic T cell differentiation factor (CTDF) present in the crude supernatant of Con A stimulating spleen cells is required for CTL-P to differentiate. Such factor was found to be different from gamma-interferon or from IL-1. The second report was published by Raulet and Bevan (150) who showed exactly the same results.

In addition, it should be mentioned that in contrast to the classical activation of CTL by whole MHC differences where Lyt.1$^+$ Th cells are needed to produce soluble factors, it is the Lyt.2$^+$ cells that release soluble factors upon activation with H-2K/D alloantigens. In this case Lyt.2$^+$ T helper factors act to induce Lyt.123$^+$ CTL (151).

B.1. In vivo Th cells

The role of Th cells in vivo is not as clear as their role in vitro. This may be due to the difficulty of studying these cells in vivo. Zinkeragel et al (152) as well as others (153) using bone marrow reconstituted radiation chimeras found a necessity of I-region compatibility between donor cells and host resistant thymus cells to generate antigen specific CTL activity. I region compatibility reflects the ability to sensitize Th cells which provide IL-2 that in turn allows the proliferation of CTL-P. Wagner et al (154) showed that injection of allogeneic cells and IL-2 into nude mice restore the cytotoxic activity of these animals indicating first that nu/nu mice have CTL-P and second that the missing cells in these animals are those that produce IL-2. Once this helper signal is provided, CTL responses can be achieved. In addition Finberg et al (155) indicated that after s.c. injection of TNP-modified syngeneic cells, they were able to prime radioresistant Th cells which augumented the CTL responses upon incubating in vitro with TNP-modified self. These Th cells were found to be antigen specific. Whether or not these cells provide help for primary CTL responses in vivo was not determined in this work. Recently, it was found that Th activity can be substituted by Con A supernatant (156).

Keen and Forman (157) proposed that there are two helper signals provided in vivo for unprimed CTL-P. Signal 1 is provided by Th 1 and represents the differentiating signal, and signal 2 is generated by Th 2 and represents the proliferation signal by providing IL-2 which is needed by both unprimed CTL-P and memory (primed) CTL-P.

These results are in accordance with those found in vitro (149, 150). Very recently, Behforouz et al (158) indicated that Th cells produce a factor different from T-replacing factor (TRF) or IL-2 after s.c. injection of P815 into syngeneic mice (DBA/2).

These results indicated that Th cells are needed in vivo for the generation of CTL by providing helper signals for the proliferation and for the differentiation of CTL. However, Sakemi et al (47) indicated that whole body X irradiation of mice did not affect the generation of CTL upon the injection of allogeneic spleen cells. These results indicate that the differentiation of CTL-P into alloreactive CTL can be achieved without significant cell proliferation. More direct approach for such conclusion was taken by Kimura and Wigzell (159) who used density gradient (to determine the size and the density of cells) and ^{3}H-thymidine incorporation. CTL in vivo could be generated without noticeable proliferation after i.p. or i.v. injection of tumor cells or lethally irradiated spleen cells. The reason for this discrepancy between the last two reports and the previously mentioned work is not apparent yet.

4.2.2. The role of T-suppressor (Ts) cells

As indicated before, cytotoxic responses mediated by T cells are regulated by positive signals derived from Th cells as well as negative signals provided by Ts cells which down regulate the CTL responses. The field of T suppressor cells has been expanded widely. Therefore, the discussion will be restricted in this section to providing very specific information and trying to concentrate on the Ts cells generated by tumor cells, specifically P815. These tumor cells are mastocytoma cells derived from DBA/2 (H-2^{d}) mice after methycholantherene painting of the mice.

The injection of P815 into DBA/2 mice resulted in direct suppression of the immune response and the rapid metastasis of tumor cells. This was attributed to the appearance of suppressor cells which shut down the host's immune

responses leading to tumor progression and the death of the animals. These suppressor cells were found between 10-19 days after s.c. injection of P815 (160), and were sensitive to treatment with anti-theta antibody indicating that they are T cells (161). T-suppressor (Ts) cells were also generated in the spleen of C57Bl/6J mice after i.p. injection of P815 tumor cells (162). These suppressor cells led to the decrease generation of cytotoxic activity of spleen cells of mice injected with the tumor cells. It was also found that when these spleen cells were added to C57Bl/6J (B6) anti-DBA spleen cells MLC, they inhibited the generation of CTL (163). In vivo, however, the suppressor cells inhibited the proliferative response of normal B6 mice activated by either DBA/2 alloantigens or mitogens (164) as well as the graft versus host reaction (165).

Transfer of Ts cells in vivo was demonstrated by the work of Hellstrom and Hellstrom (166) who found that suppressor cells generated in animals bearing tumors can inactivate the immune response upon transfer into syngeneic mice. It was later found that these Ts cells are of Lyt.1^+ and interact with a radiosensitive T cell provided by the host leading to the enhancement of tumor growth (167). Therefore, the transferred cells were found to be Ts inducers (characterized by being CY resistant) which suppress the CTL in syngeneic recipients only upon interaction with suppressor acceptor cells (characterized by being CY sensitive) provided by the host. Such interaction led to the activation of Ts effector which are the real suppressor cells (168). Rao et al (169) using antigen-antibody complex as toleragen generated Lyt.1^+ CY-resistant Ts inducer cells which upon transfer into syngeneic recipients interact with Ly.123^+ Ts-intermediate to give rise to Ly.23^+ Ts-effectors that suppress the immune response.

Gershon's group identified another circuit in the immune suppression in which Th cells induce Ts cells which shut off the Ts inducer function. They called this circuit feedback inhibition (170, 171).

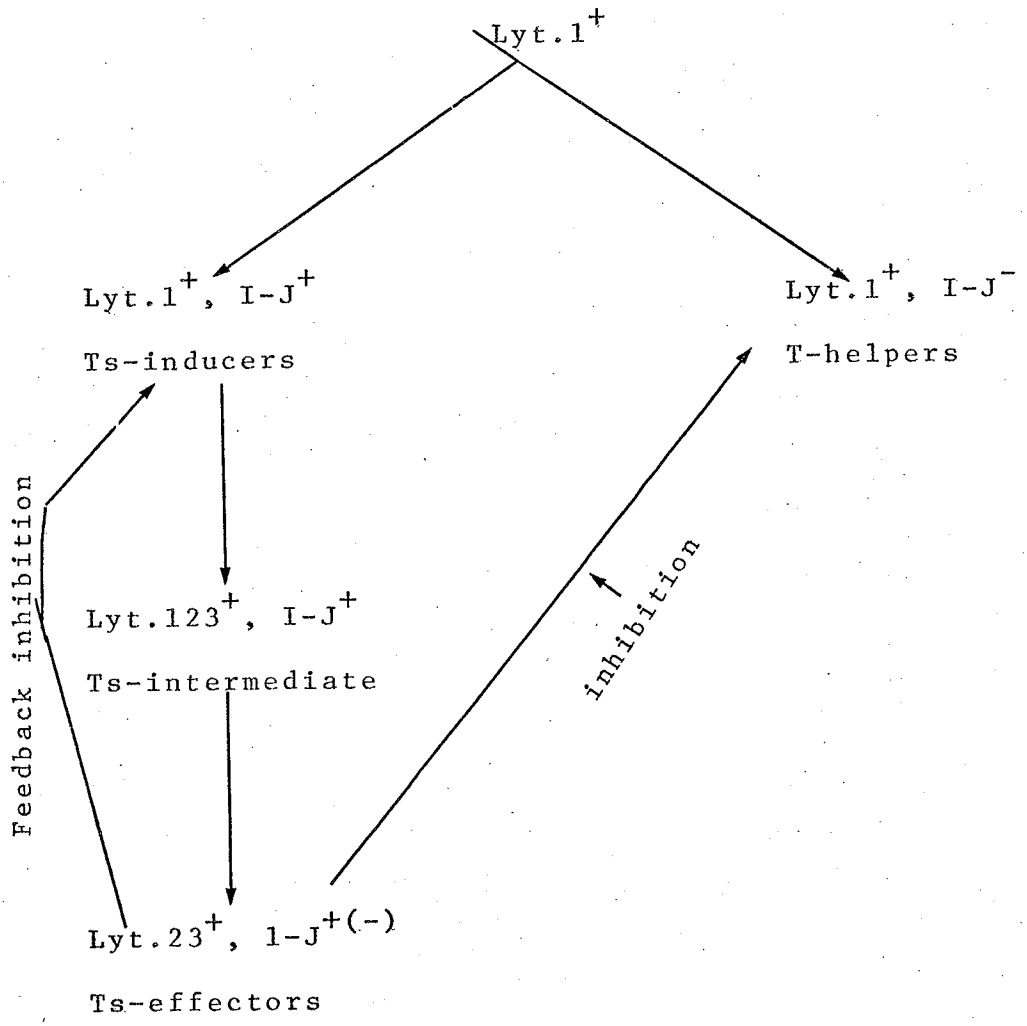


Fig. 3. Pathway of Ts Cells.

According to Gershon's group (170, 171).

Suppressor cells in general have the Ly.23^{+} phenotype and most of these cells or their products carry the I-J determinants (for review, see 172-174). Figure 3 describes the suppressor cell pathway.

Possible mechanisms of Ts Cells

As indicated before, Ts cells act by inactivating the generation of CTL through their inhibition of CTL proliferation. i.e. Ts inhibit signal 2 provided by IL-2. Schwartz et al (175) indicated that in vitro generation of CTL occurs in two steps: step one involves the differentiation of CTL-naive state (CTL-PN) into CTL-activated state (CTL-PA). This stage is under the influence of Th cells and is sensitive to Ts cells action. Whereas the second stage includes the differentiation of CTL-PA into alloreactive CTL. This stage is regulated by another Th cell which is not suppressed by Ts cells.

Mills and Paetkau(176) incubated IL-2 in suppressor culture which consist of tumor bearing (P815) splenocytes of DBA mice stimulated with P815. This system is known to contain Ts cells. However upon IL-2 addition, CTL were generated. When these IL-2 activated T cells were injected in vivo into syngeneic mice receiving P815, they reverse the state of unresponsiveness found in these mice (177). Similar results were found by Sy et al (156) who also reversed the state of CTL unresponsiveness in Ts culture by adding IL-2.

If Ts cells act by inhibiting Th cells or their products, then the question is what is the mechanism by which Ts act? Several experiments were performed to answer this question. Hardt et al (179) indicated that Ts cells (Lyt.23^{+}) release an IL-2 inhibitor factor into the serum of normal mice which acts to neutralize IL-2. These results were confirmed by the work of Klasser et al (180). Also Gautam et al (181) showed that IL-2 inhibitor appears in the serum of mice which inhibit CTL generation to modified syngeneic cells. However, when Mls disparate cells are injected into these mice, CTL to modified cells were observed (182).

This was attributed to the ability of Mls to induce T cells to produce large amounts of IL-2 which combat the inhibitory activity of IL-2 inhibitor (181).

On the other hand, Gunther et al (182) indicated that suppression of CTL in MLC is due to the consumption and/or binding of IL-2 by Ts cells which compete with the CTL for the available IL-2 present in the culture. However, Susskind et al (183) indicated that Ts cells do not compete with the already produced IL-2, rather they inhibit IL-2 production by inactivating IL-2 producer cells ($Lyt.1^+$). Those authors also showed that after the absorption of IL-2 with Ts cells, the former lost its ability to abrogate the unresponsiveness when added in culture. These results indicate that Ts cells carry receptors for IL-2. Recently, Palacios et al (184) showed that IL-2 can activate suppressor cells in the MLR of human lymphocytes.

Ptak and Gershon (185) described another pathway by which Ts cells act. In their system, Ts cells inhibit contact sensitivity reactions by suppressing the antigen presenting cells which were unable to present the antigen to Th cells. However, there is no other report to indicate that such mechanism exist in CTL responses.

4.3. The EFFECTOR PHASE

In this stage CTL inflict the damage to target cells. The initial step of target cell destruction involves adherence of CTL to target cells in immunologically specific manner (186-188). Such specific adherence involves an intermembrane interaction between the antigen recognition structures of the killer cells and the antigenic molecules on the surface of the target cells. After this specific adherence, CTL deliver the lethal hit to the target cells. Asherson et al (189) indicated that once contact between these two cells occured, killing of any target cell can be achieved by these activated CTL, i.e. non-specific killing. However, this may not be the case and killing was observed non-specifically because the above authors used plant lectins which could activate CTL

non-specifically. This latter view is supported by the work of Kuppers and Henney (190,191) who indicated that antigen non specific receptor-antigen interaction is what triggers the lethal hit mechanism. Recently, a direct proof for this latter hypothesis was provided. In this work CTL ($H\text{-}2^b$ anti-$H\text{-}2^d$) was adhered to non-specific ($H\text{-}2^b$) and specific ($H\text{-}2^d$) targets and killing was observed to the specific targets only (192). Those authors also indicated that such killing is local (i.e. restricted to where CTL contacted the specific targets) and not global (i.e. killing in any direction, to any target cells). These results indicated that antigen-specific receptors participate in the actual killing mechanism as well as serve as recognition molecules.

The nature of CTL receptor which recognize antigen was a matter of investigation by many workers. Hollander (108) indicated that anti-Lyt.2 MAb blocks cytolysis of target cells by CTL pointing to the Lyt.2/3 antigen as possible antigenic determinant responsible for recognizing target cell antigens. The effect of anti-Lyt.2 MAb is related to its interaction with Lyt.2 molecules present on the surfaces of CTL and not on target cells (194). More direct evidence for the involvement of Lyt.2 as the determinant responsible for recognizing the target cells antigen came from the work of Shinohara et al (195) who found that anti-Lyt.2 MAb inhibits the cytolysis of CTL generated in vitro in response to alloantigens. Those authors also found that such inhibition is exerted during the Mg^{++}-dependent stage of killing. This stage is known as the antigen recognition stage. In other words, anti-Lyt.2 MAb which blocks the Lyt.2 antigen on the surface of CTL, also blocks the effect of these cells during their recognition of target cells antigens. These results were recently supported by Glasebrook and MacDonald (116).

5. QUANTITATION OF CTL ACTIVITY

Chromium 51 (^{51}Cr) is the isotope widely used to label target cells and to quantitate the effect of CTL, when these are mixed with the target cells (46). ^{51}Cr is an isotope that is easily taken up by most cells by an energy-independent process (193). This isotope is firmly but non-covalently associated with cytoplasmic proteins. Once released from the cells, it is not reutilized (like many other trace-labelled metabolites). It is released in a relatively slowly diffusing form simultaneously with protein (196) and the release reflects irreversible target cell damage (lysis) (197).

Most cell types take up ^{51}Cr. However, labelled cells are often more fragile than unlabelled cells and there is always a spontaneous release of the isotope even in the absence of the effector cells. The spontaneous release varies for different target cells. Freshly explanted tumor cells or cells kept in short-term tissue culture are often unstable in vitro and may exhibit high spontaneous release. ^{51}Cr release is primarily measured in short-term assays under conditions where sufficiently strong lysis of target cells is obtained within 3-6 hours of incubation. In most lytic assays, immune lymphocytes (effector cells) are mixed with ^{51}Cr-labelled target cells in suspension. After the incubation period, lysis is measured as the fraction of isotope released from target cells with detergent or by repeated freezing and thawing. The control samples contain labelled cells with the medium only or with lymphocytes not sensitized against the target cells. These samples release isotope spontaneously which should be reasonably low (about 10% of the maximum release). If high release of the control is obtained, a more correct approximation of specific cytotoxicity is obtained by substracting the percent of release in the control samples from the percent of release in the experimental samples (198). A medium control may not necessarily be the best control, since the total cell concentration will be lower than in the lymphocyte-containing samples, resulting in an enhancement of the spontaneous isotope release from certain target cell types. The problem

can be overcome by the addition of neutral "filler" cells (199,200).

To establish the specificity of the reaction either cold-target inhibition assay is used in which unlabelled target cells are added to the mixture where they inhibit the lysis (201,202) or different target cells that have equal susceptibility to the immune lymphocytes are used (200).

To quantitate the cytotoxic potential of a given lymphocyte preparation, different numbers of lymphocytes are tested with a fixed number of target cells. Normally, the concentration of lymphocytes is more than the target cells when unfractionated cells are used. For example, effector (E) to target (T) ratio varies from 5:1 to 200:1, while similar concentrations for both E and T are used in fractionated population, where CTL are used rather than whole spleen cells or lymph node cells. Although certain numbers of lymphocyte preparations represent the actual effector cells, it is assumed that those cells are the true cytotoxic cells reflecting the cytotoxic potential of a preparation.

The ^{51}Cr-release assay allows measurement of cytotoxicity at the effector cell population level. At the cellular level, an assay has been developed where CTL specifically adhere to target cell monolayers of relevant antigenicity. The latter is achieved by mixing specifically sensitized lymphocytes with target cells in suspension under non-lytic conditions (e.g. at temperature of 15-22^{o}C). In this case, effector and target cells will form stable and specific conjugates. The latter is isolated by centrifugation and is further studied in the light or electron microscopes. The lytic activity of the lymphocytes in the conjugate can be assessed by converting into lytic conditions (raising the temperature to 37^{o}C). This method allows an assessment of the relationship between binding and cytolysis, an estimation of the minimal number of cytolytic effector cells by counting (203) and the characterization of the effector cells and their mode of interaction with the target cells (204,205).

MATERIALS AND METHODS

1. ANIMALS

C57B1/6J (B6), DBA/2, B10.AKM, B10.BR, B10.D2 and B10.M were purchased from Jackson laboratory, Bar Harbor, Maine. The mice were all males used generally at 6-8 weeks of age. The haplotypes of these mice are given in Table 1.

2. ANTIGENS

The antigens used here are mostly allogeneic antigens. Tumor P815, a DBA/2 mastocytoma was a gift from Dr. A. Greenberg (Manitoba Cancer Foundation) and is maintained in a DBA/2 mice by i.p. passage of 4×10^6 cells. Ascites was collected from these mice one week later, the cells were washed twice in HBSS (GIBCO, Long Island, N.Y.) and the cells were adjusted to the desired concentration. Spleen cells (SC) were also used as antigens.

3. ANTISERA

Anti $H\text{-}2^d$ antiserum was prepared by i.p. injection of $10\text{-}20 \times 10^6$/ml B10.D2 ($H\text{-}2^d$) spleen cells into B10.BR ($H\text{-}2^k$) mice at weekly intervals for six weeks. The mice were rested for one month and then boosted with one more injection. The serum was collected and tested for its activity in complement-dependent-cytotoxicity (CDC). Rabbit anti-mouse Ig was prepared by multiple injections of mouse Ig mixed with FCA. Anti-$I\text{-}J^k$ (B10.A(3R)anti-B10.A(5R)) was purchased from Cedarlane laboratories, London, Ontario, Canada). The anti-$I\text{-}J^b$ (B10.A (5R)anti-B10.A(3R)) was a gift from Dr. T. Delovitch (University of Toronto). Anti-Lyt.2.2 was purchased from Cedarlane and anti-Thy.1 was prepared according to Lee & Paraskevas (5).

4. SIX HOUR SERUM (6HS)

Serum was collected from B6 mice six hours after i.p. injection of either 20×10^6/ml P815 or 20×10^6/ml of DBA/2 spleen cells. Serum was also collected 6 hours after i.p. injection of 20×10^6 B10.AKM spleen cells into B10.BR or

TABLE 1 Haplotypes of the mice used.

	K	I	S	D
C57Bl/6J	b	b	b	b
DBA/2	d	d	d	d
B10.AKM	k	k	k	q
B10.BR	k	k	k	k
B10.D2	d	d	d	d
B10.M	f	f	f	f

20×10^6/ml B10.BR spleen cells into B10.AKM.

5. SPLEEN CELLS (SC) PREPARATION

SC were collected from immunized mice aseptically. The cells were teased and then washed twice with HBSS. The erythrocytes were removed by treatment with 0.83% NH_4Cl. The cells were then suspended in the culture medium which contains RPMI-1640 (GIBCO LABORATORIES, N.Y.) supplemented with 5×10^{-5} M mercoptoethenol, 100 units of penicillin, 100 µg of streptomycin and 5% heat-inactivated fetal calf serum (FCS) (GIBCO).

6. PREPARATION OF NWC-NAC

Nylon wool column (NWC)-Non adherent cells (NAC) were prepared according to the method of Julius et al (206). Three gms of NW was teased in saline to remove air bubbles and was packed in 30 cc syringe. The syringe was flushed with 75 ml RPMI + 10% FCS. Spleen cells were teased and washed in HBSS at 100 rpm for 7 minutes twice. Dulbecco's Phosphate Buffered Saline (DPBS) (GIBCO) was added to washed cells (about 3 ml). The NW column which was incubated at 37°C for 1 hr was washed free of RPMI + 10% FCS solution using DPBS + 10% FCS at 37°C until no color was left. The DPBS was removed from the top of the column and spleen cells were layered on top. The cells were allowed to enter into the upper 1/4 of the column and DPBS was layered on top. The column was left undisturbed for 15 min. at 37°C. Using DPBS + 10% FCS solution, the cells were eluted slowly every 15 min., multiple times:

1ST 15 minutes - 2 ml volume - discarded

2ND 15 minutes - 2 ml - collected

3RD 15 minutes - 2 ml - collected

The non adherent cells were washed twice with a large volume of HBSS and centrifuged at 1000 g for at least 15 min. Viability test showed that usually 95% of the cells were viable.

7. TREATMENT WITH ANTI-LYT.1.2. AND ANTI-LYT.2.2 + COMPLEMENT

7.1. Cytotoxicity medium:

The medium was prepared by adding 0.3 gm of bovine serum albumin (BSA, Miles Laboratories Inc. USA) into RPMI-1640 (GIBCO) to make the final concentration 0.3%.

7.2 Treatment with the antibodies:

Spleen cells were passed through NWC and then treated with 0.83% NH_4Cl to remove the erythrocytes. Anti-Lyt.1.2 or anti-Lyt.2.2 was added to a final concentration of 1: 20 (1×10^7 cells were usually used). The suspension was mixed and incubated for 60 minutes at 4^oC. After this time the mixture was centrifuged and the supernatant was discarded. Cedarlane Low-Tox-M rabbit complement (Cedarlane) was added in 1:20 dilution to the original volume of cells. The mixture was incubated for 60 minutes at 37^oC. The cells were washed twice and then counted in trypan blue exclusion test. Cytotoxicity Index was calculated from the following formula:

$$CI = \frac{\% \text{ cytotoxicity in experiment (Ab+C')} - \% \text{ cytotoxicity in complement alone}}{100\ \% - \% \text{ cytotoxicity in complement alone}}$$

Anti-Lyt.1.2 kill about 80% of NWC-NAC

Anti Lyt.2.2 kill about 67% of NWC-NAC

8. RECONSTITUTION OF MICE WITH LYT.1, LYT.2 AND NWC-NAC

Mice were lethally irradiated (850-900 rads, Cobalt 60, Atomic Energy of Canada), and twenty four hours later were injected i.v. with $15\text{-}20 \times 10^6$ of either NWC-NAC, $Lyt.1^+$ or $Lyt.2^+$ cells. Twenty four hours after reconstitution, they were injected with the corresponding antigens and serum was collected 6 hours after antigenic stimulation.

9. PREPARATION OF THE IMMUNOADSORBENTS

9.1. Preparation of rabbit anti-mouse Ig

Rabbit anti-mouse Ig serum (17.5 ml) was added to 17.5 ml of ammonium sulphate (50%). The mixture was stirred for 5-10 minutes and was spun at 10,000 rpm for 10 minutes. The supernatant was discarded and the precipitate was dissolved in 5-10 ml of 0.15 M NaCl which was made 40% in $(NH4)_2\ SO_4$. The suspension was centrifuged at 1000 rpm for 10 minutes and the precipitate was dissolved again in 5 ml of NaCl (0.15 M) and dialyzed in 2 liters of 0.1M NaHCO3+0.5 M NaCl overnight.

9.1.1. Coupling procedure

One gm of Sepharose-4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was placed in 50 ml round bottom tube and washed 4-5 times with 40 ml of 0.001 N HCl. It was centrifuged at 2000 rpm for 15-20 minutes at room temperature. The supernatant was discarded and the gel was washed once with 0.1 M $NaHCO_3$+0.5 M NaCl. To each gm of washed gel, 4 ml of the antibody preparation was added. The sample was centrifuged at 10,000 rpm after overnight dialysis. After centrifugation, the supernatant was removed and the O.D. was measured to determine the amount of protein coupled to the gel. After two washes with $NaHCO_3$-0.5 M NaCl buffer, five ml of 1M ethanolamine made in 0.1M $NaHCO_3$-0.5M NaCl was added to the gel which was left for 2 hrs under shaking. Finally the gel was washed three more times with a) 0.1M acetate buffer + 0.5 M NaCl (pH 3.7); b) 0.15 M borate saline + 0.5 M NaCl (pH 7.9); and c) 0.15 M borate saline (pH 8.0). Normally, 3 ml of the 6HS was passed through 10 ml of packed gel. Passing the same amount of normal serum through the same column was found to remove all the Ig activity as tested by the immunoelectrophoresis. Therefore, it is suggested that Rabbit Anti-mouse Ig sepharose 4B can remove all the Ig activity from the 6HS.

9.2. Preparation of anti-H-2^d column

Anti-H-2^d (B10.BR anti-B10.D2) antiserum was coupled to Cyanogen bromide activated Sepharose-4B according to the above method.

10. ASSAYS FOR THE ENHANCING FACTOR

The enhancing activity of the 6HS was assayed according to the following protocol: a) B6 mice; i.v. injection of 0.3 ml 6HS-P815 followed two hours later by an i.v. injection of either 1 or 5 x 10^6/ml P815.

b) B6 mice; injection of 0.3 ml 6HS-DBA/2 SC followed two hours later with a challenge of 1 x 10^6/ml DBA/2 SC.

c) B10.AKM mice; injection with 0.3 ml 6HS-B10.BR SC followed by a challenge two hours later with 1 x 10^6/ml B10.BR SC.

d) B10.BR mice; injection with 0.3 ml 6HS-B10.AKM SC challenged two hours later with 1 x 10^6 B10.AKM SC.

Normal mouse serum (NMS) was used as control. The mice were left for 1 and 2 weeks and then tested for CTL activity.

10.1. Test of specificity for 6HS enhancement

a) B10.AKM mice injected with 6HS-B10.BR SC were challenged with either 1 x 10^6/ml B10.BR SC or B10.M SC.

b) B10.BR mice injected with 6HS-B10.AKM SC were challenged with either 1 x 10^6/ml B10.AKM SC or B10.M SC.

