Waters, Stanley Joseph

CHARACTERIZATION OF RESTRICTION ELEMENTS UTILIZED BY ANTIGEN-SPECIFIC HELPER T CELL CLONES AND HYBRIDOMAS

City University of New York PH.D. 1984

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CHARACTERIZATION OF RESTRICTION ELEMENTS UTILIZED BY ANTIGEN-SPECIFIC HELPER T CELL CLONES AND HYBRIDOMAS

by

STANLEY J. WATERS

A dissertation submitted to the committee on Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the subject of Biomedical Sciences, The City University of New York.

1984

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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I. ABSTRACT

CHARACTERIZATION OF RESTRICTION ELEMENTS
UTILIZED BY ANTIGEN-SPECIFIC HELPER T CELL
CLONES AND HYBRIDOMAS

by

Stanley J. Waters

Advisor: Constantin A. Bona, M.D., Ph.D.

This study was designed to analyze the restriction elements utilized in the activation and effector function of antigen specific helper T cells. Our first aim was to generate monoclonal T cell populations reactive to keyhole limpet hemocyanin (KLH). Two approaches were employed to clonally expand these antigen specific T cells: (1) non-transformed T cell clones were isolated from antigen primed CB6/F_1 mice which exhibited proliferation to KLH in the presence of irradiated syngeneic F_1 spleen cells. (2) T cell hybridomas were also generated by the fusion of KLH reactive lymph node cells from CB6/F_1 mice with an AKR derived T lymphoma (BW5147). Antigen recognition was measured by the amount of Interleukin-2 (IL-2) secretion by the T cell hybridomas. The panel of monoclonal T cells produced in this manner were characterized in terms of their antigen specificity, surface phenotype, and function. These cells provided a simple, well defined model system to study MHC-restricted T cell interactions. Experiments focused on the genetic restriction of these cells included mapping of restriction elements using recombinant inbred strains of mice, and blocking activation of T cells with monoclonal antibodies specific for polymorphic

MHC encoded class II antigens. Functionally active T cell clones proved useful in the study of genetic restriction between T cells and B cells.

Our second aim was to prepare monoclonal antibodies specific for those T cell clones and hybridomas used as immunogens, which could identify idiotype-like determinants on their antigen receptor. One of these antibodies specifically bound to only the immunizing T cell hybridoma FN1-18, but failed to inhibit the antigen specific release of IL-2 by this hybridoma. A second antibody S3a.6-18, raise against a KLH specific T cell line, bound to both hybridoma FN1-18 and a T cell clone A12.11. This antibody prevented KLH recognition by both monoclonal T cell populations. Antibody S3a.6-18 also specifically inhibited the ability of clone A12.11 to cooperate with TNP primed B cell and generate anti-TNP plaques. This data confirms at the level of T cell function, that antibody S3a.6-18 binds to the antigen receptor on clone A12.11. These results also support the hypothesis that the antigen receptor on T cells displays a similar degree of idiotype diversity as immunoglobulin molecules.

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I would also like to thank the members of my committee for the stimulating discussions and insight they contributed to my thesis. Dr. Constantin Bona, I would particularly like to thank for the guidance, support and constant encouragement for the years I spent in his laboratory. Finally, my sincere thanks go to my advisor and friend, Dr. Robert Winchester, for his immeasurable patience and guidance in my career.

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V. INTRODUCTION

The vertebrate immune system is capable of responding to a multitude of antigenic determinants, and immunocompetent cells are responsible for the specific immune responses against this antigenic library. The various types of immunocompetent cells express receptor molecules capable of binding to foreign antigenic determinants, as well as to autologous antigens. The two classes of antigen-specific lymphoid cells, T (thymus-derived) and B (bone marrow-derived) lymphocytes, differ fundamentally in their modes of antigen recognition. T cells exhibit a requirement for simultaneous recognition of foreign antigen along with autologous MHC (major histocompatibility complex) encoded determinants on the surface membrane of cells. In contrast, B cells can recognize and bind foreign antigen without these MHC encoded determinants. The T cell repertoire also varies from B cells in that it contains a high frequency of cells specific for cell surface molecules of allogeneic individuals. These differences in antigen recognition are a consequence of the types of antigen receptor molecules utilized by these two cell populations.

The differences in the modes of antigen recognition by T cells and B cells is related to the important effector functions these two cell types play in response to infectious agents. The effector T cells are particularly important in responses to intracellular viral or parasitic infections. The requirement for antigen recognition in association with cell-surface MHC determinants preferentially focusses the effect of these T cells on cell bound antigens. In contrast, the ability of B cells and secreted antibodies to react directly with foreign antigens allows the focussing of this effector system on those extracellular infectious agents which would not be susceptible to T cell recognition. Apparently, the differences in the types of receptors and their requirements for antigen recognition results in efficient division of effector responses

by immunocompetent cells.

The cellular and molecular events that result in an antigen-specific response are not completely defined. The clonal selection theory stands as a central concept, however, in the understanding of lymphocyte activation and expansion. This theory proposes that during the development of an organism, there arises a large repertoire of lymphocytes, the progeny of each clone bearing a membrane associated receptor, which recognizes a unique antigenic determinant. The immune response is antigen specific because antigen entering the organism selectively binds to cells bearing the appropriate receptors. As a result of antigen binding, the lymphocytes are then activated to undergo a series of events leading to cell division and differentiation. Differentiation entails the development of effector functions, such as antibody production for B cells and effector or regulabory T cells.

Evidence supporting the concept of clonal selection based on variations in the antigen binding receptors of lymphocytes was provided by the discovery that idiotypic determinants found in the variable regions of immunoglobulin molecules were also found on the antigen receptor for B cells (1). The idiotypic determinants associated with B cells appear to be as diverse as the antigenic determinants recognized by these cells. A single clone of B cells which could be expanded by exposure to a particular antigen, all bear the same idiotypic markers. The idiotypic determinants associated with a particular antigenic specificity by B cells have provided insight into the diversity of immunoglobulin binding sites and the role these determinants may play in the regulation of the immune response.

It was later shown that antibodies induced by the same antigen in different individuals of an inbred strain of mice, as well as myeloma

proteins specific for the same hapten can share a cross-reactive or regulatory idiotype (2). Serological and structural data have shown that a single antibody molecule of myeloma protein can express both an individual idiotype and a cross-reactive idiotype (3). This indirect evidence suggested that idiotypes may have a role in regulation of the immune response, forming a network of interactions between secreted immunoglobulins, B cells, and possibly T cells.

The original concept of idiotype network addressed the problems of activation and regulation of lymphocytes at the clonal level by applying a directionality to idiotypic recognition (4). Therefore, idiotype specific B cells or suppressor T cells or their products could decrease the frequency and activity of cells displaying the appropriate idiotype, while clonal activation and expansion of lymphocytes could be encouraged through idiotype recognizing helper T cells or their products. These elements focussed through variable region--variable region interactions are considered to form the cellular and soluble components of the network.

According to the network concept, the frequency of idiotypically distinct antibody expressed during an immune response reflects the B cell clones which populate the lymphoid tissue and subsequently differentiate into effector cells. The marking of B cells at the clonotype level as candidates for expansion on suppression is considered to be related to the interactions with idiotype-specific helper T cells, suppressor T cells and/or anti-idiotype antibody.

Research involving the idiotypes of immunoglobulins and the structure of these molecules was radically changed by the isolation of monoclonal B cell myelomas and the development of B cell hybridoma technology. This was primarily because it offered a homogenous population of antibodies produced by a single B cell clone. Isolation of such proteins resulted

in the discovery that the antigen receptor on the surface of B cells has the same conformation and primary amino acid sequence as the antibodies secreted by plasma cells derived from a clone of B cells.

Structural characterization of the T cell antigen receptor has been hampered in the past by this same lack of monoclonal T cell populations. The development of T cell cloning techniques (5) and immortalizing T lymphocytes by fusion with tumor cells (6) has been the first major step toward structural analysis of the T cell antigen receptor and examination of the phenomena of MHC restriction. Progress toward isolation of the receptor occurred recently with the generation of monoclonal antibodies which reacted with cell surface T cell antigen receptors (7). The receptor molecule has already been characterized, and consists of a heterodimer, composed of two disulfide-like polypeptide chains, α and β. These two chains range in molecular weight from 42 to 45 kilodaltons in mice, and from 39 to 49 kilodaltons in man (8). Comparative peptide maps of α chains and β chains demonstrate that, like immunoglobulin heavy and light chains, they have constant and variable peptides. These studies, however, have not provided information on the primary structure of α and B polypeptide chains because of the difficulty in preparing a sufficient amount of purified receptor protein.

Molecular biology techniques have provided the final key to the structure of the antigen receptor on T lymphocytes. The genes for the receptor were identified by subtractive screening methods to isolate cDNA clones that appear to encode the α and β chains (9).

A model for the molecular structure of the T cell receptor has recently been proposed by Saito, et al. (10) based on the predicted amino acid sequences of the α and β chains. The receptor molecule is made up of two polypeptide chains, each with two extracellular immunoglobulin-

like domains, an amino-terminal and a carboxy-terminal constant domain. Each domain is stabilized by an S-S bond between cystein residues that are separated in the linear sequency by 60-70 residues. There appears to be a striking similarity between immunoglobulin V, D, and J regions and the N-terminal domain of the α and β chain.

Two cysteine residues located at positions 234 of the α chain and 236 of the β chain probably form a single inter-chain disulfide bond, close to the cell membrane. This would allow a stable association between the two different chains and may be required for an effective antigen MHC binding region. The contribution of these two chains to form a single combining site, characteristic of immunoglobulins, would result in a combinatorial variability likely to contribute to structural and functional diversity.

The receptor is anchored to the cell membrane by hydrophobic transmembrane peptides, which include 21-22 amino acids from each of the two chains. There is a cysteine residue located in the cytoplasmic domain of the α chain. This residue may provide a site for attachment to other molecules within the cell membrane necessary for triggering an altered state of the cell when antigen binds the receptor (10).

The constant region of the receptor appears to be encoded by distinct gene segments from the variable regions. As with immunoglobulin molecules where the constant regions define the class and effector function, it can be speculated that the constant region of the T cell receptor may provide some function. The current reports are unclear regarding whether different functional subsets of T cells use the same constant region, and whether these different constant region gene products play a role in the activation or execution of the cell's effector function.

Since the variable region of the T cell receptor provides the site

for antigen binding, it seems there may be different V gene segments utilized by different T cell subsets because the antigen repertoires seem to be quite different. For example, cytotoxic T cells recognize foreign antigen in association with class I MHC determinants and helper T cells recognize antigen in the context of class II MHC determinants.

The molecular weights of the α and β chains calculated by predicted amino acid sequences are each about 10 kilodaltons less than the molecular weight observed by the isolated protein in gel electrophoresis. This difference is due to glycosylation of the chains. There appears to be four possible sites for glycosylation on the β chain and none on the α chain. The exact sites of glycosylation have not been determined (10).

Structural analysis alone does not provide insight into the mechanism of MHC restriction and antigen binding. The rationale of our experimental design has been based on the knowledge our laboratory group has gained on idiotypic markers on immunoglobulin molecules and the role these determinants may play in lymphocyte regulation. We applied this body of knowledge toward generating both syngeneic and semi-syngeneic monoclonal antibodies specific for determinants on the antigen receptor of T lymphocytes. Selection for these reagents were based on the ability of an antibody to bind a single T cell clone from our panel of MHC restricted T cell clones and hybridomas. The binding studies were followed by a series of function studies with the clones to identify which antibodies would alter the activity of the clones. These studies included blocking of proliferation, plaque formation and IL-2 release of the antigen specific clones. The strategy has been to select antibodies which block the recognition of antigen or the ability of T cell to cooperate with B cell to generated plaque forming units as candidates for reagents which bind to the receptor. Based on our results, we could

demonstrate that the antigen receptor of T cells bears several antigenic determinants. This data, when considered in the light of the known structural data on the T cell receptor, shows the idiotypes on T cells to be as diverse as immunoglobulin idiotypes. Utilization of monoclonal T cell populations as immunogenes to produce syngeneic monoclonal anti-idiotype antibodies will provide a probe for analysis into the domains on the receptor involved in foreign antigen binding and MHC binding.

Eventually, it will be possible to study the function of the T cell receptor proteins by transferring the cloned genes into cells and seeing how this will affect their specificity for foreign antigen and self-MHC. The monoclonal antibodies developed in this study can be used in conjunction with the DNA studies to help map the domains of the receptor playing a role in antigen binding and function.

MATERIALS AND METHODS

A. Mice CB6/F_1, BALB/c, C57BL/6, C3H/He, A/J, RIII, B10.D2, SJL, and AKR 6-8 wk old were purchased from the Jackson Laboratory, Bar Harbor, ME. B10.A(2R), B10.A(4R) and B10.A(5R) were purchased from the Mayo Clinic, Rochester, MN. BALB/K, B10.S(9R), ATFR5, and BALB/c x SJL/F_1 mice were bred at the animal facility, New York University School of Medicine. BXD recombinant inbred strains were derived from the cross between C57BL/6 (H-2^b, Mls^b) and DBA/2 (H-2^d, Mls^a). All BXD strains were supplied by Dr. Benjamin Taylor of Jackson Laboratory. Ab.Y mice were obtained from the colony of Dr. David Meruelo of the New York University School of Medicine. BALB.B and BALB.K were provided by Dr. Maurice Zauderer, Columbia University, N.Y. A list of complete H-2 haplotypes for all strains of mice used in this study is shown in Table I.

B. Antigens Keyhole limpet hemocyanin (Calbiochem-Behring Corp., San Diego, CA) and Ovalbumin (Sigma Co., St. Louis, MO) were conjugated separately with 2,4,6 trinitrobenzene sulfonic acid (Eastman Kodak Co., Rochester, NY) in 0.083M sodium bicarbonate buffer and then dialyzed against 0.1M potassium bicarbonate buffer. The degree of conjugation was determined by relative optical densities to be ten TNP groups per 100,000 mol. wt. KLH (TNP-KLH) and 42 TNP groups per molecule of Ovalbumin (TNP-Ova). Horse cytochrome C (Sigma Co., MO) and an isolate of type A influenza virus, PR8 were used as controls in determining the antigen specificity of T cell clones.

C. Monoclonal Antibodies Hybridoma cell lines with defined specificities for I-E(17-3-3s), I-A^b (25-9-3s) and I-A^d (MK-D6) were purchased from the American Type Culture Collection, Rockville, MD. Ascites of anti-Lyt-1.2 and anti-Lyt-2.2 were purchased from New England Nuclear,

TABLE I

Strains used as stimulators in MLR

Strain	H-2	Igh-1	Mls
A/J	a	e	c
BALB/c	d	a	b
BALB.K	k	a	b
C57BL/6	b	b	b
BALB.B	b	a	b
B10.A	a	b	b
AY13			
B10.D2	d	c	b
B10.Q	q	b	b
B10.S	s	b	b
CB6/F_1	b/d	b/a	b
C3H/HeJ	k	a	c
C3H.Q	q	a	c
CE/J	k	f	c
PL/J	u	a	*
RIII/J	r	g	a
SJL/J	s	b	c
SWR/J	b/q	c	c
CBA/N	k		

*Not determined

Inbred and Genetically Defined Strains of Laboratory Animals, Part I, Mouse and Rat, Compiled and edited by Philip L. Altman and Dorthy Dittmer Katz, Federation of American Societies for Experimental Biology, 1979.

Boston, MA. Ascites of hybridoma 30-H12 (anti-Thy-1.2) and 10.2-16 (anti-I-A^k) originally defined by Drs. Ledbetter and Herzenberg were generously provided by Dr. Benvenuto Pernis, Columbia University, NY. Antibody Y-17 (I-E^k) was provided by Dr. C. Janeway, Yale University, New Haven, CT. Antibodies reacting to human MHC antigens, such as ILR-2 was provided by Dr. D. Capra, University Texas Southwestern Medical School, Dallas, TX; and 109d6 reacting with MT3-like determinants and 22c6 specific for common human Ia were obtained from Dr. R. Winchester. All IgG antibodies were purified from ascites on Protein A-Sepharose 4B column. The IgM antibodies were precipitated with 50% saturated ammonium sulfate and purified by ion exchange chromatography.