11. AUGMENTATION OF CTL RESPONSES

11.1. In vitro incubation for 24 hours

SC were assayed for CTL activity after culture in vitro for 24 hours (207). SC of mice injected with 6HS, NMS or P815 only were divided into two aliquots; one tested for CTL activity immediately upon removal of the cells from the animals while the second was incubated in vitro in a Marbrook system (Bio Research Glass, Vineland, N.J.) Three ml of cells (2 x 10^7/ml) suspended in culture medium were placed in the inner vessel while the outer vessel contained 25 ml of the culture medium. The cultures were incubated at 37°C in 5% CO_2 incubator, for 24 hours. At the end of the culture period, the cells were pelleted, washed in the medium and their viability was determined by the trypan blue exclusion test. Usually, 50-75% of the cells were viable. These cells were then tested for CTL activity.

11.2. Treatment of mice with anti-I-J-antisera

The method of Green et al (208) and Perry et al (209) was followed with some modification. Four µl of the antisera (anti-I-J^b and anti-I-J^k) in 0.2 ml of distilled water were injected i.v. into B6 mice for two days. On day three, the mice received 20 µl of the antiserum in 0.2 ml distilled water. Twenty four hours later, the mice were injected with the serum as previously explained.

11.3. Treatment of mice with Cyclophosphamide (CY)

Cytoxan (Bristol laboratories, Belleville, Canada) was injected i.p. in B6 mice in a dose of 100 mg/kg body weight, two days before the antigenic stimulation.

12. RECONSTITUTION OF THE CY-TREATED MICE WITH NSC

B6 mice which have received CY at day -2 were injected i.v. with 40-50 x 10^6 NWC-NAC according to the methods of Rollinghoff et al (210) and others (211). Four to five hours later the mice were treated with either 6HS-P815 or NMS. These mice were termed CY-NWC-6HS and CY-NWC-NMS respectively.

13. RECONSTITUTION OF THE 6HS-TREATED MICE WITH NSC

In this group of B6 mice, 0.3 ml of 6HS-P815 or NMS was injected i.v. on day -2. On day 0 the mice received 40-50 x 10^6 NWC as above. Four to five hours later the mice were challenged with the antigen (P815). These mice were termed 6HS-NWC and NMS-NWC respectively.

14. ENRICHMENT OF SUPPRESSOR CELLS

The method of Chiu et al (212) was used. Briefly, P815 tumor cells were heat-inactivated at 56°C for 10 minutes and after cooling were injected i.v. into B6 mice at different concentrations. Five to seven days later, 50 x 10^6 SC from mice injected with heat-treated (HT) P815 were treated with mitomycin-C and transferred into B6 mice which were then challenged with 5 x 10^6 P815. Control mice received saline instead of the HT-P815.

15. THE EFFECT OF THE 6HS ON THE INDUCTION OF SUPPRESSOR CELLS

6HS-P815 or NMS (0.3 ml) was injected i.v. into B6 mice which immediately received HT-P815 cells. SC were transferred as shown above.

16. THE EFFECT OF THE 6HS AT THE EFFECTOR STAGE OF SUPPRESSOR CELLS

B6 mice which have received 50 x 10^6 cells (as above) were challenged with 0.3 ml of 6HS-P815 or NMS immediately after the transfer of the spleen cells. For specificity, mice received 0.3 ml of 6HS collected after the injection of 5 x 10^8 SRBC (14).

17. ASSAY FOR THE PRESENCE OF ANTI-SUPPRESSOR CELLS

B6 mice were divided into 4 groups and were treated as follows:

a) received 20 x 10^6 HT-P815 and served as a source for Ts cells;

b) received 0.3 ml 6HS-P815;

c) received NMS and

d) received saline.

Spleen cells were collected from these mice 5-7 days later and passed aseptically through NWC. Non adherent cells were treated with mitomycin-C and cultured in the MLR which contained 5 x 10^6 normal responder cells (B6) and 5 x 10^6 P815 mitomycin treated cells as stimulators. The cells were cultured for 5 days in Linbro tissue culture plates (Linbro Division, Flow Laboratories Inc., Hamden, Conn.) and then tested for CTL activity.

17.1. Treatment with Mitomycin-C

Cells which were used as antigen in the MLR or for adoptive transfer were treated with mitomycin-C (Sigma Chemicals. St. Louis) according to the method of Argyris (162-165). The cells were incubated with 50 µg/2ml mitomycin-C in PBS at 37°C for 30 minutes. After this time, they were washed extensively in the buffer medium for at least three times to remove all the residual mitomycin-C.

18. DETECTION OF Ig^+-CELLS

The RICA technique was used for the detection of surface Ig on lymphocytes. Details of this technique have been published elsewhere (1,2,4). Briefly, a 5S hybrid antibody with two combining sites was used. Through its anti-Ig site it binds the Ig on the cell surface, while through its other site (anti-BSA) it binds to BSA-coated SRBC, thus forming a rosette. The number of rosette forming cells were calculated by counting 1000 cells. We use the terms rosette forming cells (RFC) and Ig^+ cells interchangeably.

19. PREPARATION OF THE TARGET CELLS

The following 5 types of target cells were used:

a) P815, b) DBA/2 SC, c) B10.AKM SC, d) B10.BR SC and e) B10.M SC.

The cells were collected aseptically and resuspended in the culture medium. Spleen cells that were used as targets (5×10^6) were incubated with 5 µg/ml of Con-A (Miles-Yeda, St. Louis) in 50 ml culture flasks for 48 hrs at 37°C in 5% CO_2. After the culture, the cells were washed in the medium supplemented with alpha-methyl-D-mannoside (Sigma chemicals laboratories) to block the remaining Con-A.

20. CELL-MEDIATED-CYTOTOXICITY ASSAY

Target cells were labelled with ^{51}Cr by incubating 5×10^6 cells with 100-200 µCi of $Na2^{51}Cr$ (New England Nuclear, Boston, Mass.) in 0.5 ml of the culture medium for 1 hr at 37°C. The cells were washed three times in the same media. The assay was performed in triplicate in Linbro microtiter plates by incubating 100 µl of target cells with 100 µl of effector cells at the desired concentration. The plates were incubated in 5% CO_2 and 100 µl was aliquoted from each well and assayed for radioactivity in Beckman Gamma Counter. Percentage of cytotoxicity was calculated according to the following:

$$\% \text{ Cytotoxicity} = \frac{\text{Exp. release} - \text{spontaneous release}}{\text{Max. release} - \text{spontaneous release}} \times 100$$

Experimental release is the CPM in supernatant of target cells mixed with effector cells. Spontaneous release is CPM in the supernatant of target cells cultured alone and maximum release is CPM after lysis of target cells with either Triton-X or after 4 rounds of freeze-thawing. Spontaneous release for P815 target cells was between 8-15%, whereas spontaneous release for SC was between 16-28% of the total release.

21. STATISTICAL ANALYSIS

Data shown in Tables and Figures represent the means of triplicate determinations and are representative of at least seven separate experiments. Statistical analysis were performed using a Student T test and an Olivetti Programma 101 electronic desk computer. Each point represents the mean $\pm$ S.E.

RESULTS

1. THE INDUCTION OF THE ENHANCING FACTOR

1.1. The induction of the 6HS using allogeneic tumor cells

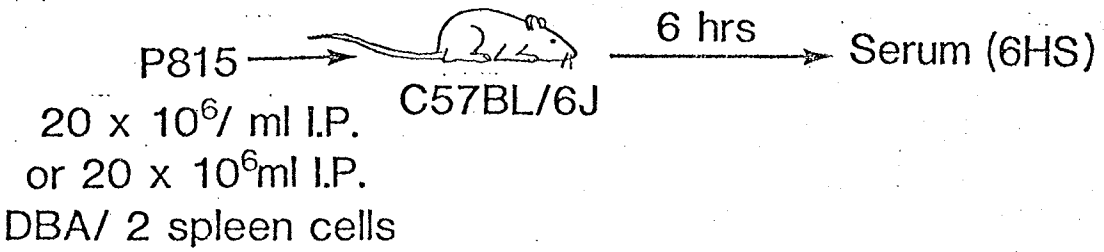
Six hour serum was induced by i.p. injection of 20 x 10^6 P815 (H-2^d) into B6 (H-2^b) mice. (Fig. 4A). Although different tumor cell concentrations were shown to produce a 6HS which contains an Ig "cytophilic" for T cells, only 20 x 10^6 dose was used throughout this study. For the detection of the "cytophilic" Ig, the serum was incubated with normal spleen cells (NSC) from B6 mice and the uptake of Ig by these cells was tested by the RICA method. As shown in Table II, cells which were incubated with the 6HS showed an increase of Ig, but no increase was detected in cells incubated with NMS. Extensive studies carried out previously in this laboratory has documented that the increase of Ig^+ cells is due to the uptake by T cells (which are Ig^-) of a "cytophilic" Ig present in 6HS.

1.2. 6HS collected after allogeneic tumor injection enhances CTL response

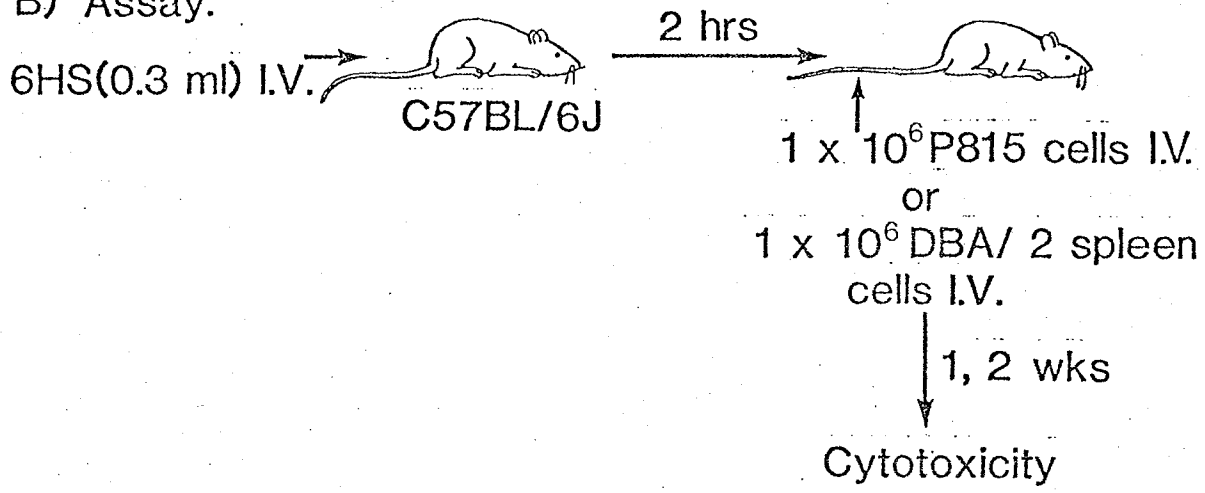
It has been shown that 6HS collected after SRBC stimulation enhanced the production of the IgG antibody (14). The question was therefore asked whether the 6HS collected after allogeneic stimulation will enhance the CTL response. Fig. 4B shows the protocol used to test 6HS for possible enhancing activity for CTL. B6 mice were injected i.v. with 0.3 ml of the 6HS while control groups received 0.3 ml of either saline or NMS. All animals were challenged 2 hours later with an i.v. injection of 1 x 10^6 P815 cells. Fig. 5 shows that only mice injected with the 6HS show enhancement of CTL. This effect was demonstrated both at one and two weeks after the challenge with tumor cells. Enhancement of the CTL response was also observed when 5 x 10^6 P815 cells were used instead of 1 x 10^6 (Fig. 6). Injection of 6HS alone (without challenging with alloantigen) was unable to enhance the CTL response (data not shown).

Fig. 4
PRODUCTION AND ASSAY OF THE ENHANCING FACTOR

A) Production:



B) Assay:



Controls for B:

1) Normal mouse serum is injected instead of 6HS
2) P815 or DBA/ 2J spleen cells injected alone

TABLE II

THE INDUCTION OF CYTOPHILIC I_G BY ALLOGENEIC TUMOR CELLS

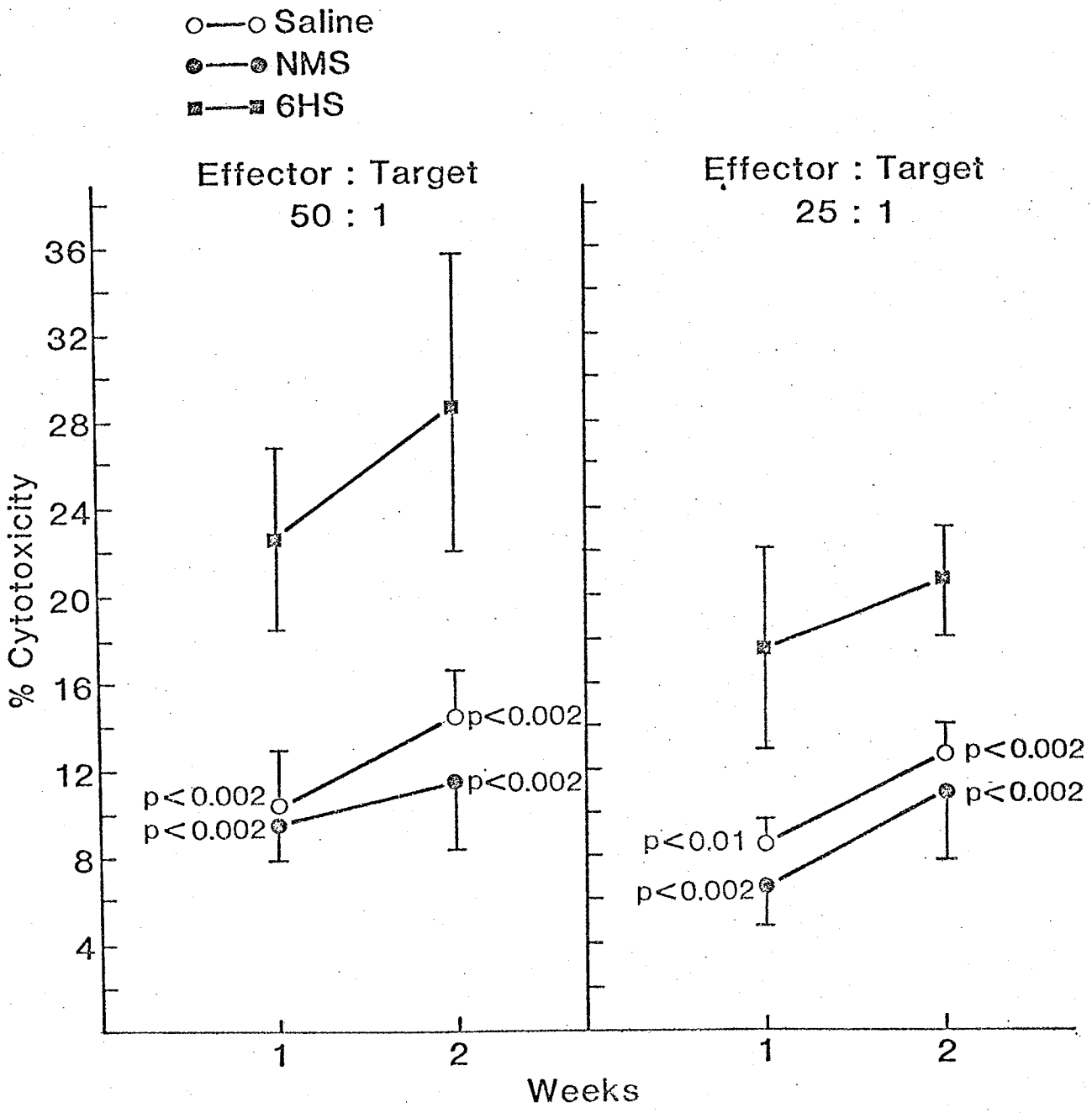
	I_G+ CELLS/1000 SPLEEN CELLS	
TREATMENT	EXP. 1	EXP. II
A) NONE	320	310
B) NMS	324	325
C) 6HS	410	425

6HS IS COLLECTED FROM B6 MICE AFTER THE INJECTION OF 20×10^6 P815 TUMOR CELLS. THE SERUM WAS INCUBATED WITH NORMAL SPLEEN CELLS (B6) AND THE UPTAKE OF I_G WAS TESTED BY THE RICA METHOD.

CONCLUSION: INJECTION OF ALLOGENEIC TUMOR CELLS GENERATES A CYTOPHILIC IG WHICH IS TAKEN UP BY NORMAL SPLEEN CELLS.

Fig.5

IN VIVO CTL RESPONSE AFTER STIMULATION WITH 1 x 10^6 P815

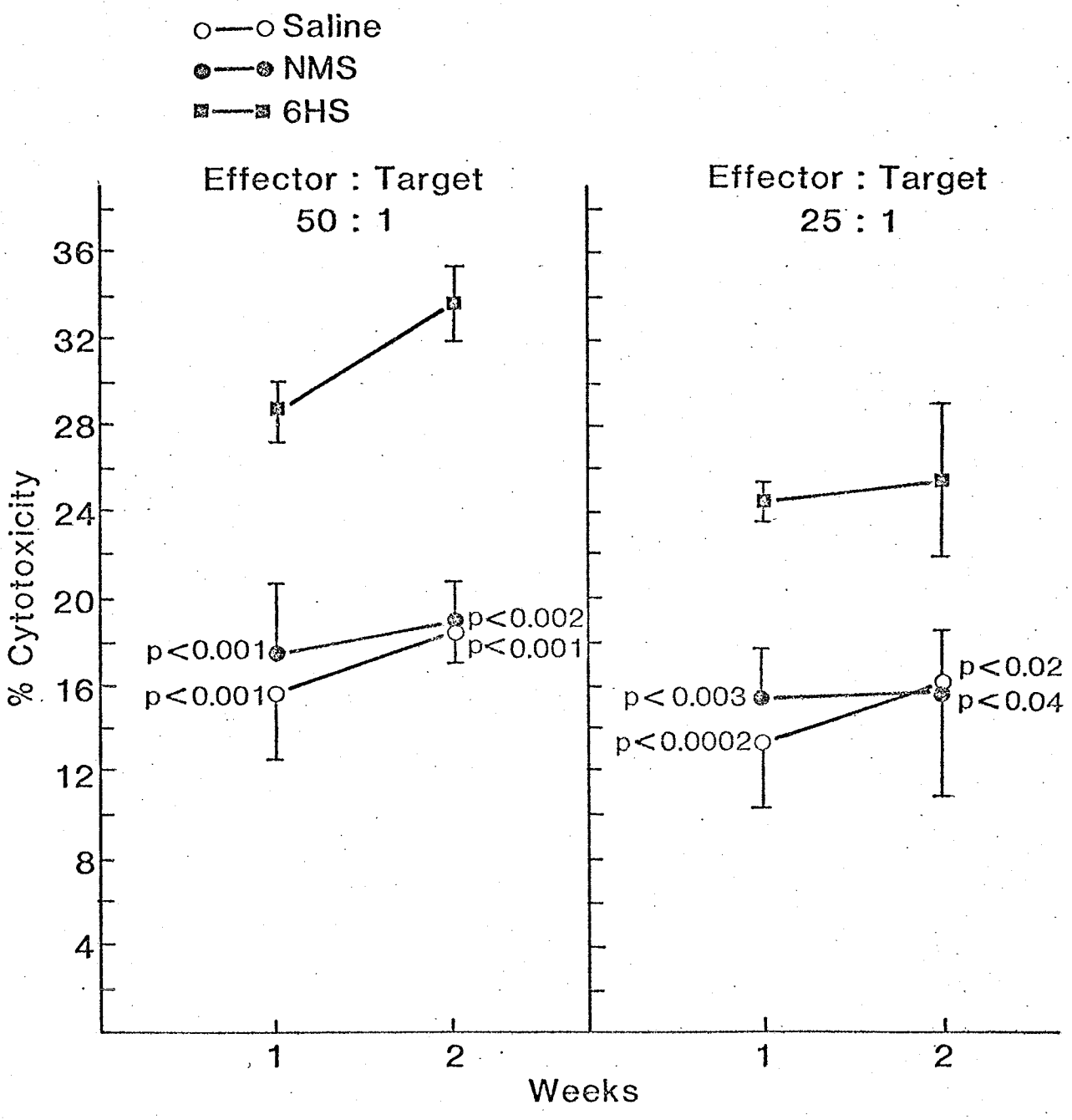


LEGEND FIG. 5:

6HS COLLECTED FROM B6 MICE AFTER STIMULATION WITH 20 x 10^6 P815 WAS INJECTED I.V. (0.3 ML) INTO NAIVE B6 MICE (■). CONTROL MICE RECEIVED 0.3 ML OF EITHER SALINE (○) OR NMS (●). TWO HOURS LATER THE MICE WERE INJECTED I.V. WITH 1 x 10^6 P815.
TARGET CELLS = P815. SPONTANEOUS RELEASE = 10%.

Fig. 6

IN VIVO CTL RESPONSE AFTER STIMULATION WITH 5 x 10^6 P815



LEGEND FIG. 6:

SEE LEGENDS OF FIG. 5. A 5×10^6 P815 DOSE OF ANTIGEN WAS USED AS CHALLENGE INSTEAD OF 1×10^6.

1.3. The induction of 6HS using allogeneic cells

We have also demonstrated that not only allogeneic tumor cells but also injection of allogeneic normal SC can induce a "cytophilic" Ig. Twenty x 10^6 DBA/2 SC (H-2^d) were injected into B6 mice and six hours later the serum was collected. This serum was incubated with NSC from B6 mice. As shown in Table III, NSC incubated with the 6HS but not with NMS showed an increase of Ig^+ cells as detected by RICA.

1.4. 6HS collected after allogeneic stimulation enhances CTL response

Since the 6HS induced by allogeneic NSC contained a "cytophilic" Ig, it was tested whether it will also enhance the CTL response. The protocol of the assay is the same as that used for 6HS of allogeneic tumor cells. As shown in Fig. 7 the 6HS enhanced the CTL response following a challenge with a low dose (1 x 10^6 cells) of normal DBA/2 SC.

1.5. The induction of 6HS using only H-2D-region coded determinants

We have asked the question if the induction of the 6HS can occur to MHC antigens coded by only one region. To answer this question we have used two congenic strains of mice which differ at the D-region only. Either 20 x 10^6 B10.AKM (k k k k q) SC were injected i.p. into B10.BR (k k k k k) mice or 20 x 10^6 B10.BR SC were injected into B10.AKM mice. Six hours serum collected from B10.BR after stimulation with B10.AKM SC was shown to contain a "cytophilic" Ig when it was incubated with B10.BR NSC (Table IV). Similar results were obtained with the 6HS collected from B10.AKM mice after stimulation with B10.BR SC (Table V). These results indicate that D-region coded determinants can induce "cytophilic" Ig for normal spleen cells.

1.6. 6HS produced by H-2D-region coded determinants enhances the CTL response

To test whether the 6HS collected after stimulation with H2-D antigens 0.3 ml of 6HS collected from B10.BR after stimulation with B10.AKM SC, enhanced the CTL when injected i.v. into B10.BR mice as compared to mice receiving NMS

TABLE III

THE INDUCTION OF CYTOPHILIC Ig BY NORMAL ALLOGENEIC CELLS

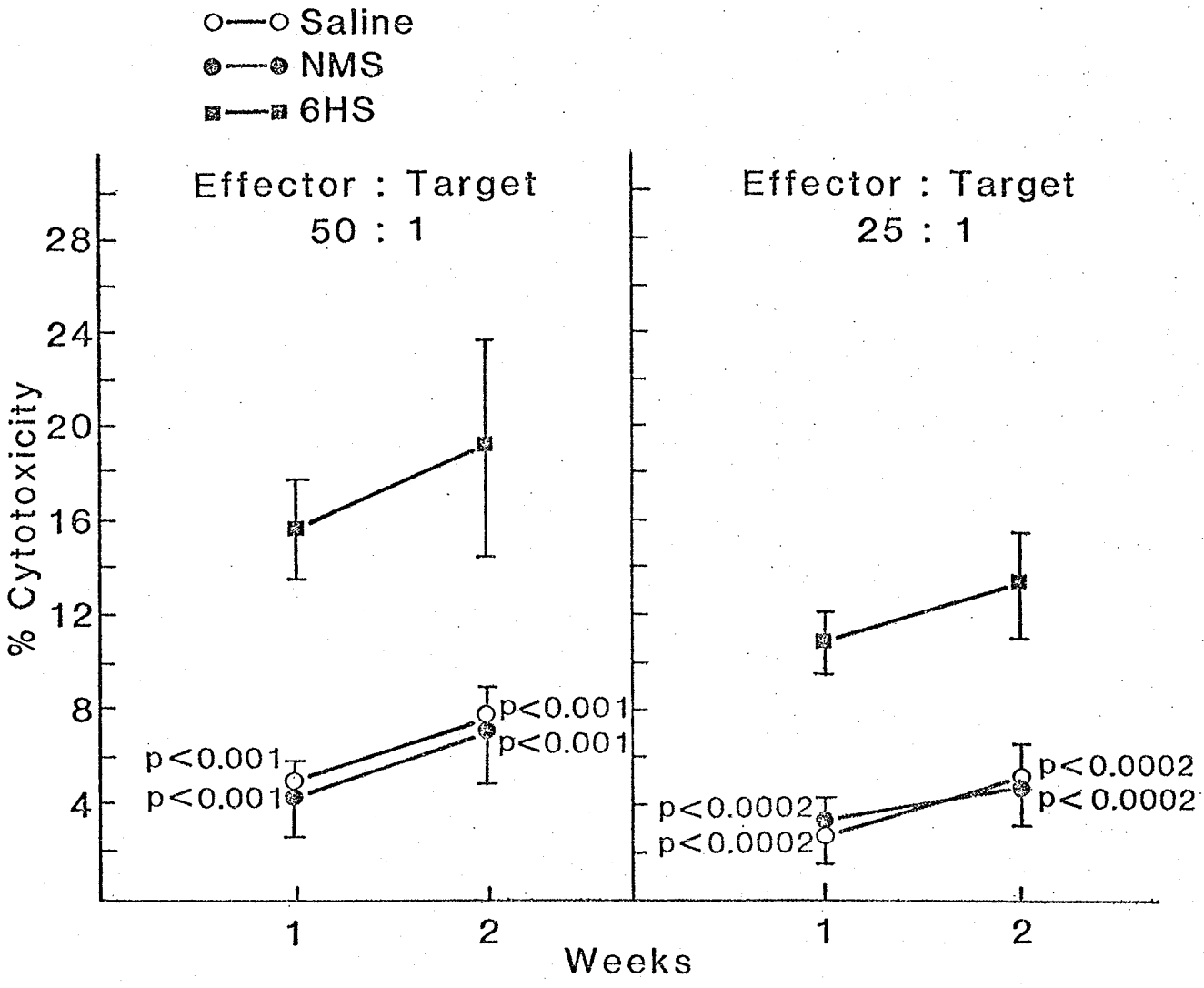
	Ig^+ CELLS/1000 SPLEEN CELLS	
TREATMENT	EXP. I	EXP. II
A) NONE	280	280
B) NMS	300	305
C) 6HS	400	405

6HS IS COLLECTED FROM B6 MICE AFTER THE INJECTION OF 20×10^6 DBA/2 SPLEEN CELLS. THE SERUM WAS INCUBATED WITH NORMAL SPLEEN CELLS (B6) AND THE UPTAKE OF Ig WAS TESTED BY THE RICA METHOD.

CONCLUSION: INJECTION OF NORMAL ALLOGENEIC CELLS GENERATES A CYTOPHILIC Ig WHICH IS TAKEN UP BY NORMAL SPLEEN CELLS.

Fig. 7

IN VIVO CTL RESPONSE AFTER STIMULATION WITH 1 x 10^6 DBA /2 SPLEEN CELLS



LEGEND FIG. 7:

6HS COLLECTED FROM B6 MICE AFTER STIMULATION WITH 20×10^6 DBA/2 SC WAS INJECTED I.V. (0.3 ML) INTO NAIVE B6 MICE (■). CONTROL MICE RECEIVED 0.3 ML OF EITHER SALINE (○) OR NMS (●). TWO HOURS LATER THE MICE WERE INJECTED I.V. WITH 1×10^6 DBA/2 SC.

TARGET CELLS = CON-A BLAST DBA/2 SC. SPONTANEOUS RELEASE = 20%.

TABLE IV

THE INDUCTION OF CYTOPHILIC Ig BY D-REGION DIFFERENCES

	Ig+ CELLS/1000 SPLEEN CELLS	
TREATMENT	EXP. I	EXP. II
A) NONE	320	322
B) NMS	327	330
C) 6HS	419	425

6HS IS COLLECTED FROM B10.BR (K K K K K) MICE AFTER I.P. INJECTION OF 20 X 10^6 B10.AKM(K K K K Q) SPLEEN CELLS. THE SERUM WAS INCUBATED WITH NORMAL SPLEEN CELLS (B10.BR) AND THE UPTAKE OF Ig BY THESE CELLS WAS TESTED BY THE RICA METHOD.

CONCLUSION: 6HS COLLECTED AFTER STIMULATION WITH CELLS WHICH DIFFER ONLY IN D-REGION CONTAINS A CYTOPHILIC Ig WHICH IS TAKEN UP BY NORMAL SPLEEN CELLS.

TABLE V

THE INDUCTION OF CYTOPHILIC IG BY D-REGION DIFFERENCES

TREATMENT	IG+ CELLS/1000 SPLEEN CELLS EXP. I	EXP. II
A) NONE	319	315
B) NMS	328	330
C) 6HS	415	435

6HS IS COLLECTED FROM B10.AKM (K K K K Q) MICE AFTER I.P. INJECTION OF 20 X 10^6 B10.BR (K K K K K) SPLEEN CELLS. THE SERUM WAS INCUBATED WITH NORMAL SPLEEN CELLS (B10.AKM) AND THE UPTAKE OF IG WAS TESTED BY THE RICA METHOD.

CONCLUSION: 6HS COLLECTED AFTER STIMULATION WITH CELLS WHICH DIFFER ONLY IN D-REGION CONTAINS A CYTOPHILIC IG WHICH IS TAKEN UP BY NORMAL SPLEEN CELLS.

or saline (Fig. 8). The enhancement was again detected 1 and 2 weeks after challenge. Fig. 9 shows the reverse experiment. In this case, 6HS collected from B10.AKM mice after stimulation with 20×10^6 B10.BR SC enhanced the CTL response when injected i.v. (0.3 ml) into B10.AKM two hours before challenging with 1×10^6 B10.BR SC.

2. SPECIFICITY OF THE 6HS ENHANCING ACTIVITY

In the humoral response it was shown that the 6HS enhancing activity was antigen specific. Since inbred strains of mice even of different H-2 haplotypes share public specificities we decided to examine the specificity of allogeneically induced 6HS by limiting the H-2 differences possibly to only one H2 region. As shown in Table VI section I-A 6HS collected from B10.AKM mice after stimulation with B10.BR can enhance the CTL response of B10.AKM mice against B10.BR SC but not against the unrelated H2 antigens of B.10M SC (Section I-B). Similar results were obtained with the reverse combination. Thus the 6HS induced in B10.BR mice against the B10.AKM SC enhances specifically the CTL response against the B10.AKM SC (Section II-A) but not against the B10.M (Section II-B). These results suggest that the CTL enhancing activity is specific to the alloantigens used for its induction.

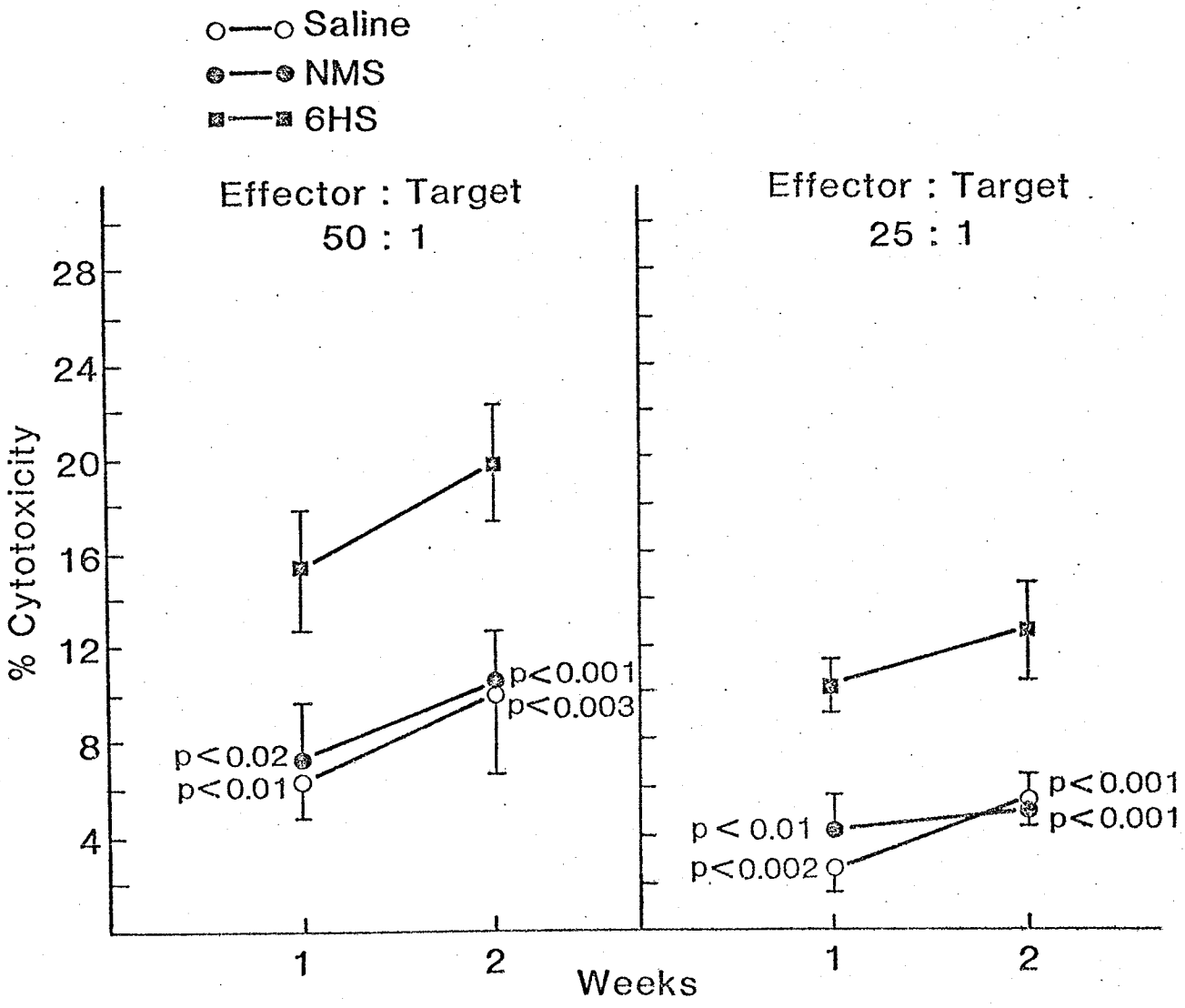
3. COMPOSITION OF THE ENHANCING FACTOR

3.1. 6HS enhancing factor contains Ig

It was previously found that the enhancing factor in the 6HS collected after antigenic stimulation with foreign antigens contains Ig (15). Therefore, it was important to determine whether the CTL enhancing factor in the 6HS collected after allogeneic stimulation also contains Ig. Six hour serum was collected after stimulation with 20×10^6 P815 and passed through rabbit anti-mouse Ig-sepharose 4B column. As control the serum was passed through normal

Fig. 8

IN VIVO CTL RESPONSE AFTER STIMULATION OF BIO·BR MICE WITH 10^6 BIO·AKM SPLEEN CELLS (D-REGION DIFFERENCE)



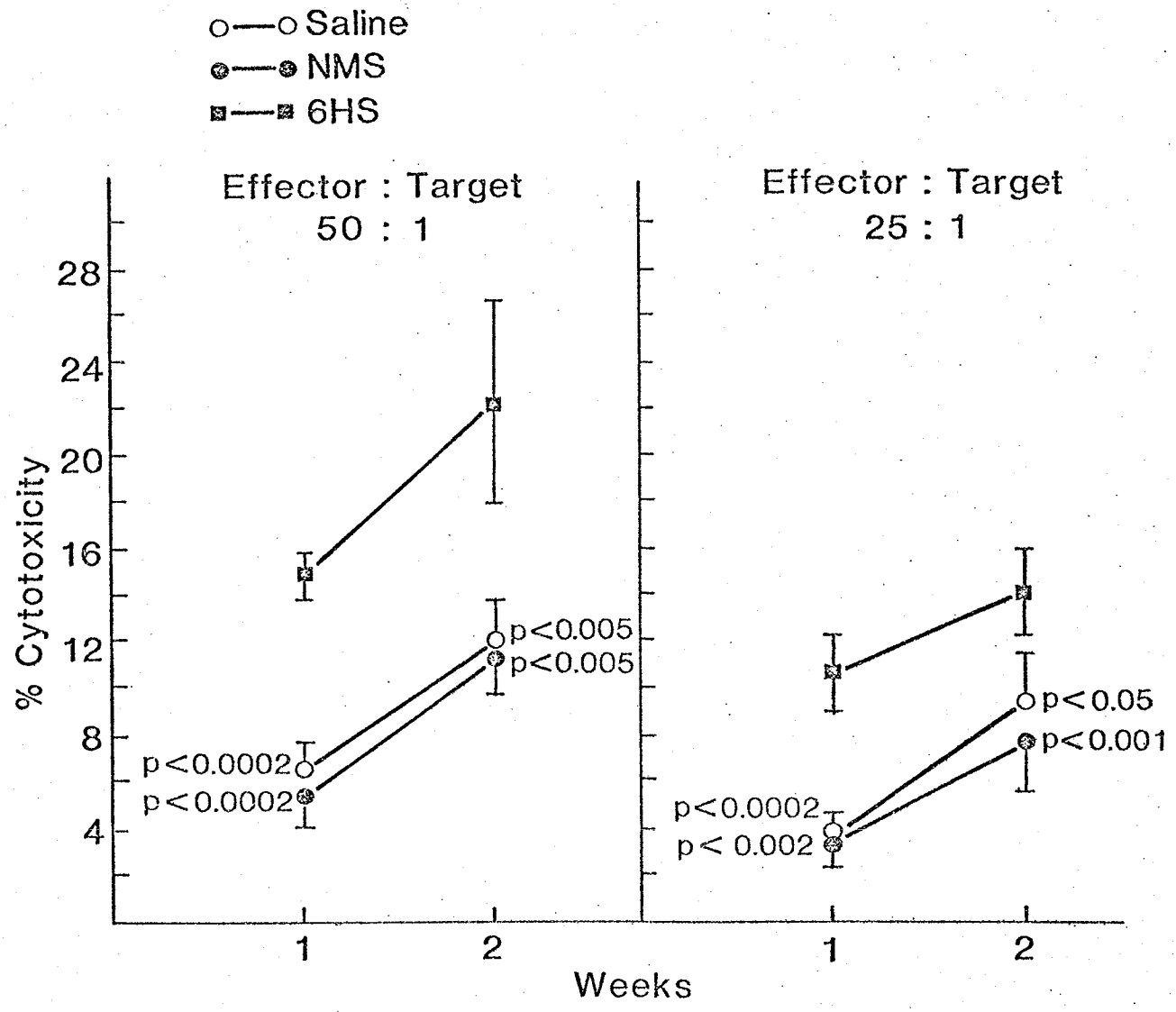
LEGEND FIG. 8:

6HS COLLECTED FROM B10.BR (K K K K K) MICE AFTER STIMULATION WITH 20 x 10^6 B10.AKM (K K K K Q) SC WAS INJECTED I.V. (0.3 ML) INTO NAIVE B10.BR MICE (■). CONTROL MICE RECEIVED 0.3 ML OF EITHER SALINE (○) OR NMS (●). TWO HOURS LATER THE MICE WERE INJECTED I.V. WITH 1 x 10^6 B10.AKM SC.

TARGET CELLS = CON-A BLAST B10.AKM SC. SPONTANEOUS RELEASE = 27-30%.

Fig. 9

IN VIVO CTL RESPONSE AFTER STIMULATION OF BIO·AKM MICE WITH 1 x 10^6 BIO·BR SPLEEN CELLS



LEGEND FIG. 9:

6HS COLLECTED FROM B10.AKM MICE AFTER STIMULATION WITH 20 x 10^6 B10.BR SC WAS INJECTED I.V. INTO B10.AKM MICE (■). CONTROL MICE RECEIVED 0.3 ML OF EITHER SALINE (○) OR NMS (●). TWO HOURS LATER THE MICE WERE INJECTED I.V. WITH 1 x 10^6 B10.BR SC.

TARGET CELLS = CON-A BLAST B10.BR SC SPONTANEOUS RELEASE = 27.5%

TABLE VI

SPECIFICITY OF THE 6HS ENHANCING ACTIVITY

CE	SERUM	CHALLENGE	% CYTOTOXICITY			
			1 WK		2 WKS	
			E : T		E : T	
			50:1	25:1	50:1	25:1
10.AKM	NONE	B10.BR(SC)[A]	6.1 ± 1.4	4.1 ± 0.8	12.0 ± 1.3	8.7 ± 1.9
10.AKM	NMS	B10.BR(SC)	5.1 ± 1.1	4.1 ± 1.0	11.2 ± 1.8	6.9 ± 1.5
10.AKM	6HS.B10.BR[B]	B10.BR(SC)	14.7 ± 1.3	10.8 ± 1.8	22.2 ± 4.4	16.1 ± 4.0
10.AKM	NONE	B10.M[C](SC)	6.4 ± 0.4	4.5 ± 0.6	12.7 ± 4.5	9.8 ± 5.0
10.AKM	NMS	B10.M (SC)	6.0 ± 1.0	3.6 ± 0.5	10.5 ± 2.9	7.5 ± 2.2
10.AKM	6HS.B10.BR	B10.M (SC)	7.2 ± 1.0	4.5 ± 1.5	12.5 ± 2.8	9.0 ± 3.2
10.BR	NONE	B10.AKM(SC)	5.5 ± 1.3	4.5 ± 1.8	9.8 ± 2.3	3.5 ± 1.5
10.BR	NMS	B10.AKM(SC)	4.2 ± 1.3	2.1 ± 0.8	8.3 ± 1.5	3.0 ± 0.9
10.BR	6HS.B10.AKM[D]	B10.AKM(SC)	17.3 ± 2.6	12.8 ± 3.1	20.0 ± 2.1	15.9 ± 2.2
10.BR	NONE	B10.M (SC)	6.3 ± 1.1	4.6 ± 0.7	10.2 ± 2.2	10.2 ± 2.2
10.BR	NMS	B10.M (SC)	5.2 ± 1.2	3.6 ± 0.9	10.2 ± 3.0	3.1 ± 1.0
10.BR	6HS.B10.AKM	B10.M (SC)	6.4 ± 2.1	4.5 ± 1.7	11.9 ± 2.1	4.1 ± 2.2

A) SC = SPLEEN CELLS

B) 6HS IS COLLECTED FROM B10.AKM (K K K K Q) MICE AFTER STIMULATION WITH 20 X 10^6 B10.BR (K K K K K) SC

C) 1 X 10^6 B10.M (F F F F F) SC WERE USED TO CHALLENGE THE MICE AFTER THE INJECTION OF THE SERUM.

D) 6HS IS COLLECTED FROM B10.BR MICE AFTER STIMULATION WITH B10.AKM SC.

IN I, B10.AKM MICE WERE INJECTED WITH 0.3 ML OF EITHER 6HS COLLECTED FROM B10.AKM AFTER STIMULATION WITH B10.BR MICE (HENCE THE SERUM IS CALLED 6HS B10.BR) (C). SALINE (A) OR NMS (B). THE MICE WERE CHALLENGED WITH THE SPECIFIC ANTIGEN USED TO PRODUCE THE 6HS, I.E. B10.BR(SC) (SECTION A) OR UNRELATED ALLOANTIGEN, I.E. B10.M(SC) (SECTION B). TARGET CELLS WERE CON-A BLAST B10.BR(SC) OR CON-A BLAST B10.M(SC) RESPECTIVELY.

IN II, THE OPPOSITE EXPERIMENT IS PERFORMED. TARGET CELLS WERE CON-A BLAST B10.AKM(SC) OR CON-A BLAST B10.M(SC).

CONCLUSION: 6HS ENHANCES CTL RESPONSE SPECIFICALLY.

rabbit Ig (NRG)-sepharose 4B column (Fig. 10). As shown in Table VII, after passing through anti-Ig column the 6HS lost its "cytophilic" Ig as tested by RICA (Table VII.c), whereas the NRG column had no effect (Table VII.d). The 6HS after removal of the "cytophilic" Ig by the anti-Ig column was tested for its CTL enhancing activity as described before (see Fig. 4). As shown in Fig. 11 the 6HS which was passed through anti-Ig column lost its ability to enhance the CTL response. These results indicate that the enhancing factor for CTL contains Ig and are similar to the results obtained in the humoral responses against foreign antigens.

3.2. 6HS enhancing factor contains antigenic determinants

Based on the findings of humoral response which showed that 6HS collected after SRBC stimulation contains antigen (15), we decided to determine whether the 6HS collected after allogeneic stimulation also contains the antigenic determinants of the stimulating antigen ($H\text{-}2^d$). Six HS collected as shown in Table II was passed through anti-$H\text{-}2^d$ (B10.BR anti B10.D2) sepharose -4B column and then tested for the presence of "cytophilic" Ig by RICA as well as for CTL enhancing activity in vivo. The results show that this treatment removed the "cytophilic" Ig (Table VIII). It was also shown that the serum was unable to enhance the CTL response when injected into B6 mice (Fig. 12). The data indicate that the enhancing factor present in the 6HS collected after allogeneic stimulation contains both Ig and antigen. Since the immunoadsorbent specific for the antigen removed the "cytophilic" Ig it is suggested that Ig and antigen are probably associated in some form of a complex as shown previously. The term "cytophilic" Ig and "cytophilic" complexes will be used interchangeably.

4. THE LYT PHENOTYPE OF THE PRODUCING CELLS

4.1. The phenotype of the cells that produce the enhancing factor after allogeneic stimulation

It was previously found that the factor present in the 6HS is released from T cells (8) and is taken up by another T cell subpopulation (4). Recently,

Fig. 10
NATURE OF THE ENHANCING FACTOR

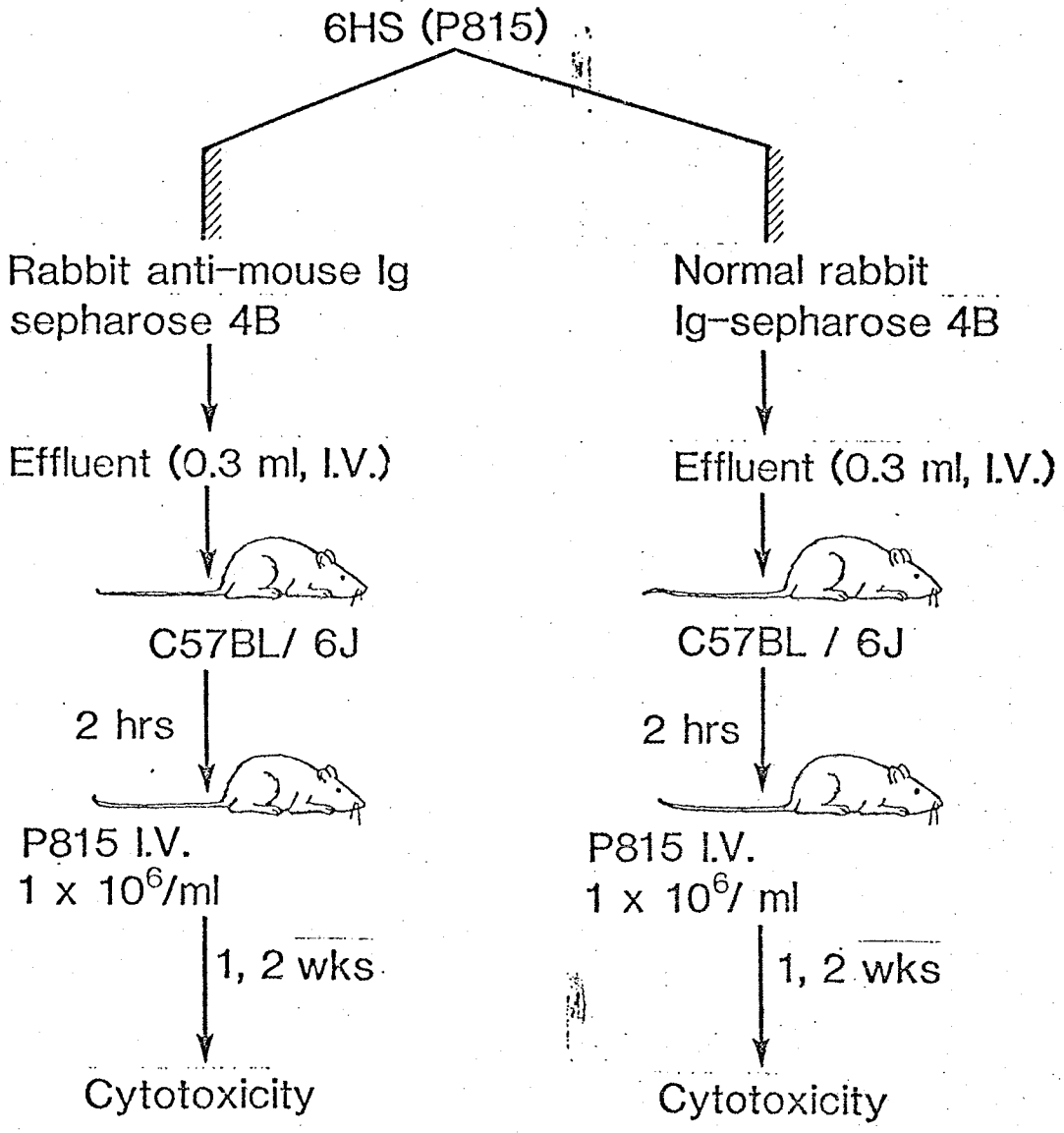


TABLE VII

REMOVAL OF CYTOPHILIC IG BY ANTI-IG SERUM

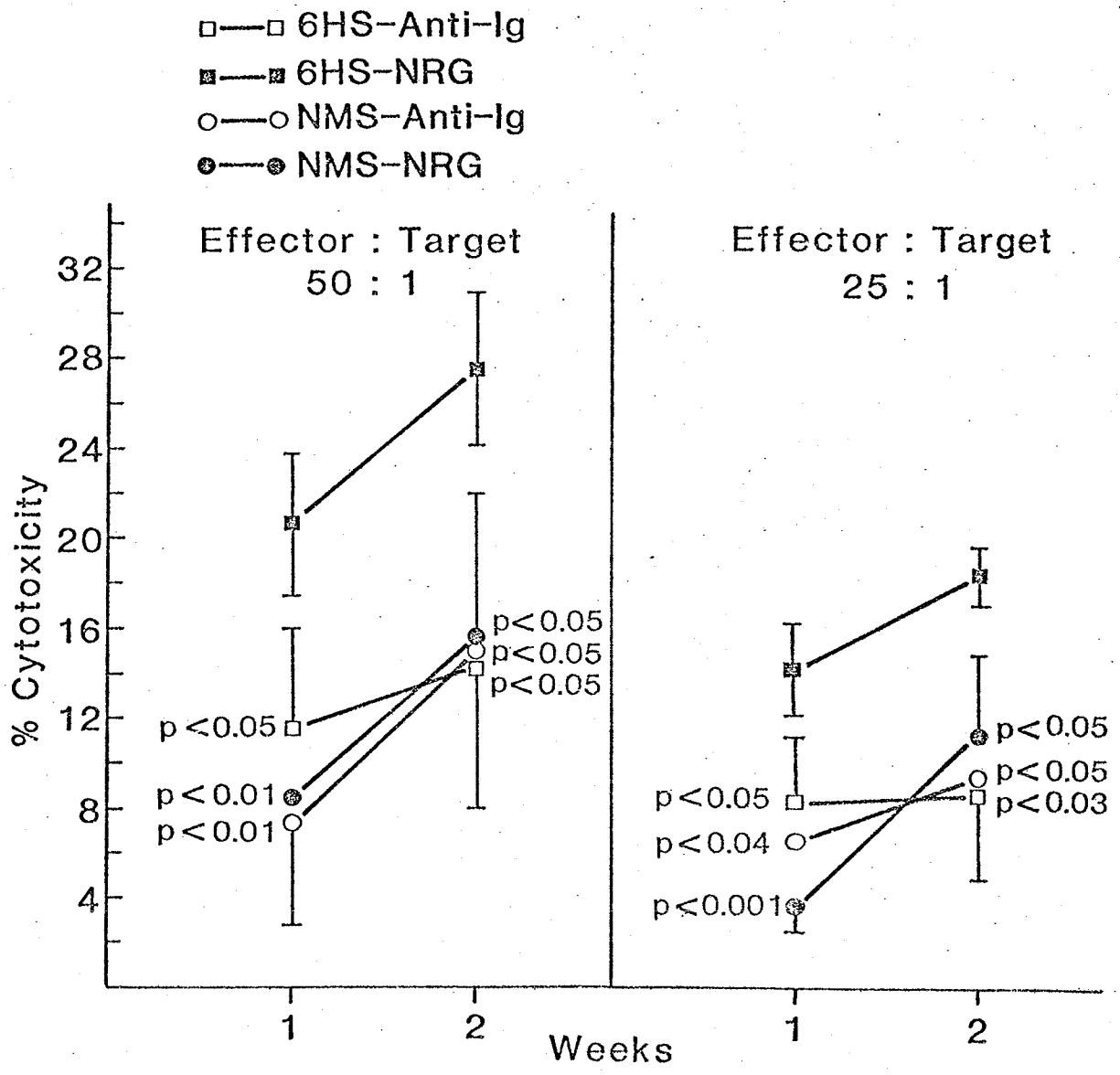
TREATMENT	IG/CELLS/1000 SPLEEN CELLS EXP. I	EXP. II
A) NONE	290	315
B) NMS	305	325
C) 6HS-ANTI-IG	310	300
D) 6HS-NRG	410	420

6HS IS COLLECTED AS IN TABLE II AND WAS PASSED THROUGH ANTI-IG OR NORMAL RABBIT IG (NRG)-SEPHAROSE 4B COLUMNS AS DESCRIBED IN THE MATERIALS AND METHODS. THE SERUM WAS INCUBATED WITH NORMAL SPLEEN CELLS (B6) AND THE UPTAKE OF IG BY THESE CELLS WAS TESTED BY THE RICA METHOD.