D. Immunization Schedules Induction of KLH T helper cells: CB6/F_1, C3H/He, or B10.Q female mice were given a single immunization subcutaneously at the base of the tail and in the hind footpads with keyhole limpet hemocyanin 100 µg in complete Freund's Adjuvant (CFA). Seven to ten days later, the lymph nodes were removed.

Induction of TNP-Ova primed B cells: Mice were primed with either TNP-Ova or TNP-KLH (100 µg) in equal volumes of CFA. Four to six weeks later, the mice received a second injection intraperitoneally with 10 µg of antigen in saline. One week after the boost, the spleens were removed and depleted of T lymphocytes according to a technique previously described (11).

E. Preparation of T cell clones Lymph node T lymphocytes were obtained from CB6/F_1 mice primed with 100 µg KLH in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). The mice were injected subcutaneously in the base of the tail and in the hind footpads. Seven days later, T cells from the periaortic, popliteal and inguinal lymph nodes were

purified on a nylon wool column (12).

The KLH reactive T cells were further enriched by culturing the nonadherent population from the nylon wool column with 50 µg KLH/ml for four days T cell medium (RPM I-1640, containing 10% fetal calf serum, Reheis Chemical Co., AZ., 12mm hepe, 100 µ/ml penicillin, 100 mg/ml streptomycin, $2x10^{-3}$M glutamine, and $3x10^{-5}$M 2-mercaptoethanol). After four days of incubation, at 37°C and 7% CO_2, lymphoblasts are isolated on a discontinued Percoll density gradient (13). The enriched blasts were established as long-term T cell lines by serial restimulation in vitro as described by Kimoto and Fathman (14).

F. Preparation of T cell Hybridomas Lymph node T lymphocytes were obtained from $CB6F_1$ mice primed with 100 µg KLH in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). The mice were injected subcutaneously in the base of the tail and the hind footpads. Seven days later, T cells from the periaortic popliteal and inguinal lymph nodes were purified on a nylon wool column. The KLH reactive T cells were further enriched by culturing the nonadherent population from the nylon wool column (cell density $1x10^6$ cells/well) with 40 µg KLH/ml for four days in RPMI-1640 medium containing 10% fetal calf serum (Reheis Chemical Co., AZ), 12mm hepes 100 µg/ml penicillin, 100 mg/ml streptomycin, $2x10^{-3}$M glutamine, and $3x10^{5}$M 2-mercaptoethanol. After four days of incubation at 37°C and 7% CO_2, lymphoblasts were isolated on a discontinuous Percoll density gradient. The enriched blasts were fused with T cell lymphoma BW5147 using 40% of polyethylene glycol, mol. wt. 1,000 (Sigma, MD) and cultured in 96-well culture plates (Costar) in Iscove's medium containing 10% fetal calf serum (FCS). Twenty-four hours after, the fusion HAT medium was added to the wells. T cell

hybridomas were cloned by plating cells at a concentration of 0.25 cells/well in the presence of $5x10^5$ thymocytes. The clones were subcloned by using the limiting dilution method at 0.25 cells/well and only the subclones exhibiting the same recognition pattern were retained. FN1-18 is one of the subclone obtained from clone FN-1.

G. IL-Assay IL-2 production by murine T cell hybridoma was determined on HT-2 cell line kindly donated by Dr. J. Kappler (National Jewish Hospital, Denver, CO). In the primary culture, $5x10^4$ T hybridoma cells were cultured for two days in 0.2ml Iscove's medium containing 1% FCS with $5x10^5$ X-irradiated (2,000 R) murine spleen cells in presence or absence of KLH.

In the case of human lymphocytes, the peripheral blood mononuclear cells were separated by the method of Boyum (1968) and have been depleted of T lymphocytes by rosseting with neuraminidase treated sheep red blood cells as previously described (15). $5x10^4$ hybridoma cells were cultured for two days in the same conditions with $5x10^5$ X-irradiated (1,500 rad) human T depleted mononuclear cells.

In pilot experiments, it was determined the optimal conditions for IL-2 production with respect to the amount of antigen (40 µg/ml) and cell ratios (T hybridoma: murine spleen or human mononuclear cells 1:10).

After two days of culture, 0.1ml of supernatant was collected, serially diluted in 0.1ml RPMI1640 medium supplemented with 1% FCS and incubated with $4x10^3$ HT-2 cells. After 24 hr incubation, 1 Ci 3H-thymidine (1 mC/mole, New England Nuclear, MA) was added and the culture was harvested 16 hours later. The radioactivity incorporated by cells was measured in a scintillation spectrometer. A reference IL-2 preparation assigned a value of 100 units/ml was produced by culturing rat spleen cells ($5x10^6$ cells/ml for 20 hrs with Concanavalin A (Pharmacia 5 µg/ml). The supernatant from rat spleen cells incubated with Con A

was absorbed with α-methylmannoside (Calbiochem, La Jolla, CA). The 3H thymidine incorporation induced by this standard preparation was compared to the magnitude 3H thymidine incorporation induced in each sample and was used to flat a standard curve allowing the expression of concentration of IL-2 in units/ml according to a previously described method (16).

H. Proliferation Assays All T cell clones and subclones were tested for proliferation to a panel of soluble protein antigens and allogenic lymphocytes. Seven days after subculture without antigen, 10^4 cloned T cells and $5x10^4$ irradiated syngeneic or allogeneic spleen cells were cultured in 0.2ml microtiter wells. After three days, the cultures were pulsed with (3H)-thymidine (1 μCi/well) and harvested 18 hrs later. No exogenous IL-2 added to the cultures. All responses were measured in triplicate cultures.

I. BUdR Suicide Experiment These experiments were performed according to a previously described technique (17). Briefly, $5x10^4$ cells were incubated for 36 hrs at 37^o in medium containing KLH or allogeneic cells without or with 10^{-5M} BUdR. The cells were then exposed to fluorescent light for 2 hrs (Sylvania 40 watt F40 D light fluorescent bulbs, Sylvania Lighting Products Division, Hillsboro, NH). The cultures were placed at a distance of 20cm from the plane of the bulbs. After three washings, the cells were incubated an additional 36 hrs with KLH or allogeneic cells.

J. In Vitro Antibody Synthesis Assay TNP-Ova primed spleen cell suspensions were depleted of T cells by treatment with anti-Thy1.2 and anti-Lyt1.2 plus complement (18). A total of $3x10^6$ T-depleted primed B cells were cultured with $1x10^3$-1×10^6 lymph nodes from KLH primed mice or cloned T helper cells in 2ml of T cell media. Three 16mm

diameter wells (3524, Costar plates, Cambridge, MA) were used for each T cell concentration tested. Cultures were incubated for five days at 37°C in a 7% CO_2-humidified incubator. Cells were cultured in either the presence or absence of 0.001-10 μg/ml TNP-KLH. An additional culture containing 10 μg/ml or 0.001 μg/ml TNP-Ova was used as specificity control.

K. Plaque Forming Cell Assay Sheep red blood cells (SRBC) were coupled with TNP(TNP-SRBC) by the method of Rittenberg and Pratt (19). The above cultures were harvested, washed, and assayed for plaque forming cells (PFC) on TNP-SRBC. Both direct (IgM) and indirect (IgG) plaques were measured according to the method described by Cunningham et al.(20).

L. Preparation of Monoclonal Anti-clonotype Hybridoma Two series of immunizations were carried out in order to generate B cell hybridomas secreting antibodies specific for our panel of monoclonal T cells. One group of CB6/F_1 mice were immunized with the syngeneic KLH specific T cell line from which all the T cell clones were derived. An initial i.p. immunization with $2\text{-}10^6$ syngeneic T cell line was followed by three weekly i.v.. boosts with 10^6 T cells. The second group of CB6/F_1 mice were immunized with a semisyngeneic T cell hybridoma FN1-18. 10^7 irradiated (10,000 Rads) T cells were mixed with alumpertussis adjuvant and injected in the base of the tail and in the hind footpads. This was followed by three weekly i.v. boosts with $5x10^6$ irradiated cells. Five days after the final boost, 10^8 lymph node cells or spleen cell suspensions from the immunized mice were fused with SP2/0 myeloma cells according to a previously described technique (21). The cell suspensions were fused with the myeloma cells at a 10:1 ratio (i.e., spleen cell: myeloma cell) in 30% PEG (polyethylene glycol, M.W=4,000, J.T. Baker Chemical Co., Phillipsburg, NJ). The selective HAT

($1x10^{-4M}$ hypoxanthine, $8x10^{-7M}$ aminopterin and $1.6x10^{-3M}$ thymidine, Sigma Chemical Co.) medium was added one day later. Supernatant fluids of wells containing hybridomas were screened for anti-clonotype activity by radioimmunoassay and immunofluorescent straining on a T cell line, T cell clones, and T cell hybridomas. The hybridomas exhibiting an ability to bind to any of our panel of monoclonal T cells were then cloned by a limiting dilution method and grown as ascites in pristane primed mice.