CONCLUSION: ANTI-IG COLUMN REMOVES CYTOPHILIC IG

Fig. 11

REMOVAL OF THE ENHANCING FACTOR BY ANTI-Ig IMMUNOADSORBENT



LEGEND FIG. 11:

6HS WAS COLLECTED FROM B6 MICE AFTER STIMULATION WITH 20 X 10^6 P815 AND WAS PASSED THROUGH EITHER RABBIT-ANTI MOUSE IG OR NRG. SEPHAROSE-4B COLUMNS. A 0.3 ML OF THE FORMER (□) OR THE LATTER (■) WAS INJECTED I.V. INTO NORMAL B6 MICE. CONTROL MICE RECEIVED 0.3 ML OF NMS PASSED THROUGH ANTI-IG (○) OR NRG (●) COLUMNS. THE MICE WERE CHALLENGED WITH 1 X 10^6 P815.

TARGET CELLS = P815 SPONTANEOUS RELEASE = 12%

TABLE VIII

REMOVAL OF CYTOPHILIC Ig BY ANTIBODY AGAINST THE ANTIGEN

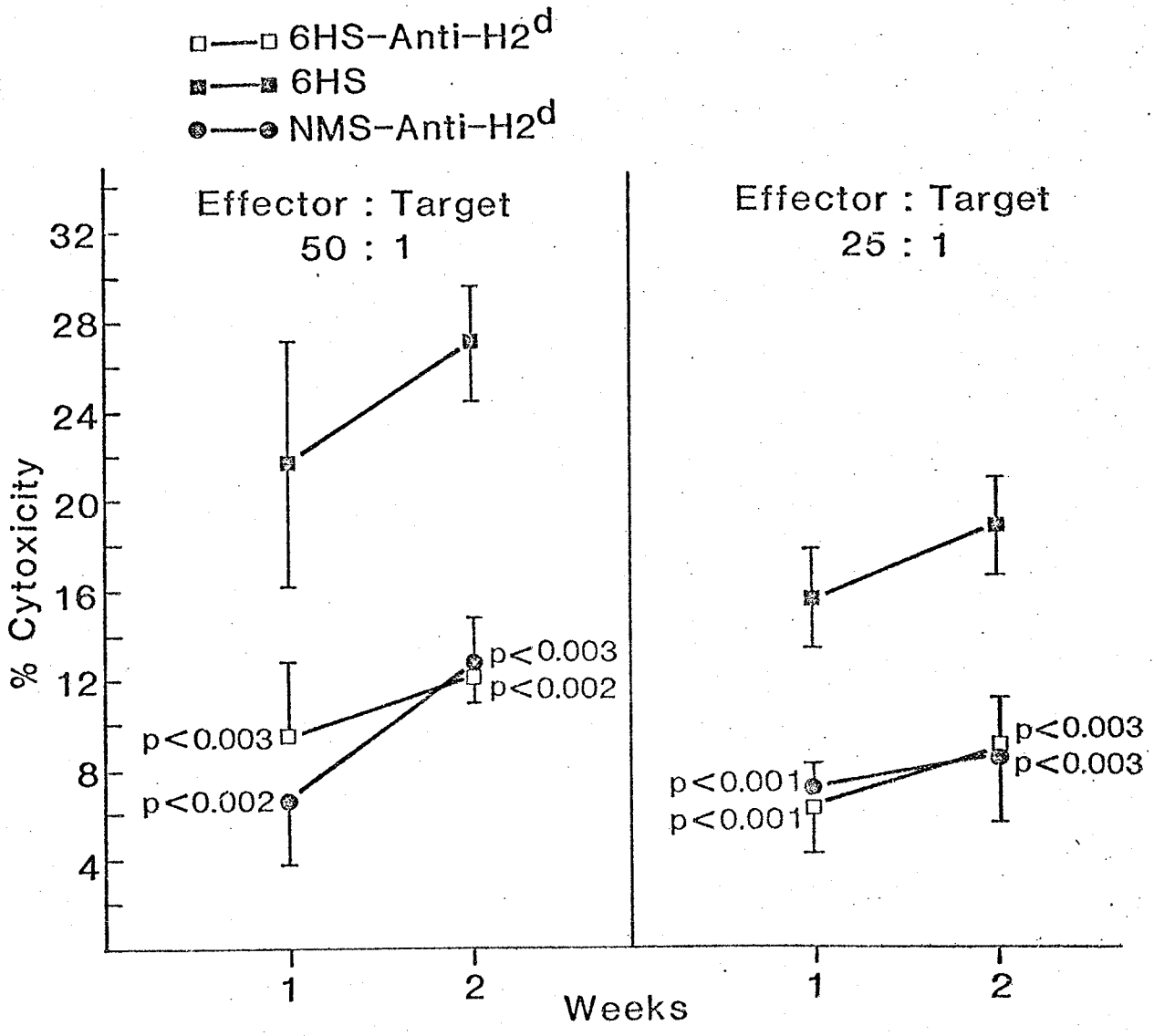
TREATMENT	IG+ CELLS/1000 SPLEEN CELLS	
	EXP. I	EXP. II
A) NONE	325	315
B) NMS	325	324
C) NMS-ANTI-$H2^D$	315	327
D) 6HS	403	405
E) 6HS-ANTI-$H2^D$	290	305

6HS IS COLLECTED AS IN TABLE II AND WAS PASSED THROUGH ANTI-$H2^D$ COLUMN AS DESCRIBED IN THE MATERIALS AND METHODS.

CONCLUSION: 6HS PASSED THROUGH ANTI-$H2^D$ COLUMN LOST ITS CYTOPHILIC IG

Fig. 12

REMOVAL OF THE ENHANCING FACTOR BY ANTIBODY TO THE ANTIGEN (ANTI-$H2^d$)



LEGEND FIG. 12:

6HS COLLECTED FROM B6 MICE AFTER STIMULATION WITH 20 X 10^6 P815 WAS PASSED THROUGH ANTI-H-2^D (B10.BR ANTI B10.D2) ANTISERA-SEPHAROSE 4B COLUMN AND INJECTED I.V. INTO NAIVE B6 MICE (□). CONTROL MICE RECEIVED 0.3 ML OF EITHER UNTREATED 6HS (■) OR NMS PASSED THROUGH ANTI-H-2^D COLUMN (●). THE MICE WERE CHALLENGED WITH 1 X 10^6 P815.

TARGET CELLS = P815 SPONTANEOUS RELEASE = 10%

it was shown that this factor which is present in the serum 6 hours after stimulation with SRBC is released from Lyt.1$^+$ cells (10). To determine the nature of the cells which produce the allogeneically induced enhancing factor, B6 mice were lethally irradiated and then reconstituted with either NWC-NAC, Lyt.1$^+$ or Lyt.2$^+$ cells. Control mice were only irradiated but not reconstituted. All mice were then injected with 20 x 10^6 P815 and the serum was collected 6 hours later. This serum was incubated with NSC (B6) and the presence of "cytophilic" Ig was tested by the RICA. As shown in Table IX, the 6HS from mice reconstituted with NWC-NAC contained a "cytophilic" Ig(d) while the 6HS from non-reconstituted mice did not (c). It was also found that 6HS from either Lyt.1$^+$ (e) or Lyt.2$^+$ (f) cells reconstituted mice contained "cytophilic" Ig and enhances the CTL responses upon injecting into naive C57Bl/6J mice (Fig. 13c and d). This result differs from previous findings which showed that "cytophilic" Ig after stimulation with foreign antigens (SRBC) is produced only in the presence of Lyt.1$^+$ but not in the presence of Lyt.2$^+$ cells. Similar results were obtained when DBA/2 SC were used to produce the 6HS (Table X).

4.2. <u>The phenotype of the cells that take up the "cytophilic" complexes formed after allogeneic stimulation</u>

B6 mice were irradiated and then reconstituted with either NWC-NAC, Lyt.1$^+$ or Lyt.2$^+$ cells while control mice were not reconstituted. One day later, the mice were injected with P815 and the serum was collected six hours later. This serum was incubated with different T cell subpopulations including NWC-NAC, Lyt.1$^+$ and Lyt.2$^+$. The uptake of Ig by these cells was tested by the RICA method. Table XI shows that NWC-NAC produce a factor 6 hours after stimulation with P815 which contains a "cytophilic" Ig which is taken up by both the Lyt.1$^+$ (A-4) and Lyt.2$^+$ (B-4). The "cytophilic" Ig produced by Lyt.1$^+$ cells was found to be taken up by both Lyt.1$^+$ (A-5) and Lyt.2$^+$ (B-5) cells while that produced by Lyt.2$^+$ cells was taken up by only Lyt.2$^+$ cells (B-6) but not by Lyt.1$^+$ (A-6) cells.

TABLE IX

THE GENERATION OF CYTOPHILIC Ig BY T CELL SUBPOPULATIONS: ALLOGENEIC TUMOR CELL STIMULATION

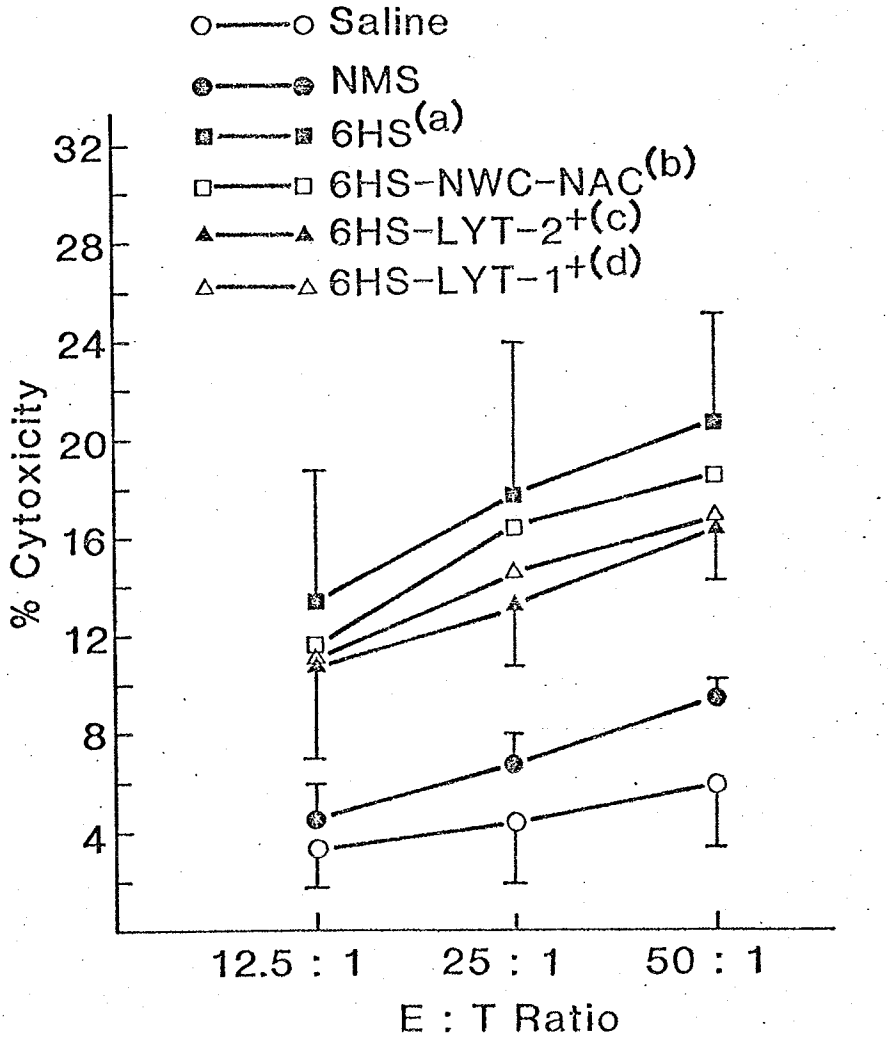
TREATMENT	RECONSTITUTION OF 6HS DONORS	Ig+ CELLS/1000 SPLEEN CELLS EXP. I	EXP. II
A) NONE	--	315	311
B) NMS	--	317	300
C) 6HS	NONE	309	256
D) 6HS	NWC-NAC	400	416
E) 6HS	LyT.1$^+$	425	416
F) 6HS	LyT.2$^+$	370	384

B6 MICE WERE LETHALLY IRRADIATED AND CHALLENGED WITH P 815 CELLS WITHOUT RECONSTITUTION (EXP. C) OR AFTER RECONSTITUTION WITH NWC (EXP. D), LyT.1$^+$ (EXP. E) OR LyT.2$^+$ (EXP. F) CELLS.
SERUM WAS COLLECTED 6 HOURS AFTER CHALLENGE.

CONCLUSION: 1) LETHALLY IRRADIATED MICE PRODUCE NO CYTOPHILIC Ig AFTER ALLOGENEIC CHALLENGE

2) BOTH LyT.1$^+$ OR LyT.2$^+$ CELLS CAN PRODUCE CYTOPHILIC Ig.

Fig. 13
ENHANCEMENT OF CTL BY 6HS PRODUCED FROM DIFFERENT T CELL SUBPOPULATIONS



LEGEND FIG. 13:

B6 MICE WERE LETHALLY IRRADIATED, RECONSTITUTED WITH DIFFERENT SUBPOPULATIONS OF T CELLS AND INJECTED WITH 20 X 10^6 P815. SIX HOURS LATER THE SERUM WAS COLLECTED FROM NORMAL (■), NWC-NAC (□), LyT.1$^+$ (△) OR LyT.2$^+$ (▲) RECONSTITUTED MICE. A 0.3 ML OF EACH WAS INJECTED I.V. INTO NAIVE B6 MICE WHICH WERE CHALLENGED WITH 1 X 10^6 P815.

TARGET CELLS = P815. SPONTANEOUS RELEASE = 15%.

TABLE X

THE GENERATION OF CYTOPHILIC I_G BY T CELL SUBPOPULATIONS: NORMAL ALLOGENEIC CELL STIMULATION

TREATMENT	RECONSTITUTION OF 6HS DONORS	Ig^+ CELLS/1000 SPLEEN CELLS EXP. I	EXP. II
A) NONE	--	325	305
B) NMS	--	320	306
C) 6HS	NONE	318	290
D) 6HS	NWC-NAC	420	395
E) 6HS	LyT.1^+	418	405
F) 6HS	LyT.2^+	417	375

B6 MICE WERE PREPARED AS IN LEGEND OF TABLE IX. THE MICE WERE INJECTED WITH 20×10^6 DBA/2 SC AND THE SERUM WAS COLLECTED SIX HOURS LATER.

CONCLUSION: AS IN TABLE IX

TABLE XI

UPTAKE OF CYTOPHILIC Ig BY T CELL SUBPOPULATIONS

TREATMENT	RECONSTITUTION OF THE 6HS DONORS	Ig^+CELLS/1000 SPLEEN CELLS EXP. I	EXP. II
A) UPTAKE BY LyT.1^+ CELL			
1) NONE	---	83	53
2) NMS	---	108	51
3) 6HS	---	187	192
4) 6HS	NWC-NAC	197	140
5) 6HS	LyT.1^+	224	196
6) 6HS	LyT.2^+	107	47
B) UPTAKE BY LyT.2^+ CELL			
1) NONE	---	94	57
2) NMS	---	90	85
3) 6HS	---	273	232
4) 6HS	NWC-NAC	210	191
5) 6HS	LyT.1^+	220	201
6) 6HS	LyT.2^+	220	166

6HS IS COLLECTED FROM IRRADIATED MICE RECONSTITUTED WITH DIFFERENT T CELL POPULATIONS AND CHALLENGED WITH P815 CELLS. THE UPTAKE OF CYTOPHILIC Ig BY LyT.1^+ AND LyT.2^+ CELLS WAS TESTED BY RICA.

CONCLUSIONS: 1)LyT.1^+ T CELLS TAKE UP CYTOPHILIC Ig INDUCED BY NWC (EXP.A-4) OR LyT.1^+ CELLS (EXP.A-5) BUT NOT LyT.2^+ CELLS (EXP.A-6)

2)LyT.2^+ T CELLS TAKES UP CYTOPHILIC Ig INDUCED BY NWC (EXP.B-4) LyT.1^+ (EXP.B-5) AND LyT.2^+ CELLS (EXP. B-6)

4.3. The phenotype of the cells that produce/take up the "cytophilic" complexes after stimulation with D-region determinants

The phenotypes of the cells which produce and take up the "cytophilic" complexes was also investigated with the congenic strains which differ only at the H2-D region. B10.AKM mice were irradiated, reconstituted with syngeneic NWC-NAC, Lyt.1^+ or Lyt.2^+ cells and then injected with B10.BR SC. Six hours later the serum was collected and incubated with different subpopulations of T cells. As shown in Table XII, "cytophilic" Ig was produced in the presence of NWC-NAC and was taken up by both Lyt.1^+ (A-3) and Lyt.2^+ (B-3) cells. It was also found that complexes were produced in the presence of Lyt.1^+ or Lyt.2^+ cells. However, the former were taken up by both Lyt.1^+ (A-4) and Lyt.2^+ (B-4) cells, while the latter were taken by only Lyt.2^+ cells (B-5). These results indicate that Lyt.1^+ or Lyt.2^+ cells are required for the induction of the "cytophilic" complexes in mice stimulated only with H2-D antigens.

5. MODE OF ACTION OF THE ENHANCING FACTOR

5.1. In vitro incubation for 24 hours

In our efforts to understand the mechanism of the CTL enhancement, we have designed methods which will increase the CTL response by eliminating interference with suppressor cells. It has previously been shown that immune spleen cells (ISC) incubated in vitro for 24 hours without added antigen before the CTL assay show increased cytotoxicity (207,213,214). B6 mice were injected with 6HS (collected as in Table II), NMS or saline and then challenged with P815 cells. Spleen cells were collected one and two weeks later. In each case the cells were divided into two aliquots: one was tested immediately for CTL activity, while the other was incubated for 24 hours in Marbrook vessels before the CTL assay. As shown in Fig. 14 after 24 hours incubation, there is an increase in the CTL activity in the control groups which received saline or NMS. In contrast, 24 hours incubation of spleen cells from mice which received 6HS did not result in an in-

TABLE XII

UPTAKE BY T CELL SUBPOPULATIONS OF CYTOPHILIC Ig INDUCED BY D-REGION DIFFERENCES

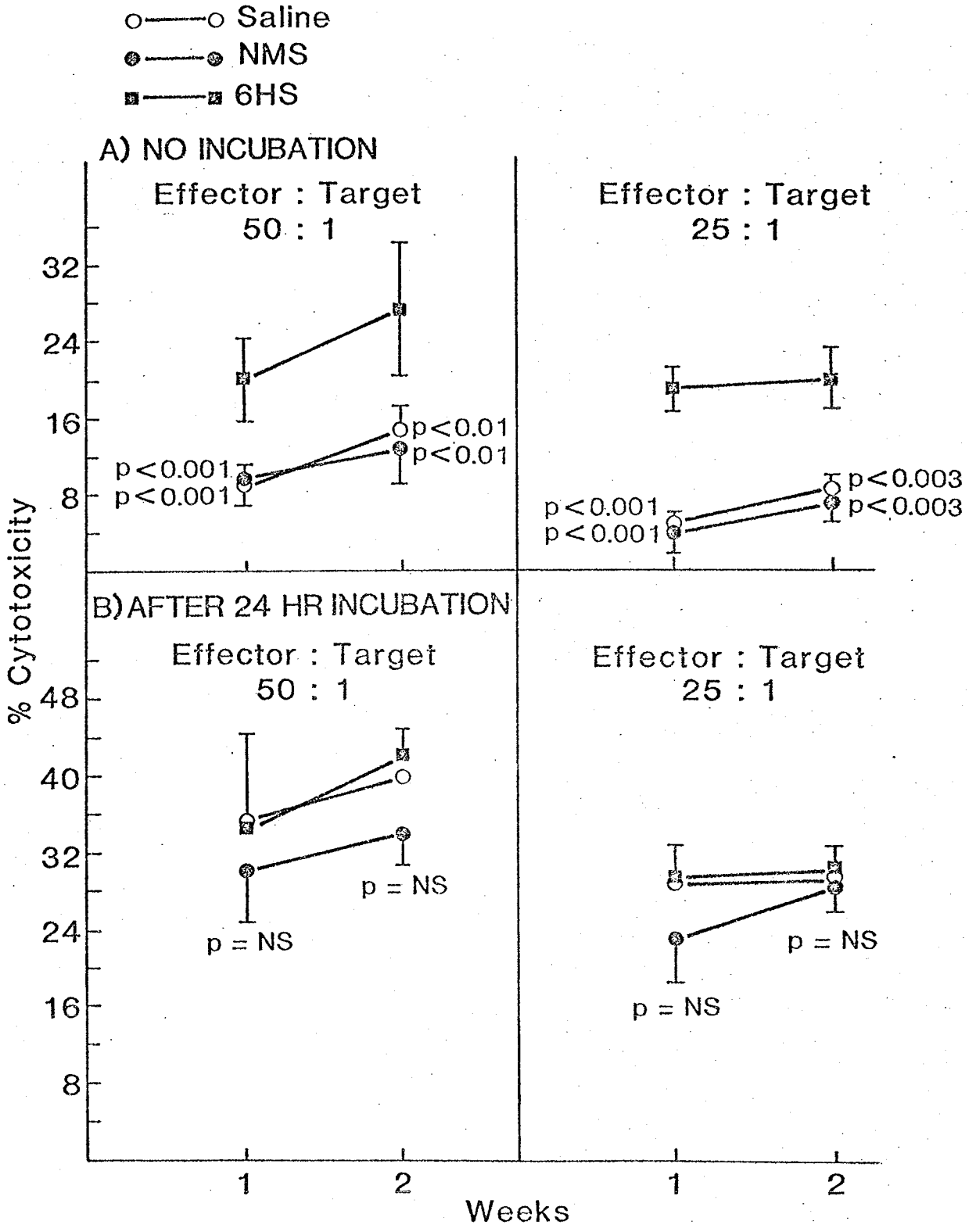
TREATMENT	RECONSTITUTION OF 6HS DONORS	Ig+ CELLS/1000 SPLEEN CELLS EXP. I	EXP. II
A) UPTAKE BY LyT.1$^+$ CELLS			
1) NONE	---	51	61
2) NMS	---	50	70
3) 6HS	NWC-NAC	170	166
4) 6HS	LyT.1$^+$	156	183
5) 6HS	LyT.2$^+$	78	86
B) UPTAKE BY LyT.2$^+$ CELLS			
1) NONE	---	70	80
2) NMS	---	60	65
3) 6HS	NWC-NAC	140	170
4) 6HS	LyT.1$^+$	170	165
5) 6HS	LyT.2$^+$	210	190

6HS WAS COLLECTED FROM B10.AKM IRRADIATED MICE, RECONSTITUTED WITH DIFFERENT T CELL POPULATIONS. THE MICE WERE CHALLENGED WITH B10.BR SPLEEN CELLS. THE UPTAKE OF CYTOPHILIC Ig WAS TESTED ON LyT.1$^+$ AND LyT.2$^+$ SUBPOPULATIONS.

CONCLUSION: D-REGION DIFFERENCES INDUCE CYTOPHILIC Ig FROM T CELL SUBPOPULATIONS WITH PROPERTIES SIMILAR TO THAT INDUCED BY WHOLE MHC DIFFERENCES.

Fig. 14

EFFECT OF IN VITRO INCUBATION OF SPLEEN CELLS ON CTL RESPONSES



LEGEND FIG. 14:

PANEL A: 6HS COLLECTED AS IN TABLE II AND TESTED AS IN FIG. 5.

PANEL B: SAME AS IN PANEL A EXCEPT THAT IMMUNE SC WERE CULTURED IN MARBOOK VESSELS FOR 24 HR. AT 37°C IN 5% CO_2.

TARGET CELLS = P815 SPONTANEOUS RELEASE = 14-16%

crease of cytotoxicity. Thus, in vitro culture for 24 hours of the spleen cells from the control groups bring the cytotoxicity to the same level as that of the 6HS treated mice. The increase in cytotoxicity observed following the in vitro incubation has been attributed to interferences with suppressor cells. It was reasonable therefore, to assume that treatment with 6HS does not allow such interference to develop normally perhaps by blocking the development of such suppressor cells. The data therefore suggested that 6HS by some unknown mechanism may interfere with suppression. To test this hypothesis, experiments were designed using methods which are better known to interfere with the function of suppressor cells.

5.2. Injection of anti-I-J antisera

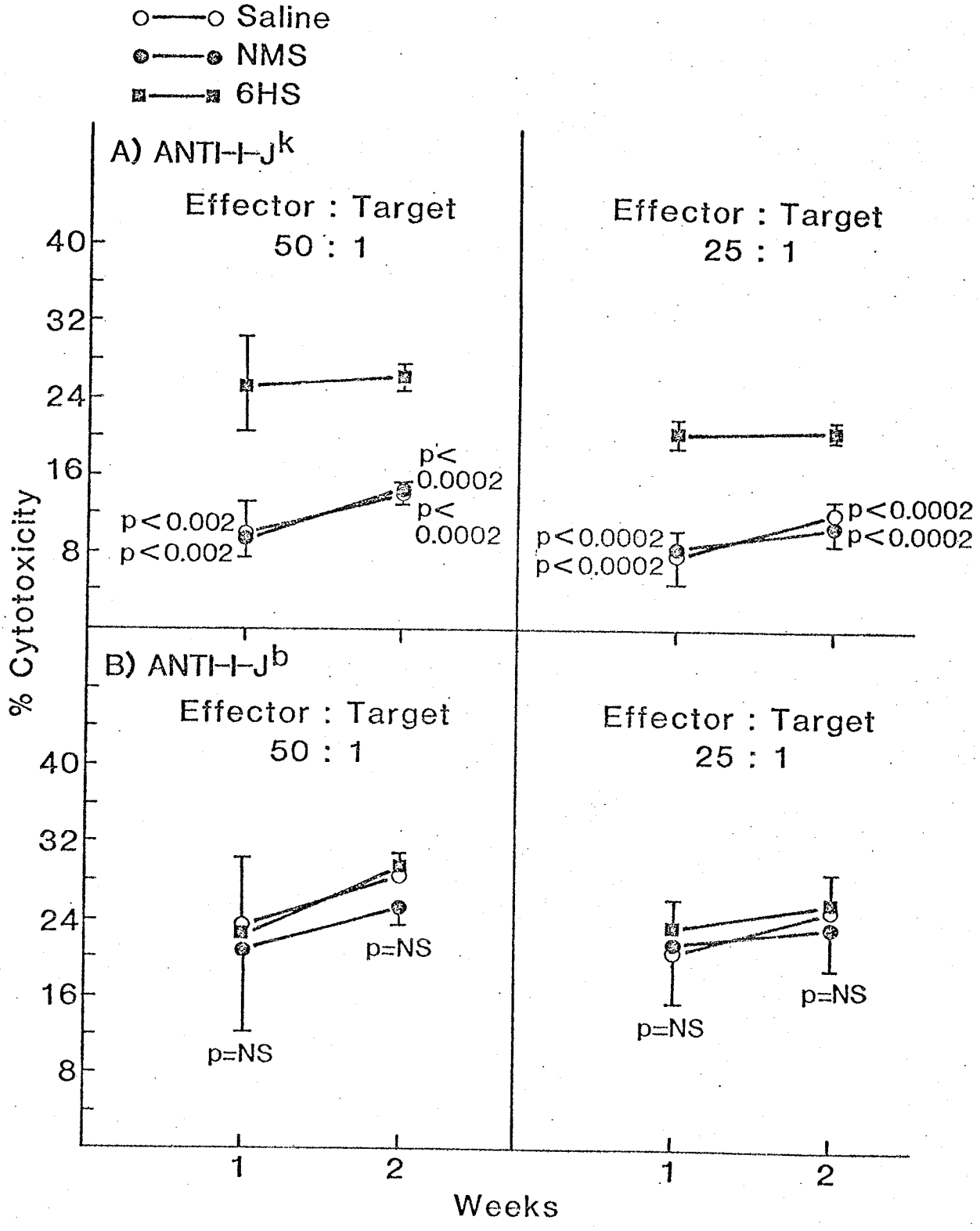
One of the known procedures for blocking suppressor cell function is the injection of anti-I-J antiserum (208,209). B6 (H-2^b) mice were injected with anti-I-J^b antiserum (B10.A"5R" anti B10.A"3R") while control groups received anti-I-J^k antiserum (B10.A"3R" anti B10.A"5R"). After this treatment, the mice were injected with 6HS, NMS or saline. As shown in Fig. 15-B, all groups of mice injected with anti-I-J^b antisera have high CTL activity. In contrast, in the control group which received the anti-I-J^k antiserum only those injected with 6HS show an enhancement of CTL (Fig. 15-A). These results demonstrate that: i) the injection of anti-I-J^b in H-2^b mice resulted in significant enhancement of CTL responses and ii) in B6 mice treated with anti-I-J^b the 6HS did not give rise to a CTL response higher than the controls. In conclusion, removal of suppressor cell function by anti-I-J treatment "masked" the significant enhancement of CTL usually observed in normal mice after 6HS injection.

5.3. Treatment with cyclophosphamide (CY)

Cyclophosphamide has been shown to abolish suppressor cell function in both humoral and cell mediated responses. We have therefore examined the effect of CY treatment on the CTL enhancement by 6HS. B6 mice were given 100 mg/kg body weight of CY two days before the injection of 6HS, NMS or saline according to the protocol depicted in Fig. 17, panel A. CY treatemnt resulted in a significant enhancement

Fig. 15

ANTI I-J ANTISERA TREATMENT ON CTL RESPONSE



LEGEND FIG. 15:

PANEL A: B6 ($H\text{-}2^B$) MICE WERE INJECTED WITH ANTI-I-J^K (B10.A(3R) ANTI B10.A(5R)) ANTISERA. THE MICE RECEIVED 0.3 ML OF 6HS (■) (COLLECTED AS IN TABLE II), NMS (●) OR SALINE (○), AND CHALLENGED WITH 1×10^6 P815.

PANEL B: SAME AS IN PANEL A EXCEPT THAT ANTI-I-J^B (B10.A(5R) ANTI B10.A(3R))ANTISERA IS USED HERE.

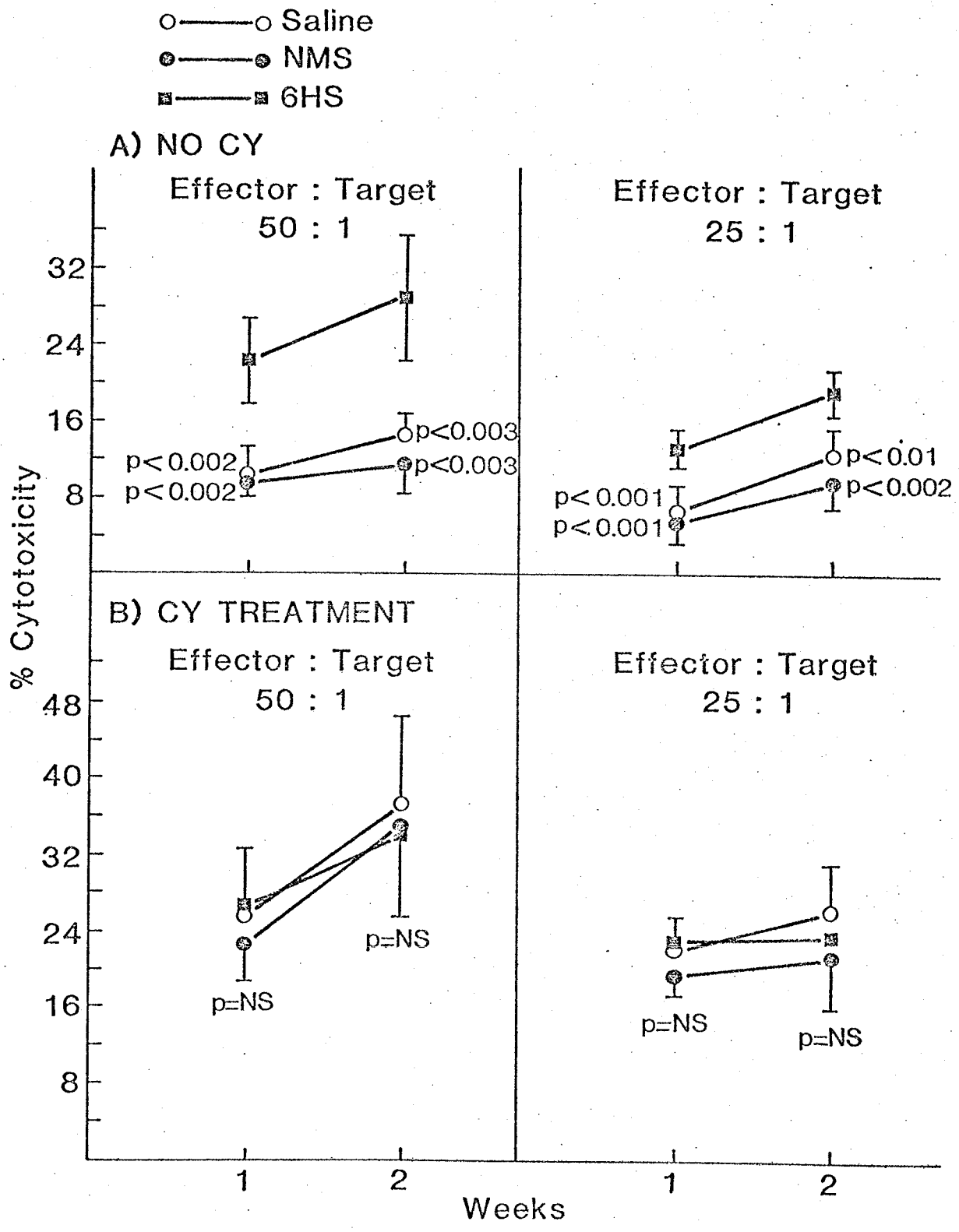
TARGET CELLS = P815. SPONTANEOUS RELEASE = 12-16%.

of the CTL response in the control groups (receiving saline or NMS) in agreement with the results reported in the literature. However, the 6HS did not enhance the CTL response in CY treated mice above the level of the controls (Fig. 16-B). These data are similar to those obtained after treatment with the anti-I-J serum. It is concluded that the enhancement of the CTL response by 6HS may be due to some form of interference with the suppressor pathway.

5.4. Reconstitution of CY-treated mice with NWC-NAC

It has been shown that reconstitution of CY treated mice with normal NWC-NAC reverses the enhancement of the immune response. B6 mice were injected with 100 mg/kg body weight of CY at day -2 and then reconstituted with NWC-NAC at day 0. Five to six hours later, the mice were injected with 6HS or NMS. These mice were termed CY-NWC-6HS (Fig. 17, panel B-c) or CY-NWC-NMS (Fig. 17, panel B-b) respectively, while another group of mice injected with saline and were termed CY-NWC (Fig. 17, panel B-a). All mice received 1 x 10^6 P815 as antigenic challenge. As shown in Fig. 18-B, the CY-NWC show no enhancement of CTL response and the same is true for the CY-NWC-NMS group. Reconstitution with NWC-NAC reversed the CTL enhancement of CY as compared to the groups which were not reconstituted (Fig. 18-A). In contrast, 6HS in the NWC-NAC reconstituted CY treated mice resulted in the usual CTL enhancement (Fig. 18-B). These results indicate that: i) the CY induced enhancement can be reversed by normal NWC-NAC cells. This effect is apparently due to the replacement of the CY sensitive suppressor cell by the NWC-NAC. ii) reconstitution with NWC-NAC did not abolish the enhancement produced by the 6HS. There are two possible interpretations of these results. Firstly, if both the CY and 6HS induced enhancement are due to the same mechanism, i.e., interference with a suppressor pathway, the two treatments may have different targets and only the CY effect is neutralized by the NWC-NAC. The cell which CY treatment eliminated is now provided by reconstitution with NWC-NAC. Secondly, the 6HS may activate an effector cell which can still neutralize the new suppressor cell precursors provided by the NWC-NAC. In this case, the suppressor function cannot be expressed and enhancement by 6HS is still observed.

Fig. 16
EFFECT OF CYCLOPHOSPHAMIDE (CY) TREATMENT ON CTL RESPONSES



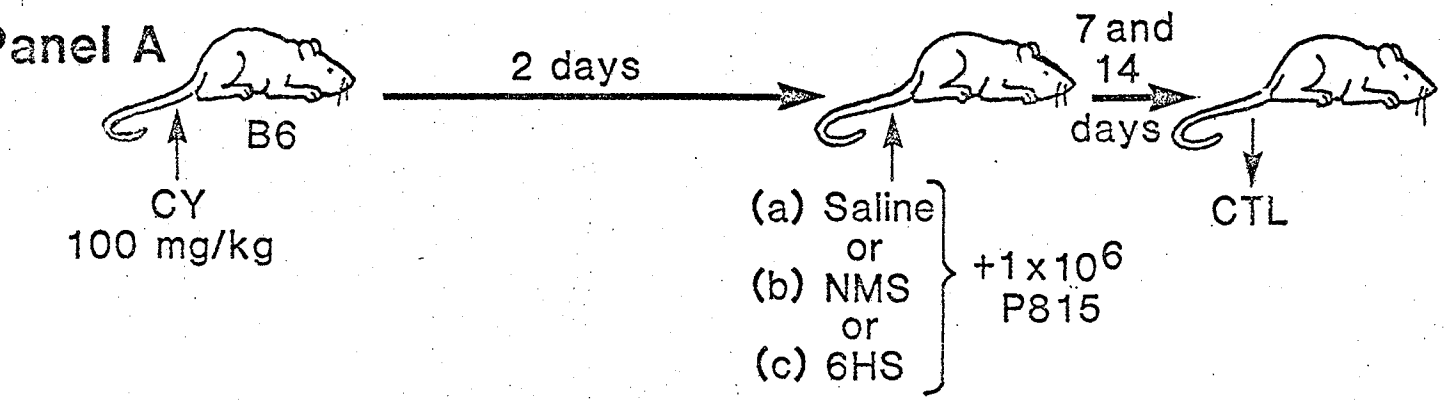
LEGEND FIG. 16:

PANEL A: B6 MICE WERE TESTED AS IN FIG. 5.

PANEL B: B6 MICE RECEIVED 100 MG/KG CY AT DAY-2. AT DAY 0, THEY WERE INJECTED I.V. WITH 0.3 ML OF EITHER 6HS (■), NMS (●) OR SALINE (○). THEY WERE CHALLENGED WITH 1×10^6 P815.

TARGET CELLS = P815. SPONTANEOUS RELEASE = 18%.

Fig. 17
RECONSTITUTION OF CY-TREATED MICE WITH NWC-NAC*

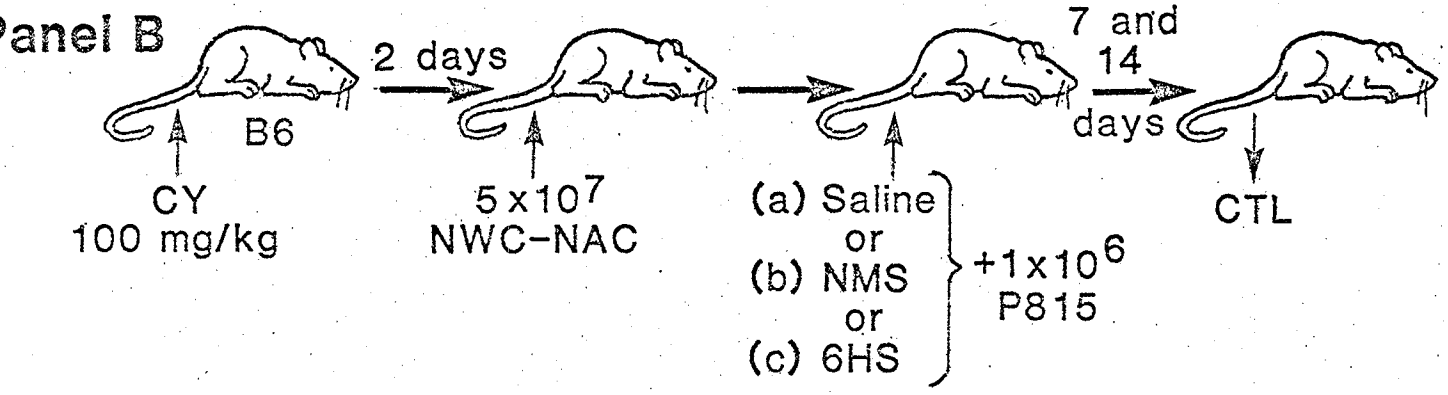


Comments

(a) Enhancement

(b) Enhancement

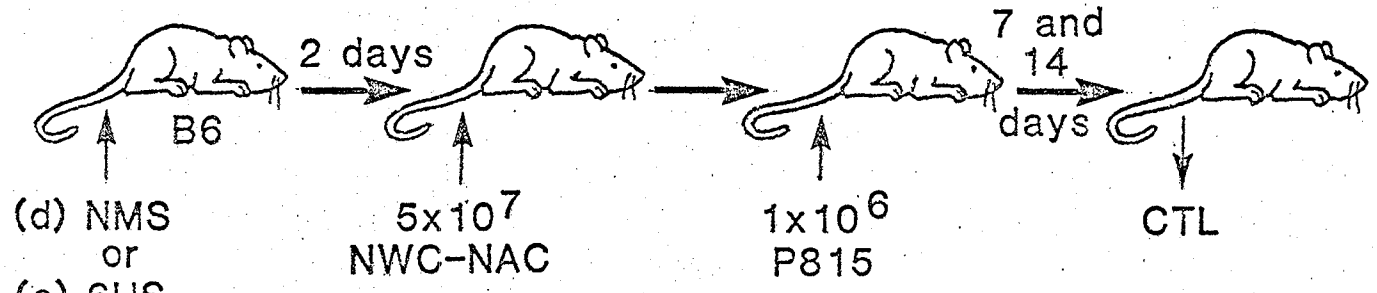
(c) Enhancement



(a) Elimination of enhancement

(b) Elimination of enhancement

(c) No elimination of enhancement

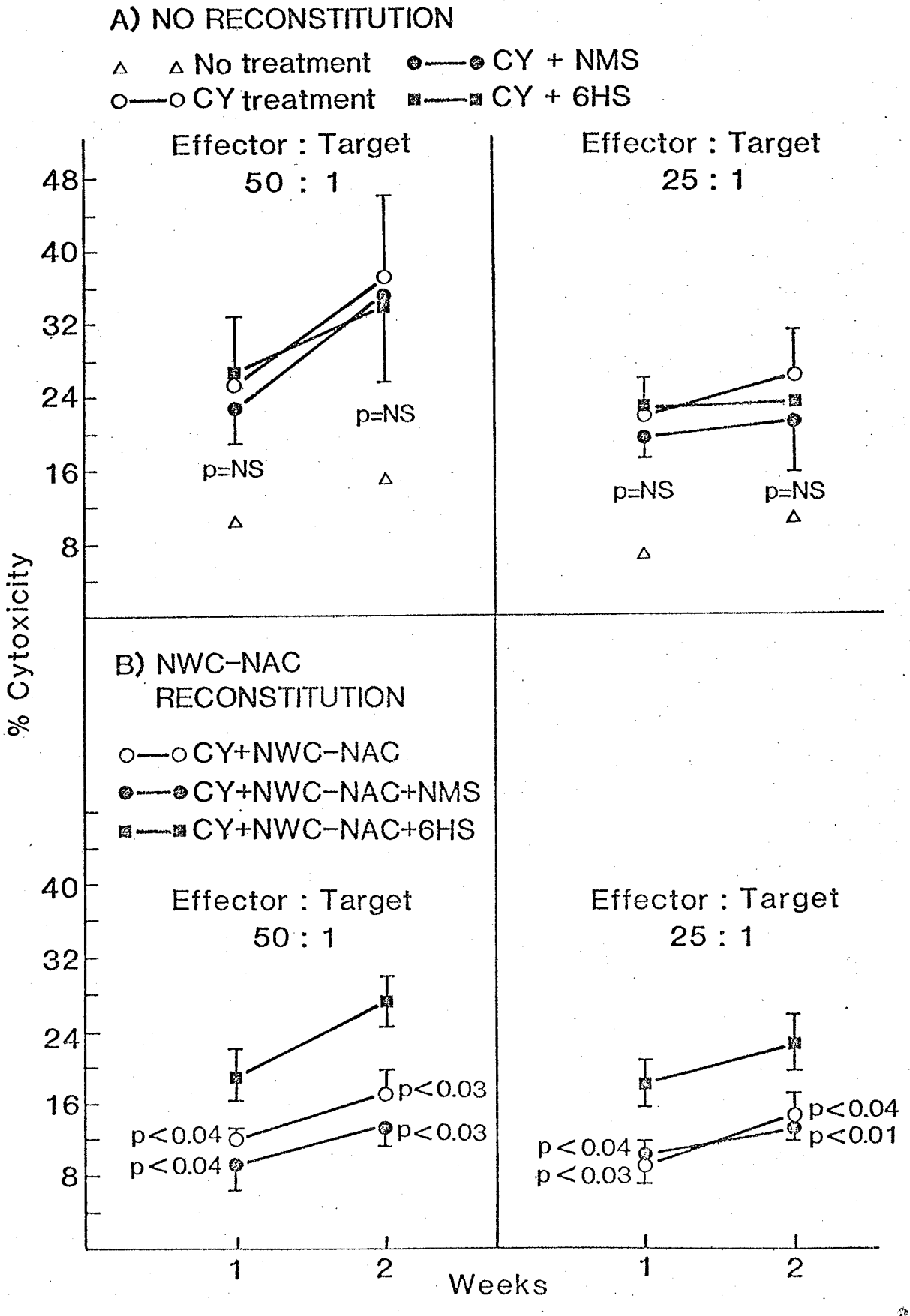


(d) Control

(e) No elimination of enhancement

* NWC-NAC = Nylon wool column- non adherent cells

Fig. 18
CYCLOPHOSPHAMIDE (CY) INDUCED CTL ENHANCEMENT : EFFECT OF RECONSTITUTION BY NWC-NAC



LEGEND FIG. 18:

PANEL A: SEE FIG. 16, PANEL B.

PANEL B: B6 MICE WERE INJECTED WITH CY AT DAY-2, RECEIVED I.V. 20×10^6 NWC-NAC AT DAY (-2). FIVE TO SIX HOURS LATER, THEY WERE INJECTED WITH 0.3 ML OF EITHER 6HS (■), NMS (●) OR SALINE (○) AND THEN CHALLENGED WITH 1×10^6 P815.

TARGET CELLS = P815. SPONTANEOUS RELEASE = 14-18%.

5.5 Reconstitution of 6HS-treated mice with NWC-NAC

In order to obtain more information on the function of the 6HS, we have asked the question whether NWC-NAC can reconstitute the 6HS induced enhancement in the absence of CY treatment. We have followed the protocol depicted in Fig. 17, Panel B,D and E. B6 mice were injected with NMS or 6HS on day -2, were reconstituted with NWC-NAC on day 0, and subsequently challenged with P815 cells. As shown in Fig. 19, NWC-NAC did not abolish the 6HS enhancement. In Fig. 19 the reversal of the CTL enhancement induced by CY is shown again. These results demonstrate conclusively that: i) both CY and 6HS enhance CTL responses and ii) only the CY enhancement can be reversed by normal NWC-NAC but not that of the 6HS.

6. THE GENERATION OF Ts CELLS

In order to determine whether the 6HS interferes with suppressor cell function we have induced T suppressor cells (Ts) according to the method of Chiu et al (212). P815 cells were heat-treated at 56^{o}C for 10 minutes, washed twice thereafter and injected in different concentrations into B6 mice. Five to seven days later, the splenocytes of those mice were passed through NWC and NAC were transferred into naive B6 mice which were challenged later with 5 x 10^{6} intact P815. As shown in Fig. 20 NWC-NAC collected from the spleens of mice injected with heat-treated tumor cells (HT-TU) suppress the CTL response of the recipient mice as compared to mice receiving NWC-NAC from saline injected mice. The suppressor cells were found to be T cells since their effect was diminished upon treatment with anti-Thy.1 + C before transfer (Fig. 20).