M. RIA used in B cell Hybridoma Screening 10^5 T hybridoma cells used as the immunogene were dispensed into each well of a 96 well polyvinyl-chloride plate (Cookes Engineering, Alexander, VA). After two washings with PBS, 100 µls of culture fluid was added to each well and incubated at room temperature for 2 hrs. Following the incubation period, the plates were washed three times with PBS to remove any unbound proteins. The presence of monoclonal antibodies bound to T cells was determined by applying 50 µls containing 50,000 cpm of ^{125}I-labeled rat anti-mouse Ig to each well. After incubation for another two hours at room temperature, and extensive washing with PBS, the wells were harvested and counted in a Beckman γ-counter.

N. Histocompatibility Typing and Immunofluorescent Straining HLA, A,B,C, DR, and MT typing was performed using the Standard National Institutes of Health Microlymphocytotoxicity assay with locally available sera. Indirect microimmunofluorescence was performed on human T-depleted mononuclears using monoclonal reagents followed by treatment with a preparation of F(Ab')2 fragments of goat anti-mouse Ig antibodies conjugated with tetramethylrhodomine isothiocyanate as second antibody (22).

O. Determination of Ig Subclasses: Subclasses of monoclonal antibodies were determined by solid phase radioimmunoassay. Briefly, microtiter wells were coated with 50 µl of 50 µg/ml of affinity-purified monoclonal antibodies overnight. After three washings with saline, they were incubated for 1 hr with 50% fetal calf serum (FCS), and then with different 3H-labeled goat antimurine IgM or IgG classes for 3 hrs. After extensive washings, the radioactivity was counted in a scintillation counter.

P. Iodination of Purified Hybridoma Proteins The rat anti-mouse Ig antibodies used in the radioimmunoassay were iodinated by the chloramine T method according to the procedure of Weir (23). 100 µg of protein was suspended in .1m. PBS, pH 7.2. Next 1mCi of I^{-125} (Amersham-Searle, Arlington Heights, IL) was added. Then .05ml of chlormine T (1mg/ml in PBS) was added. The reaction was stopped by the addition of .1ml sodium meta-bisulfite (1mg/ml in PBS).

Q. Purification of IgM Antibodies

(i) Precipitation with Ammonium Sulfate

One volume of saturated ammonium sulfate (Schwartz-Mann, Orangeburg, NY), was added dropwise to one volume of ascites fluid containing monoclonal antibodies. The suspensions were stirred for at least 10 minutes to reach equilibrium, and precipitates were sedimented by centrifugation at 10,000 rpm for one hour in a Sorvall Centrifuge, (Sorvall Instruments, Newton, CT). The precipitates then were dissolved in a minimum volume of PBS and the solutions then were dialyzed against 4 liters of 40 mM phosphate buffer (pH 8.0) overnight at $4^{\circ}C$.

(ii) DEAE Chromatography

Diethylaminoethyl cellulose (DE52) (Whatman Chemical, England) columns were packed according to the instructions provided by the manufacturer and equilibrated with 10 column volume of 40 mM phosphate buffer (pH 8.0). The nondialyzable material from step 1 was loaded onto the column and in a single step eluted with 40 mM phosphate buffer. The protein containing fractions were detected at absorbance of 280 nm. Positive fractions were pooled, concentrated with Carbowax (PEG 20,000, Fisher, Fairlawn, NJ) and dialyzed overnight against 0.9% NaCl.

R. Purification of IgG Antibodies Protein A bound to Sepharose 4B columns were equilibrated with 1.0M Tris buffer pH 8.0. Ascites was first diluted 1:1 with Tris buffer and loaded onto the column. After two hours incubation in the cold, the unbound proteins were eluted off the column with Tris buffer. The antibody was removed by an acid wash (pH 2.8). The fractions containing protein were dialyzed against Tris buffer (pH 8.0). The average yield of protein from ascitic fluid was $\sim$ 2 mg/ml ascites. The binding activity of the proteins were analyzed by RIA.

ANALYSIS OF RESTRICTION ELEMENTS UTILIZED BY KLH SPECIFIC T CELL CLONES AND HYBRIDOMAS

A. Introduction

Antigen recognition by T lymphocytes usually involves the association of antigen with products of class I or class II major histocompatibility complex (MHC) genes (24). This recognition event occurs on the surface of antigen presenting cells (25). In addition to the primary recognition of foreign antigen, MHC gene products play an important role in the collaboration of T lymphocytes with B lymphcytes and cytotoxic T lymphocytes killing of target cells modified by viruses or chemicals (26,27). The requirement for recognition of MHC determinants may be a mechanism for focusing T cell programming or regulation upon cell surfaces, rather than allowing random interactions with antigen in circulation. Subtle membrane perturbations of these determinants appear to result in establishing and maintaining homeostatic balance of immunocompetent cells (28).

The rules governing MHC regulation of T cell responses were initially described over a decade ago using heterogeneous T cell populations. However, the precise molecular interactions and underlying cellular mechanism of MHC genetic restriction are not completely defined (29). Two models have been proposed to explain the molecular basis of MHC restriction: the first model considers that the T cell receptor has two separate monofunctional receptor molecules, one for binding the nominal antigen, and another for binding an MHC product recognizing self (30). The self-receptors is probably of low affinity to prevent the immune system from being destructively autoreactive. The alternative model postulates that T cells have a single bifunctional receptor molecule which recognizes a determinant formed by the interaction of the nominal antigen with an MHC gene product (altered self) (31).

The two receptor model has been supported by experiments with chimeric mice where the mature T cell repertoire is preferentially restricted by H-2 antigens expressed on radioresistant cells present during T cell ontogeny (32). Bone marrow stem cells from H-2 heterotygous F1 mice generate T cells restricted to the parental H-2 type (maternal or paternal) of the thymus in which the F1-derived stem cell matures. These observations suggest the selection mechanism for self-recognition is determined independently of antigen recognition during the T cell maturation process. This model becomes difficult to interpret in the light of studies using T cell clones that recognize antigen in association with unique F1 specific self-determinants (33), since neither parent should be able to provide genomic material which would allow recognition of unique hybrid F1 determinants.

The one receptor theory has been supported by two lines of evicence: (1) Cytotoxic and helper T cell clones which recognize self H-2 determinants plus a different antigen (34); H-2 restricted T cell clones specific for a single antigen can also respond to a different H-2 alloantigen (35).

Hunig and Bevan (36) using cold target competition experiments described a cloned CTL specific for both H-2d plus minor H and H-2^k plus TNP-BSA. This pattern cannot be explained by cross-reactivity at either the anti-self or anti-antigen sites which have been postulated to exist in the two receptor models.

Sredni and Schwartz (37) described a B10.A (H-2^a) DNP-Ova specific T cell clone which also responds to a B10.S MHC alloantigen (H-2^s) in the absence of syngeneic antigen presenting cells. These results demonstrate that recognition by individual T cells may occur through a combination of both the anti-self MHC and antigen specific receptor (altered self), or through two completely independent sets of receptors. The latter hypo-

thesis implies a single T cell clone would be programmed for two separate functions.

Kappler et al. (38) have tried to dissect the structure of the T cell receptor by producing T cell hybridomas. After fusing two H-2 restricted T cell hybridomas, each with a distinct specificity, his laboratory could not detect reshuffling of the anti-self and anti-foreign antigen specificities. The inability to separate the two components of the T cell receptor strongly suggests the altered self receptor model.