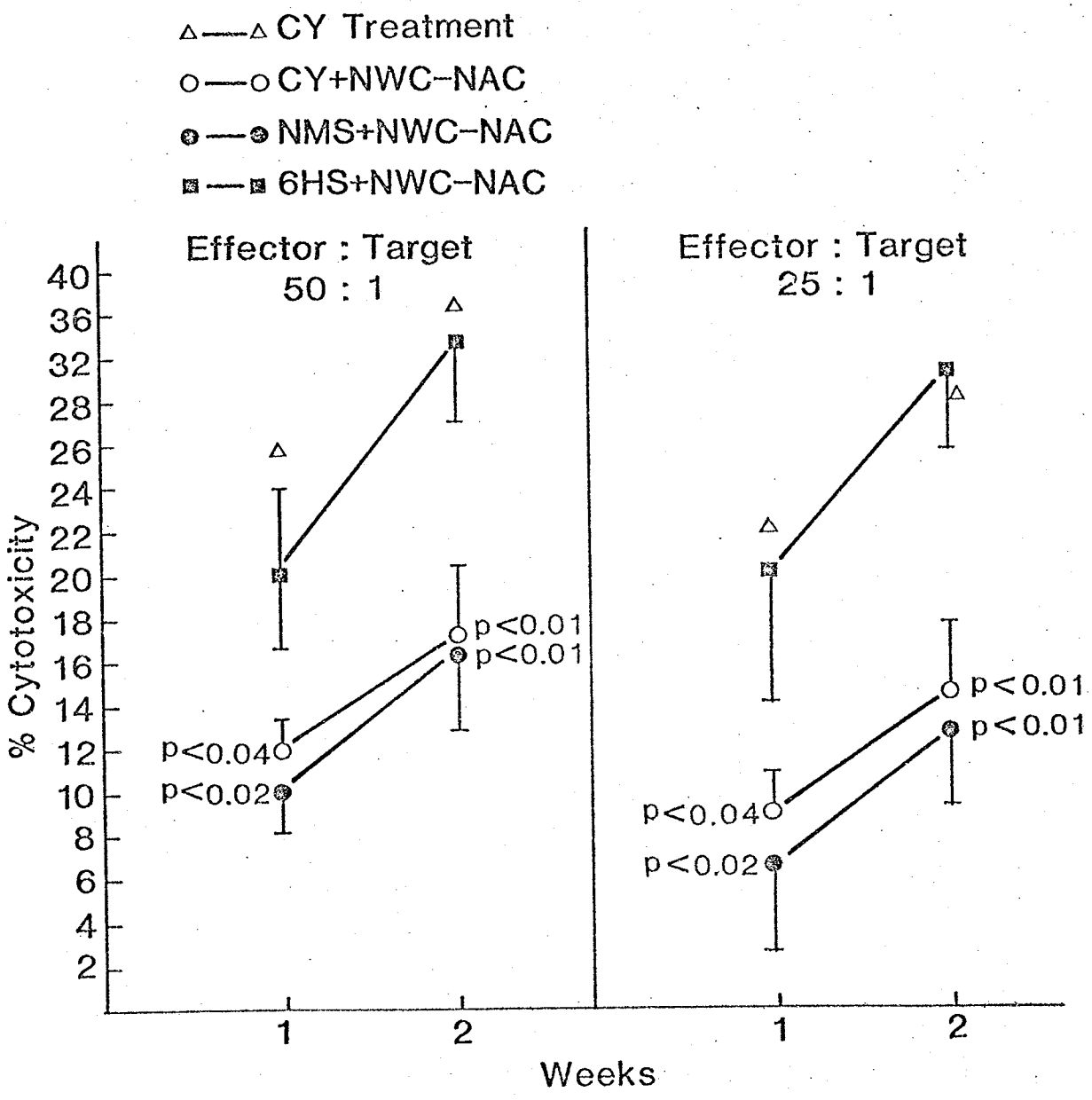
7. THE EFFECT OF THE 6HS ON Ts CELLS

7.1. Effect of the 6HS on the induction of Ts cells

After establishing that HT-TU can generate Ts cells able to suppress the CTL responses of recipient mice, we have attempted to determine whether 6HS can interfere with the induction of Ts. This was investigated according to the

Fig. 19

6HS INDUCED CTL ENHANCEMENT : EFFECT OF RECONSTITUTION BY NWC-NAC



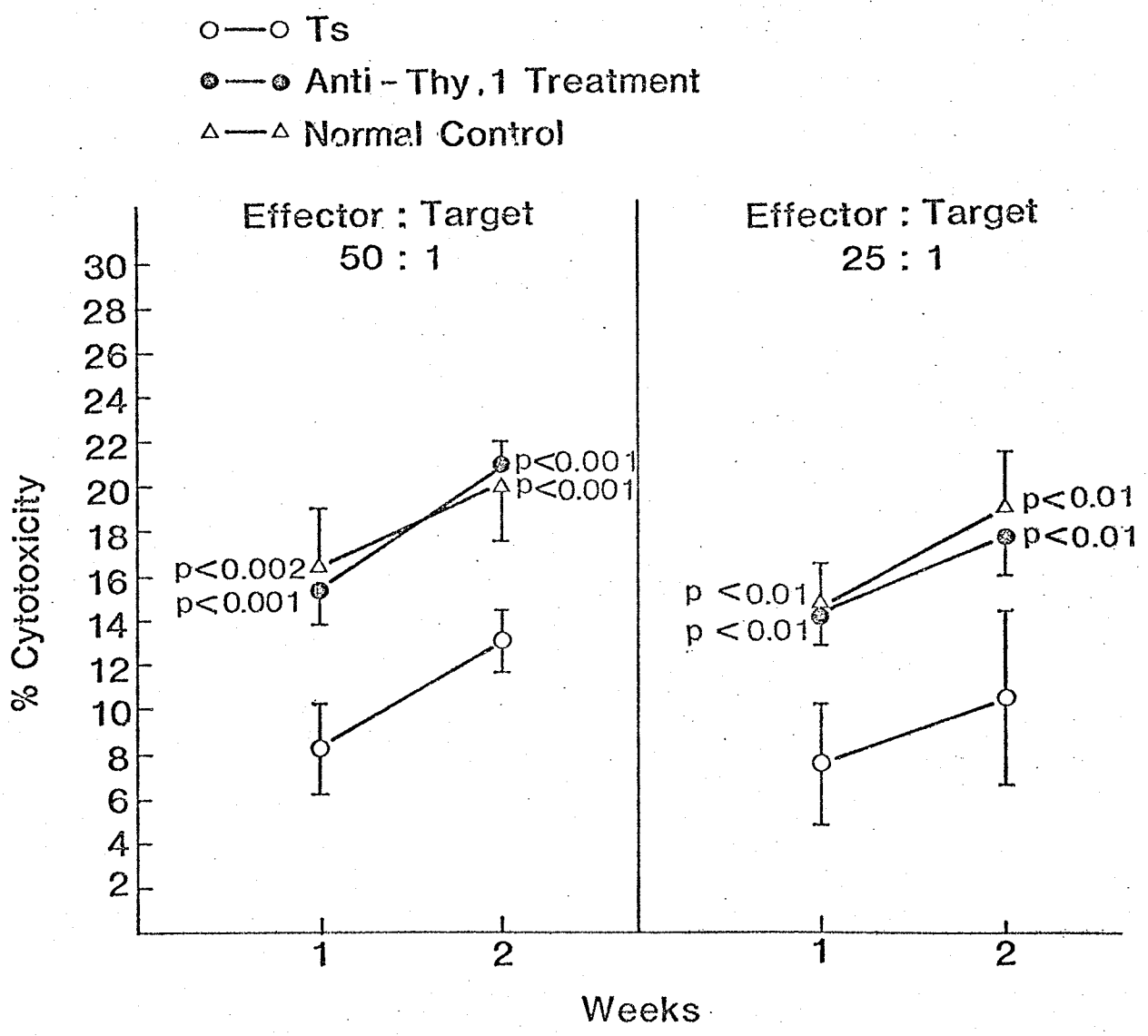
LEGEND FIG. 19:

B6 MICE WERE INJECTED WITH 0.3 ML OF EITHER 6HS (■) OR NMS (●) AT DAY-2. AT DAY 0 THEY WERE RECONSTITUTED WITH 15-20 X 10^6 NWC-NAC. THE CONTROL MICE RECEIVED CY AT DAY-2 AND WERE EITHER RECONSTITUTED (○) OR NOT RECONSTITUTED (△) WITH NWC-NAC, AT DAY 0. THE MICE WERE CHALLENGED WITH 1 X 10^6 P815.

TARGET CELLS = P815. SPONTANEOUS RELEASE = 12%.

Fig. 20

GENERATION OF T-SUPPRESSOR CELLS (Ts)



LEGEND FIG. 20:

B6 MICE WERE INJECTED I.V. WITH 20×10^6 HT-TU. FIVE TO SEVEN DAYS LATER SPLENOCYTES WERE REMOVED, PASSED THROUGH NWC AND NAC, WERE COLLECTED AND INJECTED I.V. ($15 - 20 \times 10^6$) INTO NAIVE B6 MICE (○). SOME B6 MICE RECEIVED NWC-NAC AFTER TREATMENT WITH ANTI-THY-1+C (●). AS CONTROL $15 - 20 \times 10^6$ NWC-NAC FROM MICE INJECTED WITH SALINE WERE TRANSFERRED INTO B6 MICE (△). ALL MICE WERE CHALLENGED WITH 5×10^6 P815.

TARGET CELLS = P815 SPONTANEOUS RELEASE = 10-12%

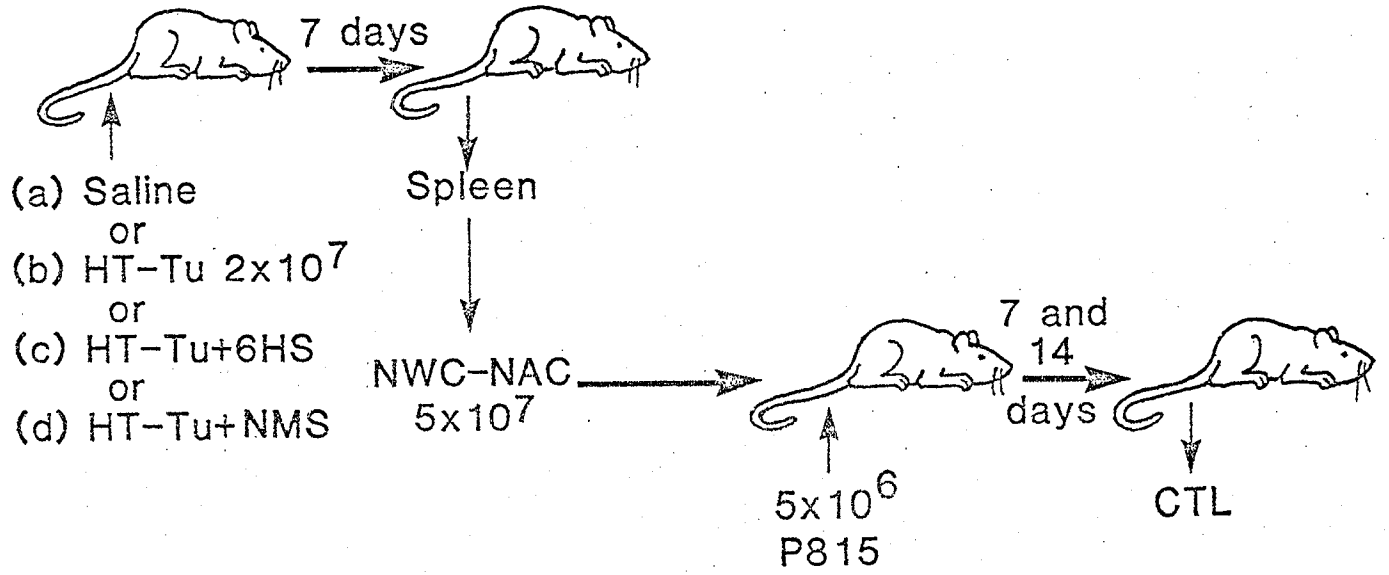
protocol shown in Fig. 21. Groups of B6 mice received saline (a), HT-TU (b), HT-TU + 6HS(c) or HT-TU + NMS(d). Spleen cells were collected 7 days later. The number of HT-TU injected was 2×10^7 cells. As shown in Fig. 22, Ts were induced by HT-TU (group b), but no Ts were detected in the spleens of mice receiving 6HS (group c). NMS (group d) had no effect on the Ts induction. An exactly similar protocol was applied in other groups of mice which received a smaller dose of HT-TU (7.5×10^6 cells). As shown in Fig. 23, the 6HS did not interfere with the induction of Ts. Collectively, the data from both experiments suggest that the 6HS interferes with the induction of Ts but this effect is antigen dose dependent.

7.2. Effect of the 6HS at the effector stage of Ts cells

In addition to its effect on the induction of Ts cells, an experiment was designed to determine whether 6HS interferes with the Ts function at the effector stage. The experimental protocol is shown in Fig. 24. Ts were induced by HT-TU as discussed previously. Spleen cells from HT-TU injected mice were collected 7 days later and the NWC-NAC were transferred to groups of normal mice which in addition received (Fig 24-2): saline (b), 6HS (c) or NMS (d). All groups were challenged with 5×10^6 P815 cells. The results using a large HT-TU dose (2×10^7) for Ts induction are shown in Fig. 25. The 6HS blocked suppression (group c) while NMS (group d) had no effect. A similar experiment was performed using the small HT-TU cell dose (7.5×10^6) and the results are shown in Fig. 26. The 6HS again blocked the Ts function. These results indicate that 6HS interferes with Ts function at the effector stage and this effect is not dependent on the antigen dose used for the induction of Ts. The conclusion which can be drawn from all the experiments presented above indicate that the 6HS interferes with Ts function at both the induction as well as effector stage. However, for the two antigen doses used, the former result is expressed only at a certain range of the immunosuppressive stimulus, while the latter result is antigen dose independent. We may

Fig. 21

EFFECT OF 6HS AT INDUCTION STAGE OF SUPPRESSOR CELLS



HT-Tu = Heat-treated P815 ($H\text{-}2^d$)

Comments

(a) Control

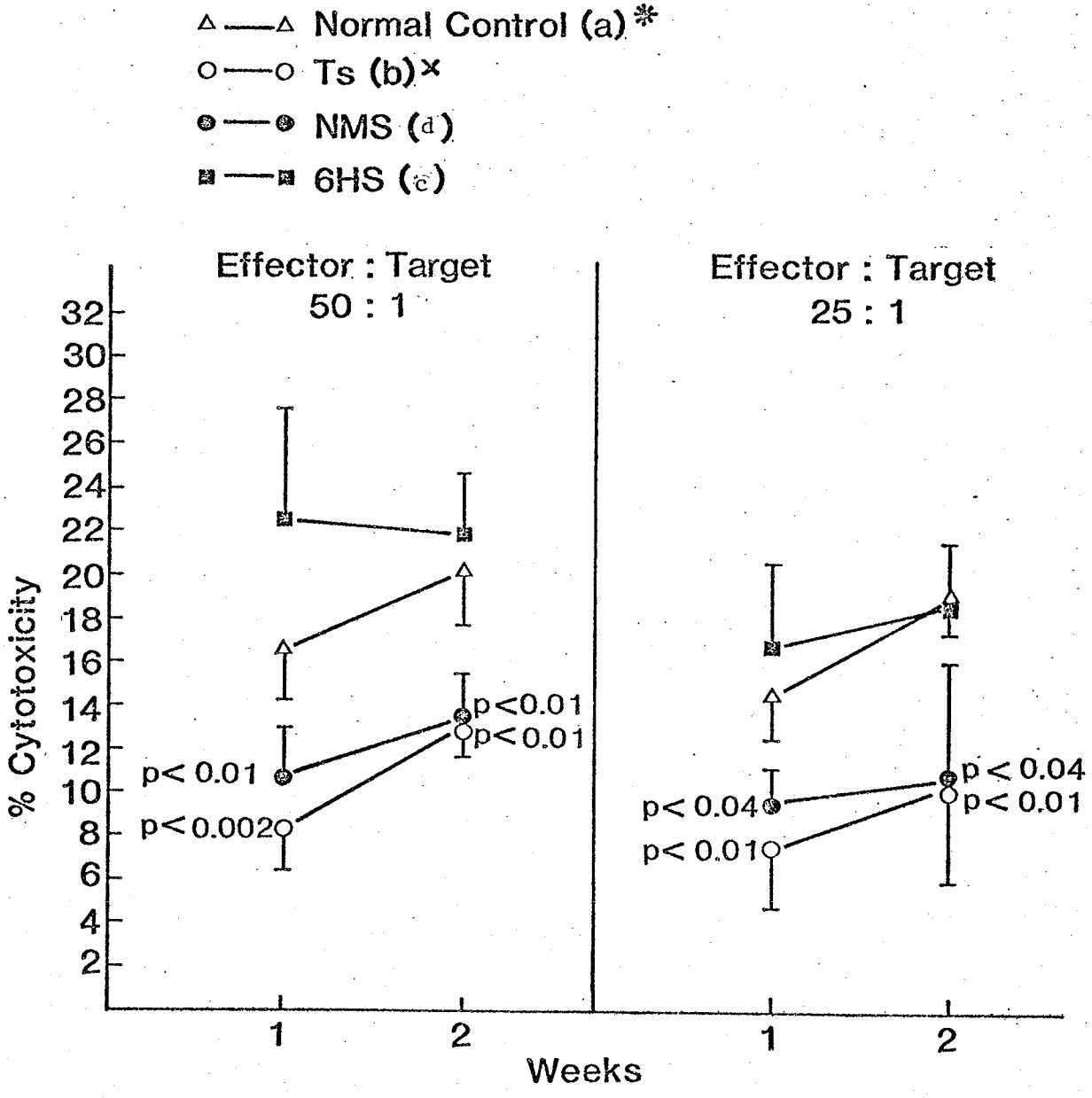
(b) Suppression

(c) Elimination of suppression

(d) Suppression

Fig. 22

EFFECT OF 6HS ON THE INDUCTION OF Ts : HIGH ANTIGEN DOSE



* See Fig. 21

× Ts=T Suppressor cells

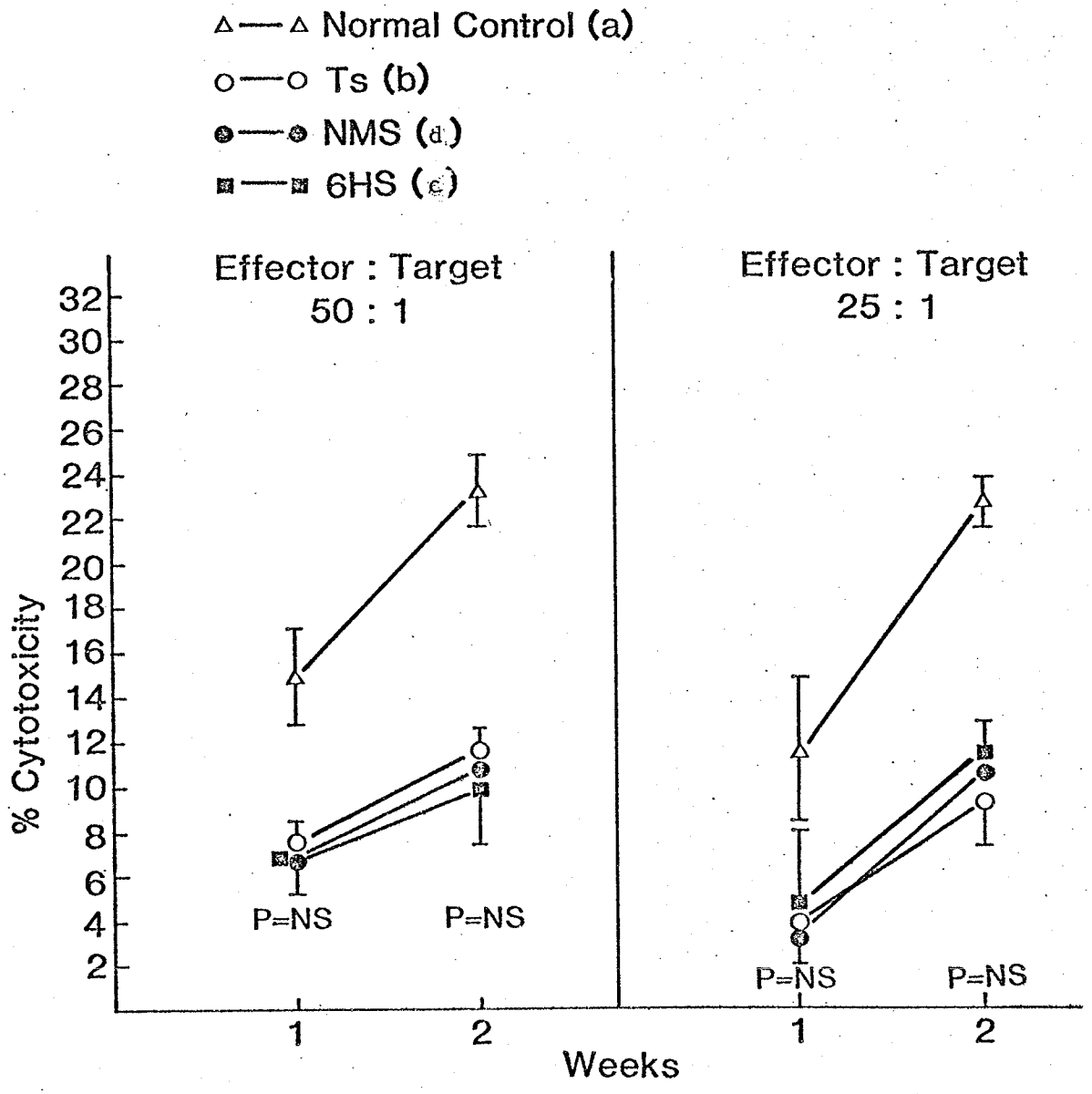
LEGEND FIG. 22:

B6 MICE WERE INJECTED I.V. WITH 20 X 10^6 HT-TU. SIMULTANEOUSLY, THEY RECEIVED 0.3 ML OF EITHER NMS (●) OR 6HS (■). CONTROL MICE RECEIVED SALINE(△). FIVE TO SEVEN DAYS LATER, NWC-NAC OF SPLENOCYTES WERE TRANSFERRED INTO NAIVE B6 MICE. THE MICE WERE CHALLENGED WITH 1 X 10^6 P815.

TARGET CELLS = P815. SPONTANEOUS RELEASE = 12-14%.

Fig. 23

EFFECT OF 6HS ON THE INDUCTION OF Ts : LOW ANTIGEN DOSE



LEGEND FIG. 23:

SEE LEGEND OF FIG. 23 EXCEPT THAT 7.5×10^6 HT-TU WERE USED HERE INSTEAD OF 20×10^6.

Fig. 24

EFFECT OF 6HS AT THE EFFECTOR STAGE OF SUPPRESSOR CELLS

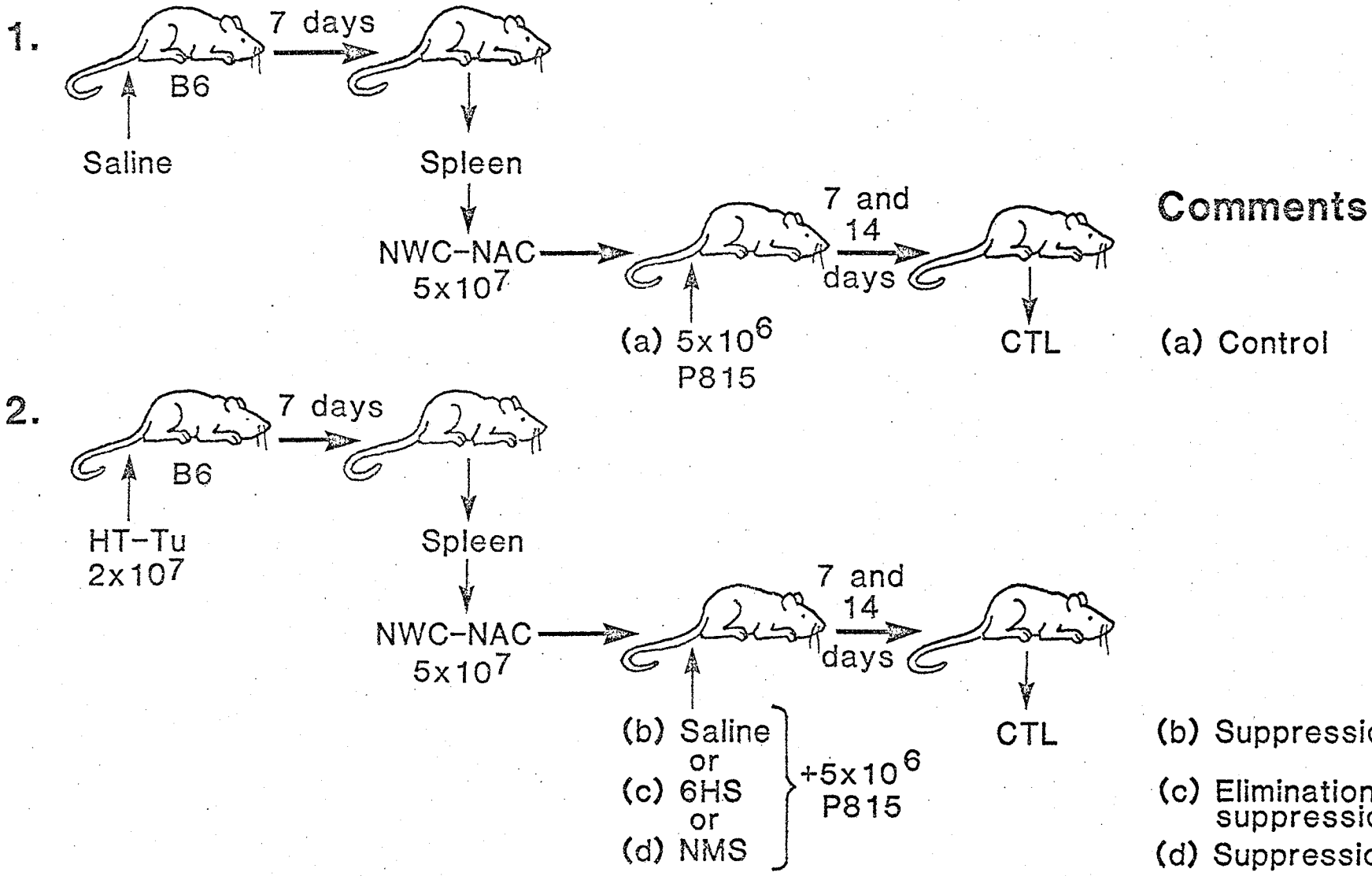
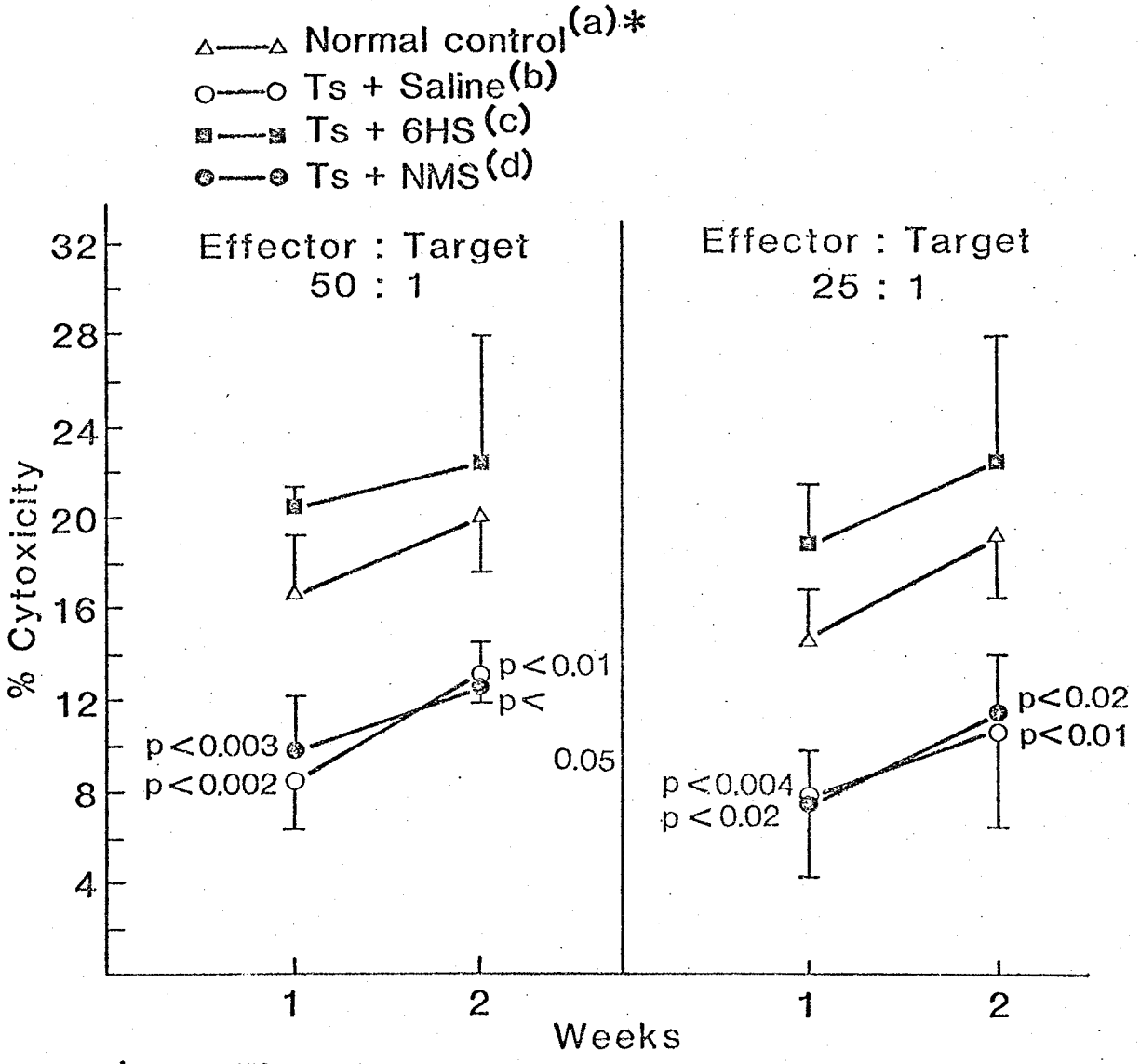