Another experimental model thought to reflect some component of self recognition is the autologous mixed lymphocyte reaction (MLR). In this reaction, Lyt-1^+ T cells from non-immunized mice can be induced to proliferate in vitro in response to Ia antigens on non-T cells from the same individual (39). Dendritic cells very rich in Ia antigen probably represents the primary stimulatory cells in auto MLR. This phenomenon has been shown to be deficient in some lympho-proliferative diseases and in autoimmune diseases (40). The acquisition of the ability of T cells to recognize the foreign antigens with self MHC encoded germ products was explained by N. Jerne as follows: he first proposed that when T cells encounter the cells of the thymic epithelium, those having a receptor that recognize the self antigens of the thymic cells are stimulated to divide. As the T cells migrate to peripheral lymphatic organs, mutations may occur to produce slight alterations in the receptor's molecular structure. This can be viewed as analogous to somatic mutations of B cells which fine tune the specificity of immunoglobulins. The autologous T cells may represent a normal component of an immunoregulatory circuit, and they may provide a useful reagent to probe high affinity anti-self receptors.

T cell hybridomas with both an autoreactive and antigen-specific phenotype have recently been isolated (41). Responding T cells in the

auto-MLR may be derived from cells with a specificity for foreign antigen in the context of self. Although foreign antigen is not required for the response of these cells, it is possible that these cells have an antigen specificity which is masked or/and mimicked by the autoreactive response. Theories on self tolerance propose that high affinity T cells specific for syngeneic Ia are eliminated (42). However, these cells may not be of sufficient affinity to interfere with the normal physiology of the host and are therefore not removed. Hybridomas with two reactivities, self-alone and self-plus antigen, could have also been derived from the fusion process; that is, the acquisition of two unrelated receptors. This is probably not the situation because these hybridoma are as stable in their phenotype as other antigen specific hybridomas. The importance of these cells is that they may represent a more activated state than resting T lymphocytes.

Serological analysis of public Ia determinants has shown that they are shared on many allelic products (43). It is the genetically polymorphic portions of MHC molecules, however, that are recognized by the T cell repertoire. The strongest evidence is derived from the studies of Schwartz and colleagues on the fine specificity of cross-reactivy recognition of related cytochrome antigens and Ia molecules (44). Their evidence supports a determinant selection model of MHC restriction by T cells proposed by Shevach and Rosenthal. This model suggests that genetic polymorphism of the MHC molecule affects the nature and orientation of the presented antigen.

The difficulty with a determinant selection model is that it would not predict such a high proportion of alloreactive cells within a resting T cell population. Between 0.5 and 20% of T cells can respond to stimulator cells expressing foreign MHC antigens, whereas, the fraction of T cells that can respond to non-MHC antigens is probably at least 100-fold

lower (45). As a consequence of this observation, it has been postulated that at least some T cells responding to MHC alloantigens are also competent to respond to various foreign antigen (46).

Studies on the specificity of T cell clones provide direct support for this. Cytolytic T clones recognizing H-Y antigens in association with H-2D were found to respond to H-2K plus TNP-BSA (47). Furthermore, a T cell clone from B10.A mice was found to proliferate in the presence of TNP-Ova and B10.A antigen presenting cells, as well as B10.S alloantigens (37). Although these studies demonstrate single T cell clone specific for antigen in association with a syngeneic H-2 gene product can also recognize alloantigenic determinants, questions of whether there are two T cell separate recognition units, specific for the alloantigens and the foreign antigens, or a single receptor is responsible for both categories of recognition was not definitively resolved.

The observation that half of the clones having identical specificity for an insulin epitope recognized in the context of syngeneic I-A^b gene product also responded to alloantigens of the H-2^u haplotype indicated that a certain degree of mimicry exists between alloantigens and foreign antigens associated with syngeneic H-2 gene products (48). These results, however, do not exclude a possible genetic linkage between the two separate recognition units.

A critical aspect of the interactions between T cells and antigen presenting cells is that the cellular interaction resulting in an immune response is restricted by elements closely related to certain allelic forms of Ia antigens (49) or to determinants that result from combinatorial interactions between allelic products (21). The concept that particular epitopes on MHC molecules are fundamental to genetic restriction of T cells has been the result of two lines of evidence. First,

molecules were found to exist on cells of F1 generation animals and to be formed by the free combinatorial association of parental α and B polypeptides that associate noncovalently to form an Ia molecule. In the case of an H-2^b x H-2^d F1 mouse, there exists in addition to parental A_α^b A_β^b and A_α^d A_β^d molecules, hybrid A_α^b A_β^d and A_α^d A_β^b Ia molecules. T cell clones and T hybridomas have been generated which recognize antigens in association with these unique combinatorial epitopes (50). Secondly, antigen specific T cell clones, restricted to an Ia epitope formed on H-2^b strain could not respond to the same antigen in the context of spleen cell from a bm/2 strain. The A_β^b polypeptide from this strain has lost a restriction epitope as a result of either a somatic mutation or gene conversion.

To further dissect the mechanism of restriction elements in T cell activation and function, our first aim was to generate monoclonal T cell populations. Two basic experimental designs were employed: The first approach has been to generate transformed monoclonal T lymphocytes by the fusion of AKR derived T lymphoma (BW5147) cells with T cells isolated from antigen primed murine lymph node populations. These T cell hybridomas have been used to explore the mechanism of T cell activation by antigen and MHC gene products. The second approach has been to clonally expand components of the diverse T cell network providing non-transformed, biologically active T cells which are maintained with repeated antigen stimulation or T cell growth factors (IL-2). Although the hybridomas generally grow more rapidly in bulk cultures and without the need for growth factors or antigen presenting cells, the advantage of the T cell clones is that they remain functionally acgive. They can therefore be used to examine the normal physiology of T-B cell collaboration.

TABLE II

KLH-SPECIFIC PROLIFERATIVE RESPONSE OF T CELL CLONES

Responder cells	CB6 irradiated cells plus‡			
	nil	KLH(40μg)	PR8(10μg)	Cytochrome(50μg)
I(Line)	214±112*	13,781±1,789	733±62	193±40
A9(Clone)	481±22	3,582±311	986±44	1,398±94
A4.9(Subclone)	455±13	10,611±919	916±136	898±110
A12(Clone)	137±19	1,381±113	145±11	110±18
A12.11(Subclone)	497±51	16,279±287	520±58	570±173
C1(Clone)	210±27	5,481±17	182±12	189±13
C1.3(Subclone)	495±18	5,137±800	465±33	516±43
F6(Clone)	451±4	16,602±478	376±70	386±62
D18(Clone)	488±12	15,359±478	367±61	471±36
Cytochrome primed	5,315±813	ND**	ND	32,386±687
KLH primed	1,446±54	9,925±1,242	3,105±69	2,143±42

*10^4 responder cells/culture for the Parental line and all clones or subclones. Cytochrome of KLH primed lymph node cells were cultured at 10^6 cells/culture. Results are tritated thymidine incorporation of triplicate cultures presented in cpm ± S.D.

***ND-not done.

‡10^6 (cells/culture) syngeneic splenic cells were added for all clones. PR8 is a solubilized glycoprotein from an isolate of influenza virus. Optimal antigen concentrations for KLH, cytochrome C and PR8 were determined by *in vitro* proliferation.

B. Generation of KLH Specific T Cell Clones

A panel of T cell clones and subclones were isolated from lymph node cell of CB6/F_1 mice primed with KLH. These clones were maintained in culture for a period longer than two years. Subclones were established from the parent clone (i.e., subclone A12.11 was derived from clone A12), and each subclone was repeatedly cloned by limiting dilution (0.25 cells/wells) to maintain high affinity clones. Table II shows the proliferative response of the parental T cell line and its clones in the presence of irradiated CB6/F_1 splenic cells to KLH, cytochrome, and PR8 (an isolate of influenza type A virus). All the clones exhibited antigen specific proliferation to KLH, and did not respond to cytochrome, PR8, or syngeneic splenic cells in the absence of antigen. The magnitude of ^{3}H-thymidine incorporation varied among clones during the two years they were maintained in culture. In some cases, as with clone A12, the magnitude of proliferation to KLH was enhanced after subcloning. The clones used in this study expressed an Lyt1.2$^+$ and Thy1.2 surface phenotype.

C. Production of KLH Specific IL-2 Secreting T cell Hybridomas

Several T cell hybridoma were prepared by the fusion of lymph node cells from KLH primed CB6/F_1 (H-$2^{b/d}$) and BW5147 (H-2^k), a T cell lymphoma line. After the initial screen for reactivity to KLH, each positive hybridoma was cloned under limiting dilution parameters, and selected clones were tested for responsiveness to KLH, cytochrome and Ova in the presence of CB6/F_1 spleneic cells. The data in Table III shows two hybridomas, SW2.3 and FN1-18, produces significant amounts of IL-2 only to KLH, and not to Ova, or cytochrome in the presence of CB6/F_1 splenic cells. This antigen specificity was stable and maintained after repeated subcloning. A third clone FN13-21 was also selected because it exhibited a two-fold increase in IL-2 production in response to KLH relative to stimulation

TABLE III

KLH INDUCED PRODUCTION OF IL-2 BY T CELL HYBRIDOMA

Responder cells	nil	KLH(15μg)	OVA(25μg)	Cytochrome(50μg)
BW5147*	<10	<10	<10	<10
SW2.3	<10	325	<10	<10
FN1-18	<10	210	<10	<10
FN13-21	58[xx]	116	67	53

*$5x10^5$ (cells/culture) syngeneic CB6/F_1 spleneic cells were cultured with $5x10^4$ responder cells.

[xx]Units of IL-2

TABLE IV

GENETIC RESTRICTION OF T CELL CLONES

Responder cells	Irradiated Cells*						
	CB6/F1			BALB/c		C57BL/6	
	nil	nil	KLH	nil	KLH	nil	KLH
A9.4	75±16	4,073±22	20,644±846	3,970±410	18,826±589	3,944±66	19,714±68
A12.11	142±30**	121±10	23,685±3,116	86±8	22,740±2,170	238±26	733±283
C1.3	85±6	142±25	20,445±6,006	113±62	237±71	235±69	17,348±2,314
D18	241±76	191±7	31,963±2,528	130±26	304±50	213±19	14,549±1,755
F6	73±11	159±32	20,445±6,006	113±62	249±32	143±40	32,023±3,296

*Responder T clones (10^4 cells/culture) stimulated with syngeneic CB6/F1 or parental BALB/c, BALB/c, C57BL/6(10^6 cells/culture) and 50 µg/ml KLH.

**Tritiated thymidine incorporation of triplicate cultures presented as cpm ± S.D.

by Ova, cytochrome or spleen cells alone. The fusion parent BW5147 was included in this panel to demonstrate the antigen specific response of these hybridoma resulted from a trait acquired from the KLH primed CB6/F_1 lymph node cells and not the lymphoma parent line. Immunofluorescent staining with anti-T cell reagents also provided direct evidence that these three clones resulted from a fusion event between CB6/F_1 lymph node cells and the AKR derived T lymphoma. Greater than 98% of the cells displayed Thy1.2 antigen. In addition, they expressed Lyt1.2 antigen and lacked Lyt2.3 (data not shown). BW5147, an AKR derived T lymphoma, lacks these allelic gene products.

D. Characterization of T cells Recognizing KLH in Association with I-A Region Gene Products

The genetic restriction for the KLH response was examined using selected T cell clones and T hybridomas. In order to characterize the Ia determinant that is recognized in association with KLH by these T cells, spleen cells from parental or H-2 recombinant strains were used to genetically map the restriction. Various monoclonal antibodies with different Ia specificities were used to block the KLH response of these clones.

In the first experiments, the T cells were cultured with syngeneic CB6/F_1 (H-$2^{b/d}$), and parental BALB/c (H-2^d) or C57BL/6 (H-2^b) spleen cells with or without KLH. Clones responding to KLH can be divided into two major groups based on their pattern of reactivity with antigen presenting cells from these strains (Table IV). Clones D18, F6 and C1.3 proliferate only in the presence of CB6/F_1 and C57BL/6; whereas, clone 12.11 proliferate only in the presence of CB6/F_1 and BALB/c. These results indicated that while D18, F6 and C1.3 are restricted to H-2^b determinants, the clone A12.11 was restricted to H-2^d determinants for KLH specific proliferation. One clone A9.4 responds to KLH in the presence of both BALB/c

TABLE V

GENETIC RESTRICTION OF THE KLH RESPONSE BY T CELL HYBRIDOMA

		CB6/F_1		BALB/c		C57BL/6	
Responder	nil	nil	KLH	nil	KLH	nil	KLH
SW2.3	<10	<10	364	<10	<10	<10	434
FN1-18	<10	<10	195	<10	<10	<10	<10
FN13-21	<10	76*	85	<10	160	114	159

*Units of IL-2 produced.

and C57BL/6 cells. The genetic restriction of this clone will be addressed in a later section.

Similar experiments using T cell hybridomas cultured with syngeneic or parental spleen cells in the presence or absence of KLH were assayed by measuring the release of IL-2 into the culture supernatants. Table V shows one hybridoma, SW2.3 which secretes IL-2 when it is cultured with KLH and antigen presenting cells from either syngeneic CB6/F ($H\text{-}2^{b/d}$) or parental C57BL/6 ($H\text{-}2^{b}$) strains. These results suggest that the KLH induced secretion of IL-2 by SW2.3 is restricted to $H\text{-}2^{b}$ determinants.

The same T cell clones show a clear division into two groups based on blocking of KLH induced proliferation by anti-I-A antibodies. The T cell clones are blocked by antibody MK-D6 ($I\text{-}A^{d}$) or 25-9-3s ($I\text{-}A^{b}$), but never by both. Clones D18, F6 and C1.3 that are blocked by 25-9-3s recognize KLH in association with an A_{α}^{b} A_{β}^{b} molecule, and clone A12.11 which is blocked by MK-D6 recognizes an A_{α}^{d} A_{β}^{d} molecules. This Ia specificity is assigned based on previously reported biochemical studies on these two reagents (51).

E. Isolation of T Cell Clones with a Double Specificity for KLH in Association with Syngeneic Ia and for Allogeneic Ia Alone

The ability of syngeneic, parental, and allogeneic spleen cells to stimulate the proliferation of KLH specific T cell clones was studied in a mixed lymphocyte response (MLR). Stimulator spleen cell preparations from mice representing nine different H-2 haplotypes were used for the allogeneic MLR.

Based on the proliferative response, shown in Table VI, the KLH specific clones can be classified into two groups: (1) Clones C1.3 and D18 which do not exhibit autoreactivity or alloreactivity. (2) Clones A12.11 and F6 showed strong proliferative responses to spleen cells from mice

TABLE VI

^{3}H-THYMIDINE INCORPORATION OF T CELL CLONES STIMULATED BY SYNGENEIC AND ALLOGENIC CELLS

Responder	Stimulating Cells												
	nil	CB6/F_1	BALB/c	C57/BL/6	C3H/HeJ	CBA/N	B.10.Q	C3H.Q	B10.A	A/J	SJL	B10.S	R-III	PL/J
		H-2 b/d	H-2d	H-2b	H-2k	H-2^k	H-2^q	H-2^q	H-2^a	H-2^a	H-2^s	H-2^s	H-2^r	H-2^u
nil	—	429±35*	465±48	459±10	532±7	508±13	642±246	532±17	533±21	472±54	561±23	537±11	567±42	408±12
Cl.3	79±43	802±116	395±2	387±20	447±21	472±32	422±17	486±18	475±29	582±109	438±9	581±72	528±26	466±15
D18	188±16	488±12	453±8	446±35	491±50	475±47	508±36	438±41	485±40	511±84	369±54	495±34	525±17	487±87
A9.4	104±32	4,071±109	6,870± 2,074	4,763± 380	465±14	487±47	457±31	474±27	510±78	484±37	457±14	623±34	467±34	492±37
A12.11	121±20	497±51	539±172	431±23	69,617± 10,760	12,871± 3,070	502±59	455±24	536±42	470±19	425±55	540±27	559±55	476±51
F6	150±17	451±14	400±16	414±40	471±43	536±64	65,663± 5,996	42,289± 9,410	460±5	492±42	373±48	505±40	481±30	505±34

*cpm, mean ± SD of triplicate cutlures

bearing H-2^9 and H-2^k haplotypes, respectively. This data strongly suggest that the proliferation was related to Ia determinants and not to Mls determinants of stimulating cells. Indeed, clone A12.11 proliferated with spleen cells from C3H (H-2^k, Mls^c), CBA/N (H-2^k Mls$^-$) and not with SJL (H-2^s Mls^c) mice. Clone F6 proliferated with spleen cells from C3H.Q (H-2^9 Mls^c) and B10.Q (H-2^9 Mls^b) mice. The alloreactivity was specific for a single H-2 haplotype as can be seen by the lack of proliferation to spleen cells from mice of other haplotypes (H-$2^{b,d,r,u,s}$).