Fig. 25

EFFECT OF 6HS ON TS FUNCTION : HIGH ANTIGEN DOSE



* see Fig. 24

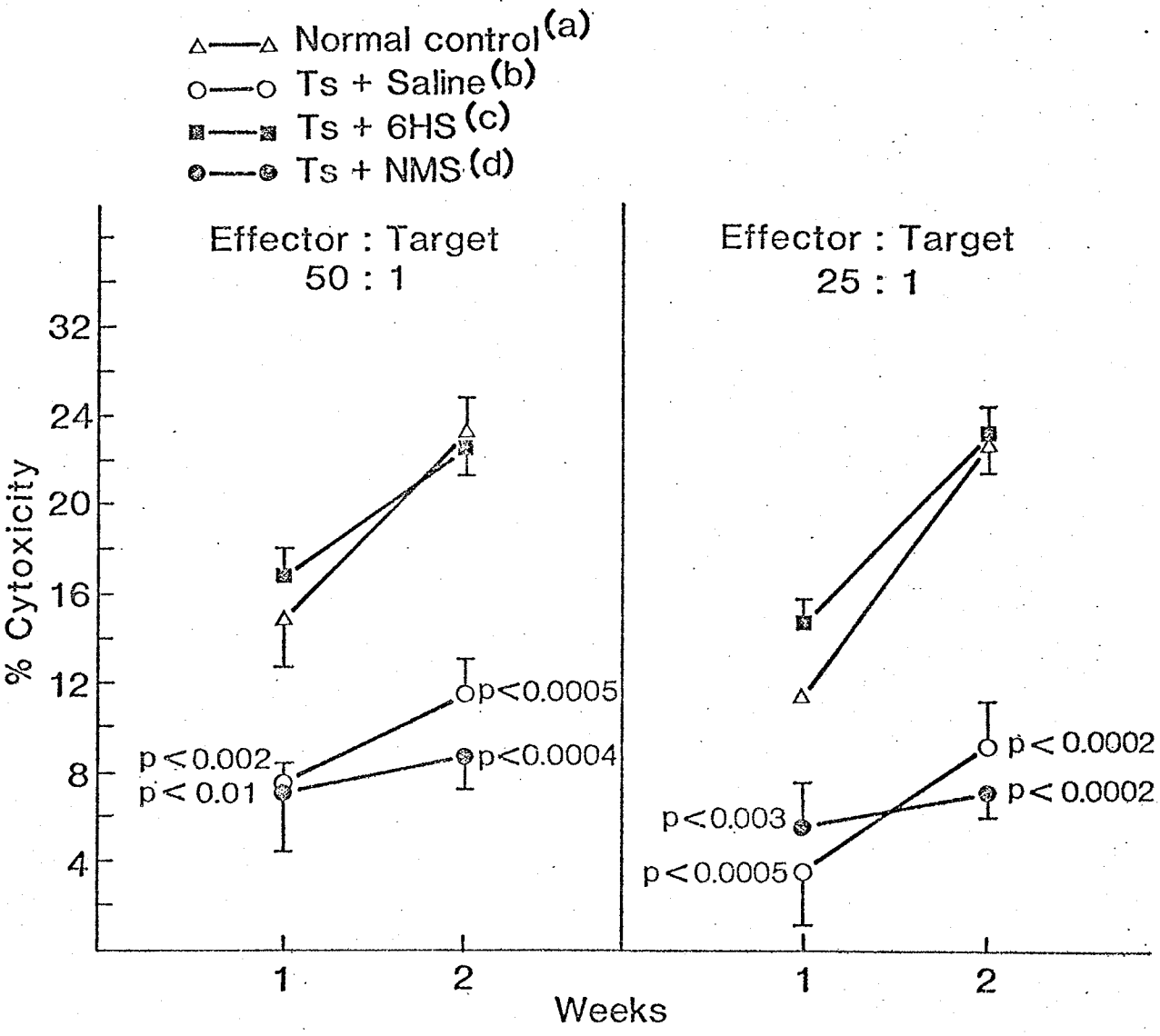
LEGEND FIG. 25:

B6 MICE RECEIVED EITHER 20 x 10^6 HT-TU OR SALINE. A 15-20 x 10^6 NWC-NAC FROM THE FORMER WERE INJECTED SIMULTANEOUSLY WITH 0.3 ML OF EITHER NMS (●) OR 6HS (■) INTO NAIVE B6 MICE. NWC-NAC FROM SALINE INJECTED MICE WERE ALSO TRANSFERRED INTO NAIVE B6 MICE WHICH SERVED AS CONTROL (△). THE MICE WERE CHALLENGED WITH 5 x 10^6 P815.

TARGET CELLS = P815. SPONTANEOUS RELEASE = 12%.

Fig. 26

EFFECT OF 6HS ON Ts FUNCTION : LOW ANTIGEN DOSE



LEGEND FIG. 26:

SEE LEGEND OF FIG. 25 EXCEPT THAT 7.5×10^6 HT-TU WERE USED INSTEAD OF 20×10^6.

therefore suggest that the CTL enhancement observed after injection of the 6HS represents an anti-suppressor effect.

7.3. Specificity of the 6HS anti-suppressor activity

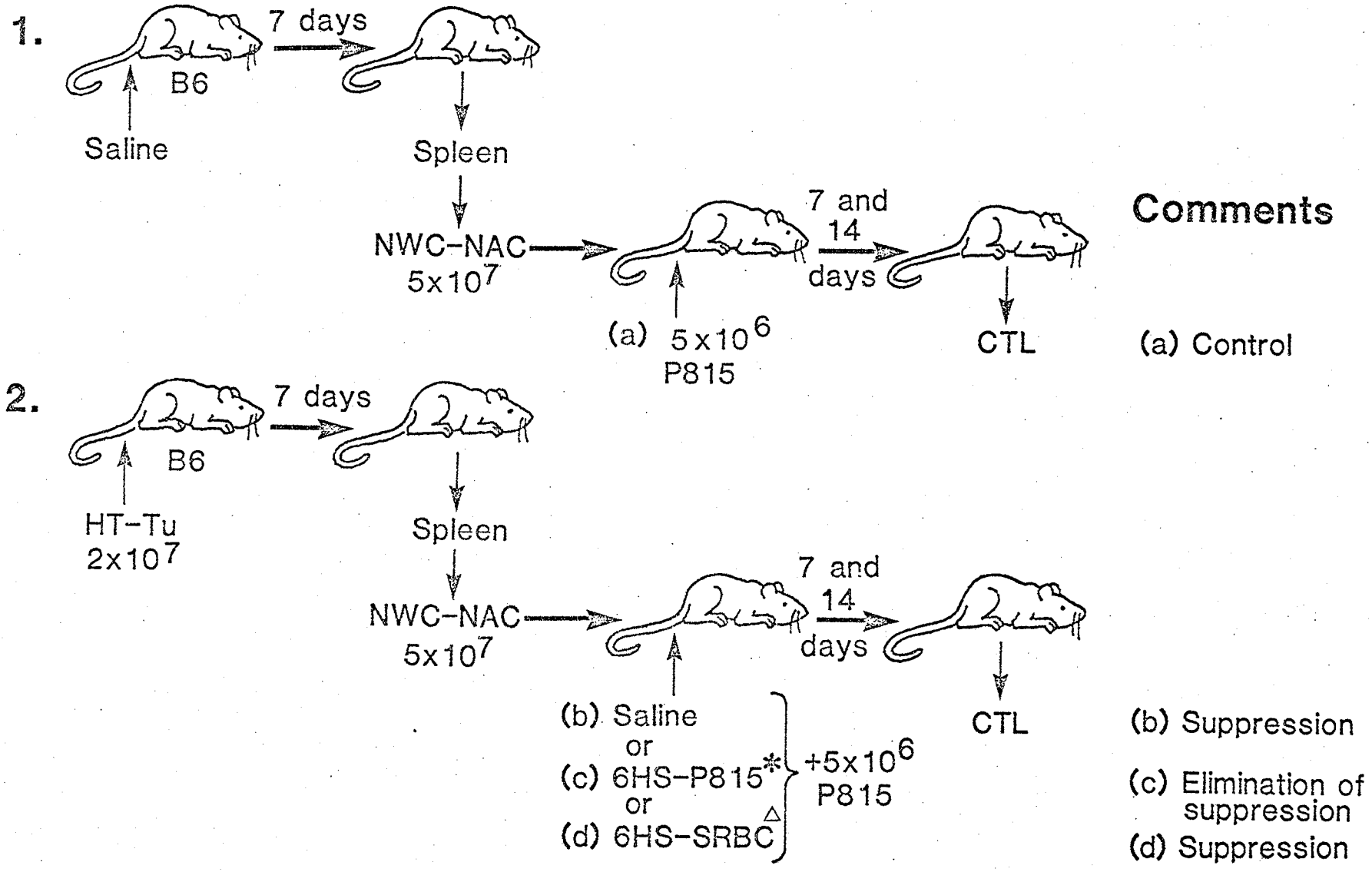
To determine whether the 6HS inhibits suppressor cells pathway specifically, B6 mice which have received NWC-NAC from mice injected with 2 x 10^7 HT-TU, therefore containing Ts, were also given 0.3 ml of 6HS collected after stimulation with either P815 or 5 x 10^8 SRBC. The protocol of this experiment is shown in Fig. 27 and is similar to that of Fig. 24. As shown in Fig. 28 only 6HS collected after stimulation with P815 and not with SRBC can interfere with the action of Ts indicating that the antisuppressor effect of 6HS is antigen specific.

7.4. Induction of anti-suppressor effector cells by 6HS

To further investigate the role of the 6HS in the induction of regulatory cells which are able to interfere with the effect of Ts cells, we performed an experiment according to the protocol shown in Fig. 29. B6 mice were injected i.v. with saline (group a), or NMS (group b) or HT-TU (group c)or 6HS (group d). Spleen cells were removed 7 days later and the NWC-NAC (1.5 x 10^7) after treatment with mitomycin-C were added to a mixed lymphocyte culture (MLC) which contains 5 x 10^6 responder cells (B6) and 5 x 10^6 mitomycin-C treated stimulator cells (P815). The cells were cultured in Linbro plates in 2 ml of the culture medium. Five days later the cells were washed and tested for CTL activity. The results from three experiments are shown in Table XIII. Addition of NWC-NAC from HT-TU injected mice caused suppression of CTL in the MLC (line 2) as compared to the addition of NWC-NAC from saline injected mice (line 1). Addition of NWC-NAC from NMS injected mice (called NMS cells) which also served as control (line 5) did not affect the normal response. The same is true for the addition of NWC-NAC from 6HS injected mice (line 6) (called 6HS cells). These results indicate that: i) HT-TU injected mice contain Ts cells which inhibit the CTL response in the MLC and ii) 6HS cells do not enhance the CTL response when added to a normal MLC (line 6). However, when 6HS cells were added to the MLC which contained Ts cells

Fig. 27

SPECIFICITY OF 6HS ANTI-SUPPRESSOR EFFECT



*6HS is collected from B6 mice after stimulation with P815.
△6HS is collected from B6 mice after stimulation with SRBC.

Fig. 28

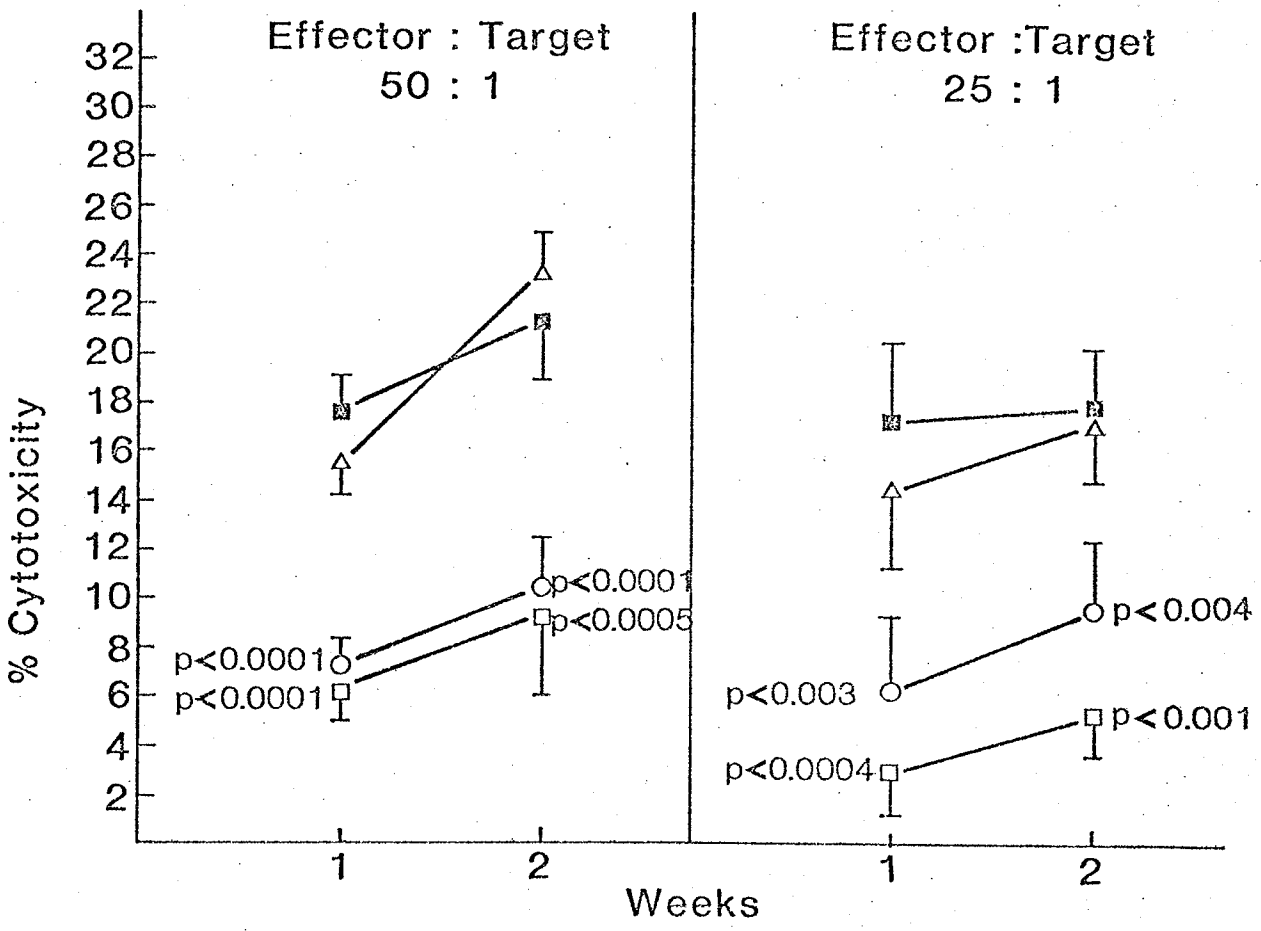
SPECIFICITY OF 6HS INDUCED ANTI-SUPPRESSOR ACTIVITY

△—△ Normal Control (a)*

○—○ Ts+Saline(b)

■—■ Ts+6HS-P815 (c)

□—□ Ts+6HS-SRBC (d)



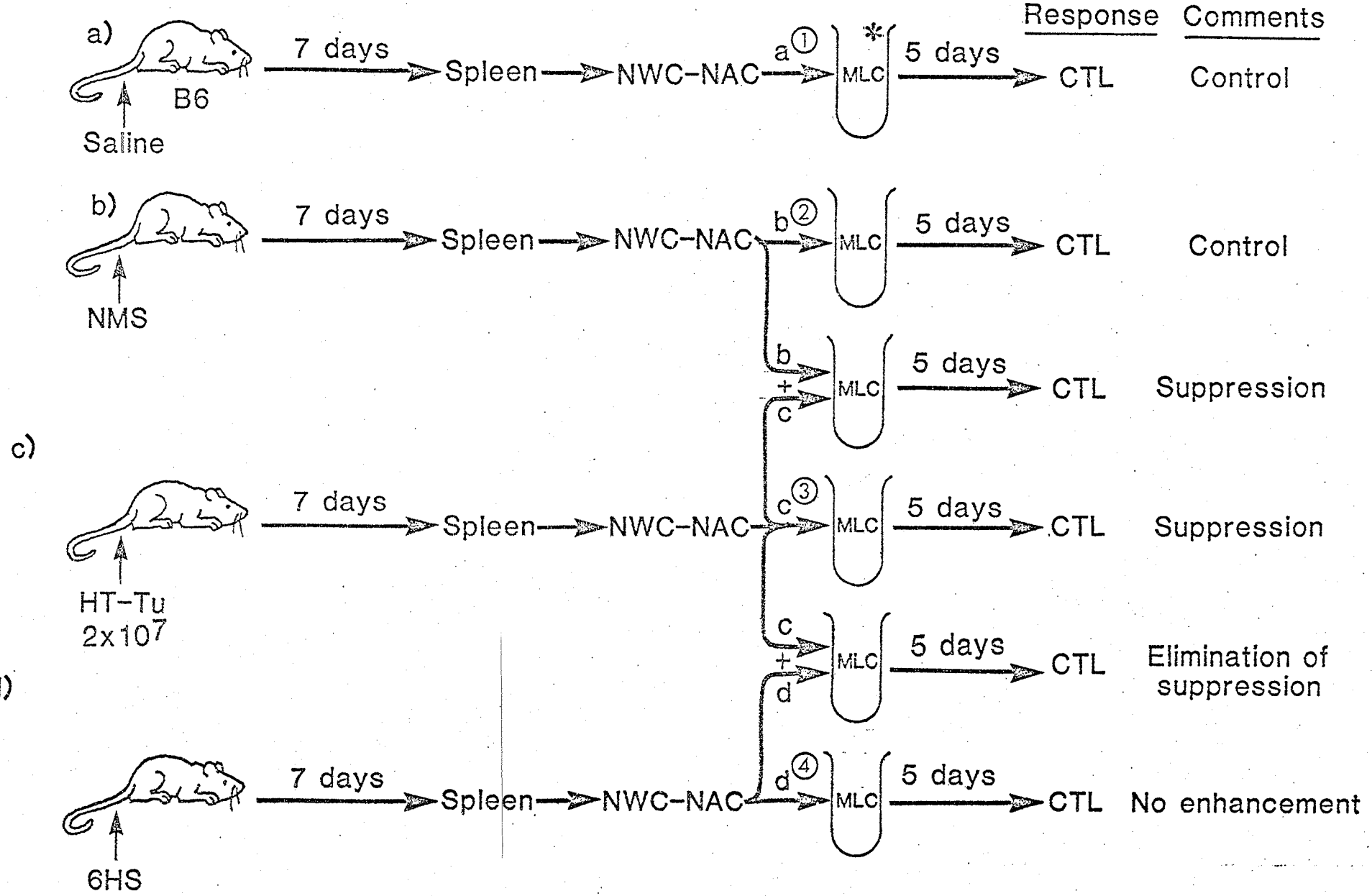
* See Fig.27

LEGEND FIG. 28:

B6 MICE WERE INJECTED WITH EITHER 20 X 10^6 HT-TU OR SALINE. FIVE TO SEVEN DAYS LATER 15-20 X 10^6 NWC-NAC FROM THE FORMER WERE TRANSFERRED INTO NAIVE B6 MICE. THESE MICE RECEIVED EITHER 6HS COLLECTED AFTER STIMULATION WITH INTACT P815 (HENCE CALLED 6HS-P815) (■) OR 6HS COLLECTED AFTER STIMULATION WITH SRBC (HENCE CALLED 6HS-SRBC) (□). AS CONTROL NWC-NAC FROM MICE INJECTED WITH SALINE WERE TRANSFERRED INTO NAIVE B6 MICE (△). ALL MICE WERE CHALLENGED WITH 5 X 10^6 P815.

TARGET CELLS = P815. SPONTANEOUS RELEASE = 12-14%

6HS INDUCES ANTI-SUPPRESSOR EFFECTOR CELL



* MLC = Mixed lymphocytes culture

① a = Normal cells ② b = NMS cells ③ c = Ts cells ④ d = 6HS cells

(line 4) suppression was abolished. This effect was not observed with the addition of NMS cells to a suppressed MLC (line 3). We have further investigated the phenotype of the T cell subpopulation which is responsible for the anti-suppression mechanism. 6HS cells were treated first with anti-Lyt.1.2 + C or anti-Lyt.2.2 + C antiserum before their addition to the MLC. As shown in Table XIII, the 6HS-Lyt.1^+ cells can interfere with Ts function (line 7) while 6HS-Lyt.2^+ cells are not able to do so (line 8).

The data presented above indicate that the 6HS acts through the activation of an effector cell which bears the Lyt.1^+ phenotype. Whether this effector mechanism represents a pathway involving more than one cell remains to be seen. Furthermore, these data provide conclusive evidence of the anti-suppressive mechanism of 6HS in the CTL enhancement. The 6HS has no effect on enhancing in a normal MLC, the CTL response. However, in the presence of Ts in a MLC, it clearly reverses the Ts function allowing a normal CTL response. The reason the 6HS enhances the CTL response in vivo in normal mice is probably due to the concomitant activation by antigen of Ts pathways, which normally restrict the full development of a CTL response. This restriction is lifted by the 6HS, the result being a net elevation of the cytotoxicity level.

GENERATION OF ANTI-SUPPRESSOR CELLS BY 6HS

NWC-NAC + MLC	EXP.1 E:T		EXP.II E:T		EXP. III E:T	
	50:1	25:1	50:1	25:1	50:1	25:1
1) NORMAL CELLS (A)*	25 ± 3.5	20.1 ± 3.4	19.2 ± 7	12.1 ± 3.9	22.6 ± 4.6	14 ± 3.2
2) Ts CELLS (C)	5.8 ± 1.0	3.8 ± 1.1	7.9 ± 1.5	5.9 ± 2.8	7.7 ± 2.7	3.9 ± 1.4
1:2 P <	0.0002	0.0002	0.0005	0.01	0.0003	0.0003
3) NMS CELLS (B) + Ts CELLS (C)	5.1 ± 1.1	2.4 ± 1.0	7.3 ± 2.0	4.7 ± 2.9	2.5 ± 0.7	1.4 ± 0.9
1:3 P <	0.0002	0.0002	0.005	0.01	0.0002	0.0002
4) 6HS CELLS (D) + Ts CELLS (C)	18 ± 3.2	13.6 ± 1.4	21.1 ± 5.4	15.6 ± 6.0	22.8 ± 8.7	15.9 ± 8.8
4:2 P <	0.001	0.001	0.0005	0.01	0.01	0.02
4:3 P <	0.001	0.001	0.0005	0.005	0.005	0.005
5) NMS CELLS (B)	17.3 ± 2.1	14 ± 1.9	15.2 ± 7.3	11.2 ± 4.6	N.D.	N.D.
6) 6HS CELLS (D)	18.2 ± 1.5	15.5 ± 1.3	15.1 ± 4.2	14.9 ± 2.4	N.D.	N.D.
7) 6HS-LyT.1$^+$ CELLS (E) + Ts CELLS (C)	N.D.	N.D.	N.D.	N.D.	15.7 ± 4.6	10.1 ± 3.0
7:2 P <					0.01	0.02
7:3 P <					0.005	0.005
8) 6HS-LyT.2$^+$ CELLS (F) + Ts CELLS (C)	N.D.‡	N.D.	N.D.	N.D.	8 ± 3.5	4.7 ± 3.1

* SEE LEGENDS OF FIG. 29

‡ N.D. = NOT DETERMINED

CONCLUSION: LyT.1$^+$-6HS INHIBITS THE ACTION OF Ts ALLOWING THE GENERATION OF CTL.

DISCUSSION

Serum (6HS) collected 6 hours after antigenic stimulation was found to contain a soluble factor which represents a complex composed of antigen, Ig and a T cell derived factor known as Immunoglobulin Antigen Complexing Factor (IACF) (8) which contains Ia antigens. IACF is produced from T cells in vivo upon stimulation with FCA or released from T cells in vitro by mercaptoethanol or in cultures containing macrophages and T cells by particulate substances such as mycobacteria. The complexes present in the 6HS are taken up by Ia^+ T cells (11) and their uptake is detected by a sensitive technique known as Reverse Immune Cytoadherence (RICA) (1). RICA utilizes a hybrid antibody made of an anti-Ig site and an anti-BSA site. Through the former, it reacts with Ig on the cell surface while through the latter it attracts BSA coated SRBC thus forming a rosette.

Paraskevas and Lee (12) have reported that the T cells which take up the complexes enhance both IgM and IgG-PFC upon transfer into sublethally irradiated recipients together with bone marrow cells. Recently, it was also found that injection of the 6HS collected after either SRBC or BSA in FCA stimulation, can enhance SRBC-PFC or BSA-PFC respectively upon injection into naive recipients along with subimmunogenic dose of the antigen. The latter seems to be mandatory in all types of enhancement generated by the 6HS. The serum by itself does not enhance the immune responses (14).

This work was undertaken to determine whether the 6HS formed after allogeneic stimulation exerts any effect on the cell mediated immunity. The mastocytoma P815 (DBA/2 origin, $H\text{-}2^d$) was used as stimulating antigen and 20×10^6 cells were injected i.p. into C57Bl/6J (B6, $H\text{-}2^b$) mice. The serum collected six hours later was found to contain a "cytophilic" for T cells Ig which was taken up by normal spleen cells (NSC) upon incubation in vitro. A similar result was also obtained when DBA/2 SC were used as stimulating antigen. D-region differences were sufficient to induce the "cytophilic" Ig. This was

shown by injecting B10.BR (k k k k k) SC i.p. into B10.AKM (k k k k q) mice and vice versa. These experiments indicate that alloantigens derived from whole-MHC or only D-region can stimulate the formation of the "cytophilic" Ig. It was previously demonstrated that the "cytophilic" Ig represents complexes of Ig and Ag. This was shown by autoradiography using radioactively labelled Ag(6) as well as affinity chromatography with antisera specific for the antigens used. We have therefore, asked the question whether the alloantigen induced "cytophilic" Ig also represents such complexes. Columns made with mouse anti-rabbit Ig (anti-Ig) or anti-H-2^d (anti-antigen) serum removed the "cytophilic" Ig. (Table VII and Table VIII). These results are similar to those reported previously for foreign antigens and suggest that following allogeneic stimulation, the 6HS contains complexes of Ig and alloantigens. As mentioned above the complexes induced by stimulation with foreign antigens enhance in an antigen specific manner the 7S antibody response (14). We have therefore asked the question whether the allogeneically induced complexes exert any effect on CTL responses. 6HS (0.3 ml) collected after allogeneic stimulation was injected i.v. in normal mice which were challenged later with allogeneic cells. The 6HS collected after stimulation with P815 enhanced the CTL when low doses of this antigen were used for challenge (Fig. 5). Similarly, 6HS collected from B6 mice after stimulation with normal DBA/2 SC enhanced the CTL response to a low dose of DBA/2 SC (Fig. 7). The same is true for 6HS collected after stimulation with D=region coded antigens (Fig. 8 and Fig.9). These results indicate that 6HS collected after stimulation with whole-MHC or only D antigens contain an enhancing factor that augments the CTL response.