Despite the fact that clone F6 and subclone A12.11 were obtained by cloning through limiting dilution method, to further demonstrate their clonal nature, BUdR suicide experiments were performed to assess whether the same cell population responding to KLH plus self, were also responding to the alloantigens. In this series of experiments, the cells were cultured in the presence of minute amounts of IL-2, KLH, or alloantigens, and on day 2, BUdR was added for 18 hrs and then the cultures were exposed to light for 2 hrs. The cells cultured for four days were reciprocally stimulated with KLH fresh irradiated syngeneic splenic cells or allotype stimulator cells. The data presented in Table VII show clones cultured with small amounts of IL-2 followed by stimulation with KLH or alloantigen, exhibited significant proliferation with the second round of stimulation. In contrast, clone F6 and subclone A12.11 when stimulated with either KLH or alloantigen on the first round incubated with BUdR and exposed to light did not mount a proliferative response when stimulated with the reciprocal antigen. The results support the clonal nature of our two T cell clones that display a double specificity.

It has been well-documented that under certain circumstances, anti-I-A antibodies are capable of blocking T cell recognition of alloantigens (52). Three monoclonal anti-I-A antibodies were used to study the proliferative

TABLE VII

RECIPROCAL STIMULATION WITH KLH AND ALLOGENIC CELLS OF T CELL CLONES WITH DOUBLE SPECIFICITY AFTER BUdR-LIGHT TREATMENT

clones	day 0	day 2	day 3	day 3	response day 4	day 7	response day 8
	nil	-	-	3H-T	439±22		
	KLH	-	-	3H-T	3,168±361		
	C3H	-	-	3H-T	3,974±74		
	nil	BUdR**	light	3H-T	320±15		
	KLH	BUdR "	light	3H-T	468±15		
	C3H	BUdR "	light	3H-T	511±34		
A12.11							
	IL-2	BUdR "	light	nil		3H-T	505±53
	IL-2	BUdR "	light	KLH		3H-T	3,717±712
	IL-2	BUdR "	light	C3H		3H-T	16,003±2,330
KLH	IL-2	BUdR "	light	nil		3H-T	392±15
KLH	IL-2	BUdR "	light	KLH		3H-T	579±153
KLH	IL-2	BUdR "	light	C3H		3H-T	473±45
C3H	IL-2	BUdR "	light	nil		3H-T	343±32
C3H	IL-2	BUdR "	light	KLH		3H-T	585±74
C3H	IL-2	BUdR "	light	C3H		3H-T	450±45
	nil	BUdR "	light	3H-T	844±170		
	KLH	BUdR "	light	3H-T	12,835±836		
	B10.Q	BUdR "	light	3H-T	16,541±1,238		
	nil	BUDR "	light	3H-T	794±341		
	KLH	BUdR "	light	3H-T	1,308±248		
	B10.Q	BUdR "	light	3H-T	2,463±457		
F6							
	IL-2	BUdR "	light	nil		3H-T	639±381
	IL-2	BUdR "	light	KLH		3H-T	4,462±1,016
	IL-2	BUdR "	light	B10.Q		3H-T	5,015±499
KLH	IL-2	BUdR "	light	nil		3H-T	260±100
KLH	IL-2	BUdR "	light	KLH		3H-T	257±223
KLH	IL-2	BUdR "	light	B10.Q		3H-T	350±154
B10.Q	IL-2	BUdR "	light	nil		3H-T	879±168
B10.Q	IL-2	BUdR "	light	KLH		3H-T	1,025±217
B10.Q	IL-2	BUdR "	light	B10.Q		3H-T	984±143

* cpm mean ± SD of triplicate cultures

** cultures contained 10^4 responding clone A12.11 or F_6 and 5×10^5 splenic filler cells on day 0. After stimulation with KLH or allogeneic stimulator cells on day 2 BUdR was added for 18 hrs followed by exposure to light for 2 hours. Fresh splenic fillers plus KLH or allogeneic filler cells were added to reciprocally stimulated cultures. Tritiated thymidine in corporation was measured on day 4 and day 8.

*** cultures contained 0.1% IL-2 instead of 2% in standard culture.

TABLE VIII

EFFECT OF ANTI-Ia ANTIBODIES ON THE PROLIFERATIVE RESPONSE OF A12.11 T CELL CLONE

Antibody added to the culture	Specificity	A12.11	CB6/F1+KLH*	C3H/HeJ
nil	-	-	130±34**	90±8
nil	-	+	19,588±2,699	24,636±386
25-9-3s	I-A^b	+	18,691±2,221	23,079±3,234
MK-D6	I-A^d	+	2,673±383	20,711±1,055
10.2-6	I-A^k	+	20,301±3,588	2,928±421

*$5x10^5$ syngeneic CB6/F1 cells were incubated with $2x10^4$ T cells in the presence of 40µg/ml KLH.

**H^3-thymidine incorporation represented in cpm.

response of subclone A12.11 to KLH plus syngeneic APC (H-2^d) and the alloreactive cells (H-2^k). The data illustrated in Table VIII show that only anti-I-A^d antibodies block the proliferative response to KLH. These data are in agreement with Table III which shows that the proliferation to KLH only occur in the context of H-2^d antigen presenting cells. The allogeneic response to H-2^k stimulator cells, however, was only blocked by anti-I-A^k monoclonal antibodies. Similar experiments were not attempted with F6 because of the lack of anti-I-A^q monoclonal antibodies. This data suggest the alloreactive determinant recognized by clone A12.11 is an A_α^k A_β^k molecule.

F. A Cloned T cell Hybridoma Generated from CB6/F_1 (H-$2^{b/d}$) Mice, Exhibits a Specificity for KLH in Association with One Parental Ia Molecule (I-A^d) and Autoreactive to the Other Parental Ia Molecule (I-A^b)

The aim of this series of experiments has been to examine the requirements for activation of T cells with a specificity for both KLH plus self Ia and to self Ia alone. The data depicted in Table IX shows that hybridoma FN13 produces significant amounts of IL-2 in the presence of CB6/F_1 or C57BL/6 cells, with or without antigen (1-100 µg/ml), and only in the presence of KLH when irradiated BALB/c spleen cells are used in culture.

The most rigorous method of ascertaining the specificity of a T cell clone is by studying the pattern of its reactivity after subcloning. Therefore, the subclone FN13-21 was recloned under rigid limiting dilution (0.25 cells/well) and the reactivity of 20 subclones were studied. The data in Table X illustrate that all T cell subclones product IL-2 subsequent to stimulation with KLH in the presence of BALB/c cells and with C57BL/6 cells in the presence or absence of KLH. Interestingly,

TABLE IX

DOSE DEPENDENT RELATIONSHIP BETWEEN ANTIGEN CONCENTRATION AND IL-2 PRODUCTION BY FN13 CELLS

Antigen	DOSE (µg/ml)	IL-2 Production in the Presence of APC from[a]		
		$CB6F_1$[b]	BALB/c	C57BL/6
nil		59[b]	0	101
KLH	0.01	57	0	170
	0.1	55	0	125
	1.0	58	0	176
	10	67	62	180
	40	111	61	88
	100	147	115	116
Cytochrome C	10	57	0	
	100	55	0	
TNP-OVA	10	54	0	
	100	55	0	

[a]APC=γ-irradiated spleen cells.

[b]Units of IL-2 produced, $5x10^4$ hybridoma cells per well.