The enhancing factor was found to act specifically. When B10.BR SC were used as stimulating alloantigen for the production of the 6HS in B10.AKM, it was found that the 6HS was able to enhance CTL response against B10.BR SC but not against the unrelated alloantigen B10.M SC (Table VI). Similar results were found when B10.AKM SC were used for the production of the factor. The nature of the enhancing factor was investigated using affinity chromatography as mentioned

above. The same immunoadsorbents which removed the "cytophilic" Ig abolished the enhancing activity (Fig. 11 and Fig. 12). These results may be accepted as evidence compatible with the suggestion that the complexes of Ig and allo-antigen constitute the CTL enhancing factor.

We have next examined the phenotype of the cell which produces the CTL enhancing factor. It was previously shown that the complexes produced after stimulation with foreign antigens (e.g. SRBC) require the presence of Lyt.1^+ cells (10). The complexes produced after allogeneic stimulation were found to require either Lyt.1^+ or Lyt.2^+ cells (Table IX and Table X). This is true whether the stimulating antigen was whole-MHC or only D-coded determinants. The reasons for the differences observed between foreign antigenic stimulation and allogeneic stimulation, in the phenotype of the T cells required for the production of the complexes, are not apparent at the present time. Both the Lyt.1^+ and Lyt.2^+ derived complexes were found to enhance the CTL responses when injected into naive recipients two hours before the challenge with the antigen (Fig. 13). Whether I-region differences can stimulate the production of the complexes was not investigated. However, D region difference is sufficient for the induction of the complexes as shown by these experiments and the concomitant existence of I region differences are not necessary since the P815 cells lack I region coded determinants. It is tempting to suggest that for CTL development two factors are required: one an antigen specific which is represented by the complexes reported here and one an antigen non-specific such as IL-2. The role of IL-2 in combination with the allogeneically induced complexes for CTL development was not investigated. Since 6HS contains full enhancing activity, one must assume that according to our hypothesis, the 6HS may contain in addition to the complexes, IL-2. The fact remains that removal of the complexes abolishes the enhancement, therefore, the complexes are necessary for CTL differentiation.

Whether the complexes produced from Lyt.1$^+$ cells are different from those produced from Lyt.2$^+$ cells is not known. The 6HS from either Lyt.1$^+$ or Lyt.2$^+$ T cell reconstituted mice contain complexes and enhancing activity. Using the RICA assay, we have shown that the complexes produced in the presence of Lyt.1$^+$ are taken up by both Lyt.1$^+$ and Lyt.2$^+$ cells, while those produced in the presence of Lyt.2$^+$ cells are taken up only by Lyt.2$^+$ cells. The functional significance of the uptake of the complexes by T cells is not known. From studies in the humoral response, it is known that T cells which have taken up the cytophilic complexes markedly enhance the antibody formation. It has also been shown that exposure of T cells in vitro to the complexes induced by SRBC, activates the T cells which then markedly enhance antibody formation (unpublished data).

Further studies are necessary to elucidate the role of the Lyt.1$^+$ and Lyt.2$^+$ derived enhancing activity in the development of a CTL response.

The relationship of these results to those in the literature is at the present time unknown. Thus Panfilli and Dutton (71) have reported that Th cells are generated against K/D differences. Furthermore, Melief et al (72) showed that it is the Lyt.1$^+$ cell which recognizes K region differences. An Lyt.1$^+$ T cell is known to release IL-2 which acts as the proliferative signal for CTL. On the other hand, Okada and Henney (85) reported that the Lyt.2$^+$ cells recognize K/D determinants and are required in the differentiation of CTL. Similarly, Raulet and Bevan have shown that D-restricted clones of CTL need the participation of Th cells (Lyt.2$^+$) which proliferate and release soluble factor upon stimulation with D but not I-region determinants (269). At the present time it is not known whether IL-2 acts alone or in concert with other soluble mediators. Our results implicate the allogeneically induced complexes in the development of the CTL response. Such complexes were produced in the presence of Lyt.1$^+$ and Lyt.2$^+$ T cells. These complexes act as an antigen specific factor while the IL-2 is known to act non-specifically.

Vasudevan et al (213) attempted to enhance the CTL response by incubating the spleen cells of mice injected with syngeneic tumors in vitro for 24 hours. They found that after in vitro incubation, the spleen cells expressed higher cytotoxicity than that detected before incubation. De Landazui and Herberman (214) also showed that spleen cells from W/Fu rats injected with syngeneic Gross virus-induced leukemia, became cytotoxic after mere in vitro incubation for 12-24 hours at 37°C. Czitrom and Gascoigne (215) found that lymph node cells from mice injected with cells with minor histocompatibility differences demonstrated a higher CTL level after incubation in vitro for 3 days without added antigen. Similar results have been found by Kamat and Henney (216) who showed that spleen cells of B6 mice injected with allogeneic tumors (P815) increased their cytolytic activity after 24 hours of incubation in vitro without added antigen. The effector cells were found to be of T cell origin. However, they failed to show any CTL augmentation when the spleen cells were incubated at 4°C. Similar results were reported by Wagner et al (207). They suggested that the reason behind this phenomenon is due to the fact the Ts cells which act in vivo to inhibit the cytolytic activity are eliminated after in vitro incubation at 37°C (217). These Ts cells appear in the thymus and the spleen of mice 24 hours after antigen exposure (218, 219). In vitro elimination of Ts cells leaves other populations to develop into effector and amplifier pathways. These results suggested that Ts cells may limit the development and/or expression of effector CTL cells. We have therefore asked the question whether the augmentation of CTL observed after injection of 6HS may (as in the case of the in vitro incubation) be related to some interference with the suppressor cell pathway. We have been able to reproduce the augmentation of CTL activity as reported in the literature after incubation for 24 hours in vitro. However, incubation of spleen cells from mice injected with 6HS did not result in further augmentation of cytotoxicity (Fig. 14-B). The interpretation of these results is difficult. If the augmentation after 24 hour incubation is due to elimination of Ts cells, one may reasonably assume that such Ts cells did not normally develop or their function could not be expressed in

the animals injected with 6HS. According to this interpretation, the augmentation of CTL observed after injection of 6HS may be due to an effect on Ts function. In order to obtain further evidence on the mechanism which underlies the CTL augmentation, we have used two other approaches known to interfere with Ts cells. It has been shown that antigens coded by the I-J subregion which was originally defined by Murphy et al (220) are present on Ts cells and factors derived from them (221). I-J coded determinants act as the restricting elements for the expression of Ts cells (222, 223). This restriction is similar to that exerted by K/D molecules acting on CTL or I-E molecules on Th cells (224). Lowy et al (260) identified an $I\text{-}J^{+}$ APC which activate a particular subset of Ts, Ts3 which share the I-J determinant with the APC. In addition, Braley-Mullen have shown that Ts cells which were generated against type III pneumococcal polysaccharide are activated when the toleragen is coupled to spleen cells which shared the I-J determinants with the responding population (261).

The restriction imposed by I-J determinants may occur not only at the activation level of Ts cells, but also at the effector stage of these cells. In a series of reports, Klein and his colleagues indicated that Ts cells which inactivate the antibody response to lactate dehydrogenase B (LDH_B), inhibit Th cells which share with the Ts cells an I-region coded determinant serologically similar to the I-J gene product as determined by anti-I-J monoclonal antibody (262, 263).

Injection of anti-I-J antisera was known to eliminate Ts cells in vivo leading to an enhanced immune response. Greene et al (208) showed that the injection of anti-$I\text{-}J^{k}$ alloantisera into A/J mice resulted in the decrease of the growth of the syngeneic tumor S1509 a. The effect of this antiserum was related to its ability to inhibit Ts cells which carry the I-J region determinants. The same antiserum was also able to decrease the growth of P815 tumor cells or 1316 ultraviolet radiation-induced fibrosarcoma upon inoculation of these tumors into syngeneic hosts again as a result of interference with the hosts Ts (209).

We have also injected anti-I-J^b antisera into B6 ($H\text{-}2^b$) mice and showed that this treatment resulted in the augmentation of the CTL responses, whereas the injection of the anti-I-J^k antisera into $H\text{-}2^b$ mice was not effective (Fig.15).

However, injection of the 6HS into B6 mice which were treated with anti-I-J^b antisera did not result in any significant enhancement of CTL above the level seen with the control groups. In contrast the 6HS significantly enhanced the CTL responses of B6 mice which were treated with anti-I-J^k antiserum. These results suggest that treatment with anti-I-J antiserum resulted in the removal of the augmentation of CTL responses,i.e. the difference in CTL observed between the control groups (receiving NMS or saline with the antigen) and the test group (receiving 6HS). Elimination of Ts by anti-I-J antiserum therefore, masks the 6HS induced enhancement.

CY was originally found to enhance the delayed type hypersensitivity (DTH) reactions in mice and guinea pigs by affecting suppressor B cells (225-227). Later work showed that CY affects T but not B cells (228). For example CY enhanced DTH and contact sensitivity (CS) (229-224). Injection of CY two days before the injection of the contact sensitizer abolishes the development of Ts cells which inhibit the transfer of CS into naive recipients (235). When CY was injected two days before the administration of SV-40 transformed tumor cells, an immune response was demonstrated against the tumor cells due to an effect of CY on Ts cells in vivo (236). Even low doses of CY (as low as 500 ug/mouse) when injected two days before the injection of 3-methylcholanthrene-induced sarcoma MC 2, resulted in the diminution of the growth of this tumor in syngeneic mice (237). We have therefore, attempted to study the augmentation of CTL by 6HS in cyclophosphamide treated animals. B6 mice were injected with 100 mg/kg body weight of CY. This treatment was found as previously described by others to result in high CTL response when low dose of antigen (1×10^6 P815) was used to challenge the mice. Schwartz et al (238) indicated that CY enhanced DTH

response only when higher but not lower doses of antigen (SRBC) were used. They indicated that low doses of antigen stimulate the amplifier cells which help the DTH-inducer cells and that these amplifiers are CY-sensitive. However, high-doses of the antigen activate inducer cells which are also CY-sensitive. On the other hand, Asherson et al (239) showed that CY treatment of mice increased the CS after painting the mice with low but not high doses of the contact sensitizer, indicating that low doses of the antigen generate Ts cells. The discrepancy between our data and Schwartz's may be due to the antigen used. For example, low doses of SRBC used in their work could not stimulate Ts cells.

Injection of the 6HS in CY-treated mice did not significantly enhance the CTL response above the level observed in the control groups (Fig. 16). Collectively, these data suggest that 6HS contains an augmenting factor for CTL and that the augmentation may be due to interference with Ts cell function.

It has been shown that reconstitution of CY treated mice with normal NWC-NAC abolishes the enhancement of the immune response. In order to determine whether the CY and 6HS act on similar targets, we have used NWC-NAC reconstitution. NWC-NAC from normal mice were transferred at day 0 into CY-treated mice which have received 100 mg/kg body weight of CY two days earlier. This reconstitution abolished the CY-augmentation of CTL responses indicating that CY-sensitive T cells are the ones responsible for the suppression of the immune responses. It should be mentioned here that the action of CY takes place shortly after its injection and that CY half-life in the circulation is between 4-6.5 hours (240). Therefore, CY has no effect on cells injected two days later. Our results with reconstitution of CY treated mice are in accordance with those of others (210, 211). Injection of 6HS simultaneously with NWC-NAC two days after CY injection did not abolish the augmentation of CTL response, whereas injection of NMS led to the inhibition of this response (Fig. 18-B). These results indicate that 6HS acts to either activate some cells in the recipients which were able to interfere with the generation of Ts cells from the NWC-NAC

inoculum, or that 6HS directly inhibits the Ts-P present in the transferred NWC-NAC. To discriminate between these two alternatives, we injected 6HS into B6 mice, then reconstituted them with NWC-NAC two days later. The results showed that control mice which received NMS at day -2 have low CTL levels after reconstitution with NWC-NAC whereas mice injected with the 6HS demonstrate significant augmentation of CTL level. Thus in contrast to CY augmentation of CTL, that caused by 6HS cannot be abolished by normal NWC-NAC.

Although both CY and 6HS may augment CTL by interference with Ts pathway, their site of action is certainly different. The former may act by eliminating a cell necessary for the Ts development and NWC-NAC can provide this cell in the reconstitution experiments thus eliminating augmentation. In contrast, 6HS augmentation may be due to the activation of a cell which can still counteract the development of Ts, therefore, NWC-NAC cannot abolish augmentation. On the other hand, it is still conceivable that the 6 hour complexes may "survive" on the surface of some cells for prolonged periods of time and thereafter released and directly inactivate Ts as they develop. Kontiainen and Mitchison (307, 308) have shown that antigen-antibody complexes can be retained on the surface of T cells for prolonged periods of time and activate antibody formation. Further evidence in favor of the first alternative is provided below. Direct evidence for interference of the 6HS with Ts function was sought by another approach, i.e. the induction of Ts cells.

Ts cells were induced by injecting mice with heat treated tumor cells. This method resulted in the generation of Ts cells that inhibited the CTL response upon transferring into naive recipients which received the antigen shortly after this transfer. Sondel et al (241) have found that heat-treatment of allogeneic cells induce suppressor cells that inactivate CTL responses in human peripheral blood lymphocytes. Chiu et al extended these observations to murine system and found that i.v. injection of heat-treated allogeneic spleen cells (242) or tumor cells (212) resulted in the generation of Ts cells which were

able to block the generation of CTL. They have also found that this suppression is not due to the presence of cytotoxic antibody which may eliminate CTL, neither to the generation of CTL which may suppress the response by acting on stimulator cells. Most likely, heat-treatment of allogeneic cells destroys the antigenic determinants which are important for the induction of CTL or Th cells but has no effect on determinants that activate Ts cells. Recently, Holan and Mitchison (243) confirmed these data in vitro. Those authors generated allospecific Ts cells upon incubation of normal cells for 4 days with heat-treated allogeneic cells. Our results differ slightly from the work cited above. Ts cells were generated in vivo and their function was also tested in vivo, i.e. Ts generated after the injection of heat-treated allogeneic cells were transferred into naive mice and CTL response was then tested in the recipient mice.

The i.v. injection of heat-treated allogeneic cells (2×10^7 or 7.5×10^6) generated suppressor cells which inhibit the CTL response when transferred into naive recipients that receive intact P815 as an antigen. The suppressor cells were found to be T cells since their effect was diminished after treatment with anti-theta plus complement (Fig. 20).

When 6HS was injected simultaneously with the high dose of the HT-P815 (20×10^6) it blocked the induction of the Ts cells (Fig. 22). However, when the serum was injected with the low dose of the toleragen (7.5×10^6) it did not inhibit the induction of these cells (Fig. 23). The difference between 6HS effects using high and low doses of the toleragen is not clear as yet. It could be due to the presence of two suppressor cells acting on two different stages of CTL generation as suggested by Sy et al (156). One cell, Ts2, suppresses Lyt.1$^+$ Th2 cells which produce IL-2 and the other, Ts1, suppresses Lyt.1$^+$ Th1 cells which provides signal 1 to CTL-P. This signal is necessary for making CTL-P susceptible to IL-2. Interleukin-2 was found to be able to reverse the effect of Ts2 but not Ts1. In this regard it should be mentioned that the effect of Ts1 disappears upon transferring into naive recipients. Therefore, it could be

argued here that a high dose of HT-TU generates Ts2, whereas Ts1 is generated by a low dose of the tolerogen. Hence, 6HS generates anti-suppressor (As) cells which act predominantly to inhibit the function of Ts2 but not Ts1 (Fig. 30).

On the other hand, 6HS was able to block the effect of Ts cells regardless of the tolerogen dose which was used to generate them (Fig. 25 and Fig. 26). These data indicate that 6HS is able to interfere with the effect of Ts cells and depending on the tolerogen dose used, it also interferes with the induction of these cells.

To further prove that 6HS generates As cells which inhibit the Ts pathway, we have designed an experiment in which 6HS was injected alone (without antigen) into B6 mice and spleen cells were collected 5-7 days later (called 6HS-cells). The 6HS cells were added to MLC with or without added Ts cells which were generated by the i.v. injection of HT-TU into naive B6 mice. The results showed that 6HS-cells did not enhance the CTL responses when added to the MLC, indicating that these cells are not helper or amplifier cells. However, when they were added to the MLC in which Ts cells were present, 6HS-cells reversed the effect of Ts cells. These results indicate that 6HS-cells act only in the presence of Ts cells and their action by some unknown mechanism interferes with the function of Ts cells. We tentatively call the cells which mediate the action of the complexes, anti-suppressor cells. Whether antisuppression is exerted by one cell or represents a pathway involving more than one cell remains to be seen.

In previous work from this laboratory, it was reported that the complexes enhance the antibody response only to low or subimmunogenic doses of antigen (14). The same results were obtained for CTL responses in our work (data not shown). These observations may have an explanation in the anti-suppressor functions of the complexes reported above. It is possible that in the response against low antigen doses suppression is dominant over immunity. Blocking of

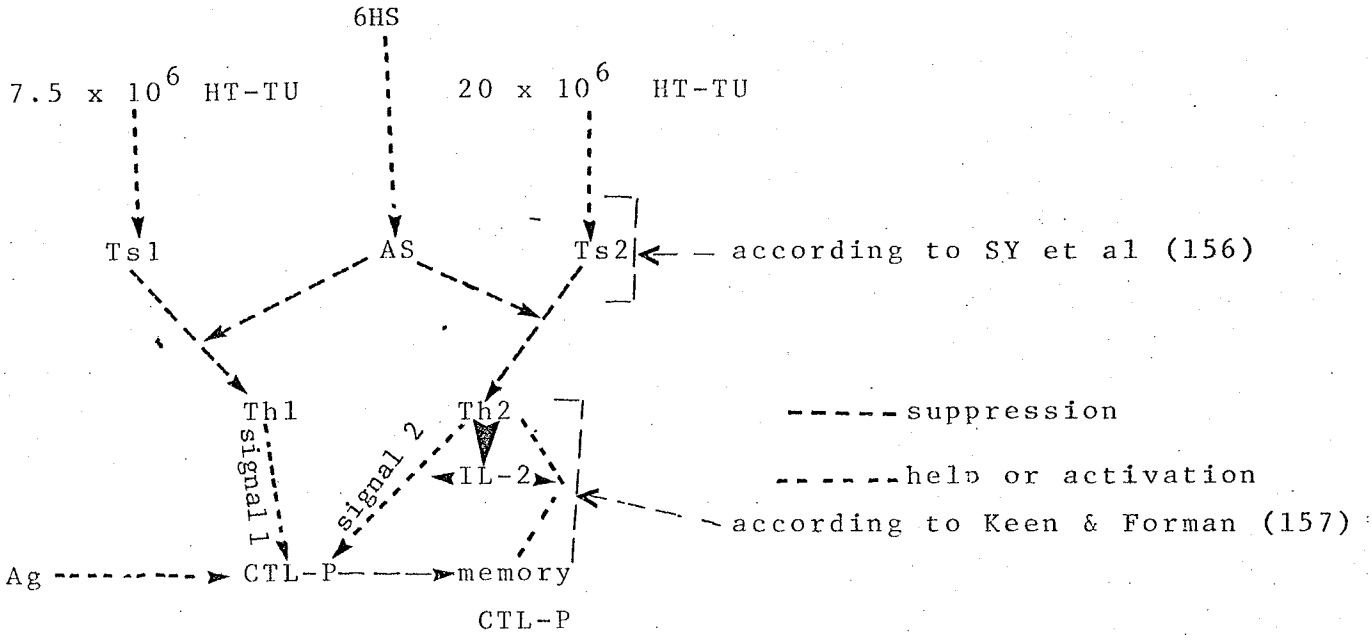


FIG. 30: Possible mechanism of action of 6HS in inhibiting the induction of Ts cells.

the Ts by 6HS will allow an immune response to develop at low antigen doses without interference by Ts. This is measured as enhancement. However, with higher antigen doses helper cell function dominates over suppression and the additional 6HS will not have any effect. Preliminary data obtained in this laboratory tend to support this explanation (unpublished data). A possible mechanism for the action of 6HS is shown in Fig. 31.

In addition to IL-2, very few factors have been found to enhance the CTL response. One of these factors is known as allogeneic effect factor (AEF) which has been previously found to enhance the antibody formation (reviewed in 244) and more recently the CTL (245). It was suggested that AEF provides a mitogenic stimulus which may overcome suppression. However, there is no indication that this factor can activate the generation of the As cells.

Recently, Gershon et al (246) described a new immunoregulatory pathway in which Lyt.2^+, I-J^+ cells produce a factor that generates Lyt.1^+2^+, I-J^+ contra-suppressor (Cs) transducer cells which activate the Lyt.1^+, I-J^+ Cs effector cells which in turn act to render Th cells resistant to the suppression by the Ts cells (247). Like our As cells, it was found that the Cs cells are not helpers (248).

Although most of the work concerning the Cs cells was done in vitro, Green and Gershon (249) have found that the Cs cells are generated in hyperimmunized animals. It was also found that the Cs inducers are present in the microenvironment (e.g. the gut) and upon interaction with the Cs-transducers they activate the Cs-effectors (250). In addition, mediators like histamine released at local sites of the immune response can activate the Cs cells when interacting with their receptors (251). Very recently, Verson et al (252) indicated that the Cs cells can protect the immune spleen cells from suppression allowing the development of DTH reactions in the recipient mice. These results indicate that Cs cells exist in vivo and they are not an in vitro artefact.

Beside being both Lyt.1^+ and both lack helper activity, we do not know if

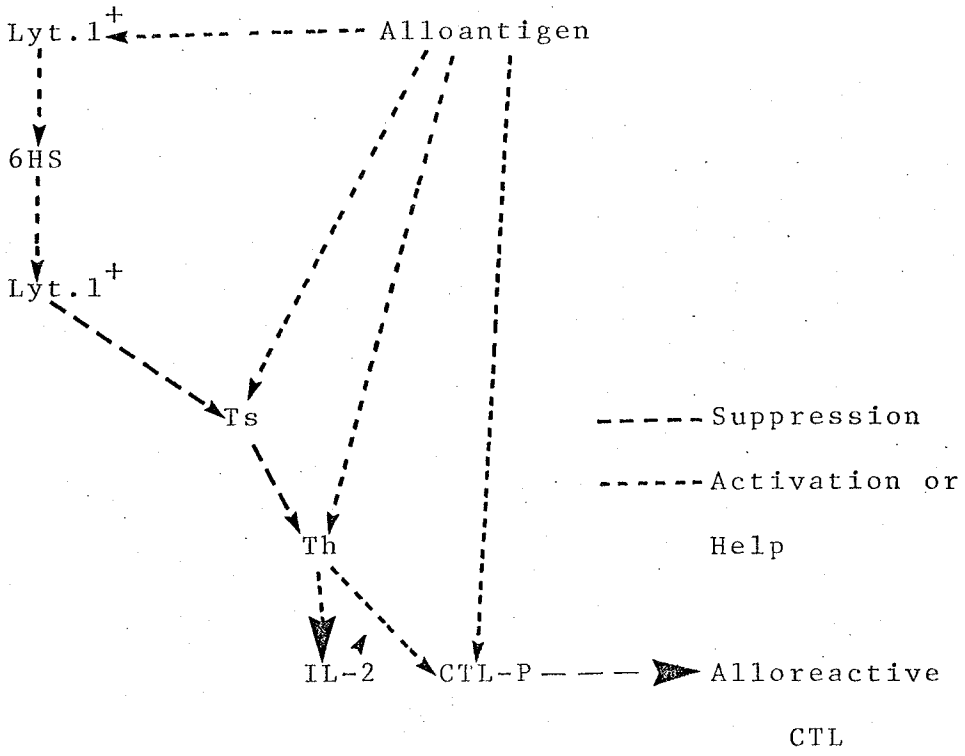


FIG. 31 (Possible anti-suppression pathway)

the As and the Cs cells are the same or different cells. Gershon's group indicated that Cs cells act to make helper cells resistant to suppression. They have developed an in vitro system in which the Th cells were sensitive to suppression by Ts cells. However, when Th cells were incubated with the Cs cells, the latter cells provide the former with ability to resist suppression. Consequently, Th cells were able to enhance the antibody formation even in the presence of Ts cells. The interaction between the As and the Ts cells must be specific since 6HS generated from SRBC stimulation (6HS-SRBC) could not inhibit the Ts generated after P815 injèction (Fig. 28). The significance of anti-suppression in the function of the immune response remains to be seen. According to data published from this laboratory, the formation of the complexes 6 hours after antigenic stimulation represents a universal response of the immune system to all antigens tested: foreign, allogeneic or syngeneic tumor cell antigens. It is probably one of the, if not the, earliest response of the immune system to antigens since the complexes can be detected as early as 3 hours after immunization. If one accepts that the fundamental function of the immune system is the self-non self discrimination the generation of the complexes may be the result of such a function since none of the self antigens tested (proteins or cells) will induce the complexes. It is speculated that the early activation of an antisuppressor pathway may be pivotal in the expression of immunity. Antisuppression may prevent the induction or expression of Ts thus allowing time for the activation of Th. If this is the case, antisuppression may be considered as the "master switch" which determines between suppression or unresponsiveness and immunity. It is attractive to speculate that some aspects of the genetic control of the immune response may be regulated at the level of induction or function of the 6 hour complexes. Thus the development of suppressor cells in non-responder mice in some systems, may be due not to their special propensity for Ts induction, but to a genetically determined inability to generate the 6 hour complexes or some interference with the function of the complexes.

These and other questions are presently under investigation and further data need to be obtained in order to evaluate the full significance of this novel immunoregulatory pathway.

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