TABLE X

IL-2 PRODUCTION BY SUBCLONES DERIVED FROM FN13-21

Subclone	APC					
	$CB6F_1$		BALB/c		C57BL/6	
	-	KLH[a] +	-	KLH +	-	KLH +
FN13-21-1	160[b]	920	<10	965	721	406
2	116	253	<10	464	221	148
3	11	100	<10	102	112	105
4	<10	118	0	111	108	121
5	14	127	0	110	125	110
6	<10	108	0	98	118	102
7	10	113	0	131	121	130
8	<10	106	0	138	123	110
9	115	424	<10	596	298	351
10	10	119	0	201	107	63
11	136	671	<10	474	254	224
12	18	45	0	123	50	39
13	139	258	<10	273	204	249
14	120	328	<10	301	262	236
15	159	191	0	175	209	169
16	12	131	0	159	131	141
17	19	37	0	57	81	60
18	111	178	<10	214	477	280
19	92	236	0	444	231	277
20	18	57	0	81	57	78

[a] 40µg/ml

[b] Units of IL-2 produced.

only 50% of the T cell subclones display an increased IL-2 production in the presence of CB6/F1 irradiated spleen cells and KLH. This same pattern of autoreactivity to CB6F1 and C57BL/6 cells has been observed after subcloning of FN13 using BALB/c cells as filler cells (data not shown).

It has been reported that autoreactivity of T cells in vitro depends on the presence of antigens in fetal calf serum in the medium (53). The effect of FCS on autoreactivity of FN13-21 was therefore compared in Iscove's medium with or without 1% FCS or 0.5% mouse serum. As shown in Table XI, both the response to KLH in association with I-A^d, and the response to I-A^b in the presence or absence of KLH, are maintained in cultures with mouse serum, or in cultures using serum free medium, in spite of the fact that the overall survival of the hybridoma cells is much lower in serum free than in FCS or mouse serum containing medium. These results show that FN13-21 can respond to the I-A^b gene product in the absence of other antigen stimulation, although the autoreactivity is much more marked in response to homozygous I-A^b cells than in response to heterozygous cells.

Since the phenotype of this hybridoma remains stable after cloning and is not effected by the source of FCS, additional studies with antigen presenting cells from various H-2 recombinant and congeneic strains were used to genetically map the restriction elements. The experiment represented in Table XII shows the responses of three hybridomas to splenic cells in the presence or absence of KLH. The autoreactivity of FN13-21 is restricted to I-A^b determinants. Since IL-2 production is stimulated in the presence of B10.A(5R) and C57BL/6 but not in the presence of B10.A (2R) and B10.A(4R). Results with hybridoma SW2.3 are included to illustrate the pattern of reactivity of a clone specific for KLH in the context of I-A^b determinants. Clones FN13-21 and SW2.3 did not exhibit any allo-

TABLE XI

RECOGNITION OF FOREIGN AND SELF ANTIGEN INDEPENDENT OF SERUM ADDED IN CULTURE MEDIUM

Serum During[b] Preculture	Serum Added During Assay	IL-2 Production[a] CB6F1[e] − KLH[f]	CB6F1[e] + KLH	BALB/c − KLH	BALB/c + KLH	C57BL/6 − KLH	C57BL/6 + KLH
FCS[c]	FCS	15	55	0	74	61	65
Mouse[d]	FCS	15	59	0	83	84	71
Mouse	Mouse	10	52	0	59	57	49
Mouse	-	<5	52	0	55	50	51
-	FCS	<5	63	0	75	74	62
-	Mouse	8	49	0	57	57	56
-	-	<5	43	0	55	48	48

a Units of IL-2 produced

b 3 days preculture

c FCS 1%

d Mouse serum 0.5%

e Origin of APC

f 40 µg/ml

TABLE XII

MAPPING OF MHC RESTRICTION TO THE I-E SUBREGION

Source presenting cells	H-2 region K	A	E	D	FN1-18 KLH −	FN1-18 KLH +	FN13-21 KLH −	FN13-21 KLH +	SW2.3 KLH −	SW2.3 KLH +
$CB6F_1$*	b/d	b/d	b/d	b/d	<10	95**	102	121	<10	85
BALB/c	d	d	d	d	<10	<10	<10	160	<10	<10
C57BL/6	b	b	b	b	<10	<10	214	159	<10	126
C3H/HeJ*	k	k	k	k	<10	70	<10	<10	<10	<10
BALB/K*	k	k	k	k	<10	59	<10	<10	N.D.	N.D.
AKR*	k	k	k	d	<10	86	<10	<10	<10	<10
A/J*	k	k	k	d	<10	58	<10	<10	<10	<10
B10.A*	k	k	k	d	<10	50	N.D.	N.D.	N.D.	N.D.
B10.A(2R)*	k	k	k	b	<10	68	<10	<10	N.D.	N.D.
B10.A(5R)*	b	b	k	d	<10	96	211	203	N.D.	N.D.
B10.A(4R)	k	k	b	b	<10	<10	<10	<10	N.D.	N.D.
B10.S(9R)	s	s	k	d	<10	<10	N.D.	N.D.	N.D.	N.D.
A.TFR5	f	f	k	d	<10	<10	<10	<10	N.D.	N.D.
BALB/c x SJL)F_1	d/s	d/s	d/s	d/s	<10	<10	N.D.	N.D.	N.D.	N.D.
RIII*	r	r	r	r	<10	<10	<10	<10	<10	<10
$DBA/_1$	q	q	q	q	<10	<10	<10	<10	<10	<10
PL/J	u	u	u	u	<10	<10	<10	<10	<10	<10

*Exhibit a cell surface determinant identified by MnAb, 17-3-3S.

**IL-2 production (units)

TABLE XIII

EFFECT OF ANTI-I-A ANTIBODIES ON IL-2 PRODUCTION BY FN13-21

Antibodies Added[a]		IL-2 Production					
		$CB6F_1$[b]		BALB/c[b]		C57BL/6[b]	
Designation	Specificity	- KLH[c]	+	- KLH	+	- KLH	+
None	None	52[d]	83	<10	57	106	57
UPC-10	β2 Fructosan	55	145	0	82	96	79
25-9-35	$I\text{-}A^b$	0	52	0	91	0	0
MK-D6	$I\text{-}A^d$	47	16	0	0	96	70

[a] 100 µg/ml of each antibody was added at the beginning of the primary culture.

[b] Source of APC

[c] 40 µg/ml

[d] Units of IL-2 produced.

reactivity to strains expressing H-2 haplotype k,s,f,r, or u. The genetic restriction of hybridoma FN1-18 will be discussed in the next section.

Further evidence bearing on the nature of the restriction elements utilized by FN13-21 were obtained by studying the effects of monoclonal anti-Ia antibodies on antigen presentation. The results of experiments summarized in Table XIII demonstrate that anti-I-A^b reagents inhibit the IL-2 production induced by C57BL/6 cells but do not alter the KLH induced IL-2 production in the presence of CB6/F_1, or BALB/c cells. Conversely, anti-I-A^d antibodies inhibit KLH induced IL-2 production in the presence of BALB/c cells. These results are in agreement with Table XI which shows the proliferation of KLH occur only in the context of I-A^d and that proliferation in the absence of KLH occur in the context of I-A^b determinants. Taken collectively, these results demonstrate that hybridoma FN13-21 exhibits a double specificity recognizing Ia determinants of one parent and KLH associated with Ia determinants of the other parent.

G. Analysis of a T cell Hybridoma Specific for KLH in Association with a Murine I-E Transcomplementation gene product: Identification of restriction element shared within the human and murine MHC

One hybridoma generated in our laboratory was found to secrete IL-2 in response to KLH presented only by CB6/F_1 antigen presenting cells. Cells from mice bearing either parental haplotype were incapable of antigen presentation to FN1-18 suggesting that the hybridoma recognized the nominal antigen in association with an F_1 determinant(Table V). IL-2 production by the hybridoma was induced by KLH and not by other unrelated antigens, or by alloantigens(Fig 1). Restriction of KLH antigen presentation to the FN1-18 hybridoma was evident only with cells from CBA/N, C3H/HeJ, BALB.K, CE/J, and AKR mice which share the H-2^k haplotype induced IL-2 secretion while mice of H-2 haplotype b,d,q,r,s, and u did not induce IL-2 secretion(Table XII). Using recombinant strains, the fine specificity of the genetic restriction

Figure 1

Antigen specificity and dose response curve for hybridoma FN1-18. Three antigens, KLH (●—●), cytochrome C (□—□), and TNP-OVA (Δ—Δ) were tested in the presence of syngeneic CB6/F1 antigen presenting cells.

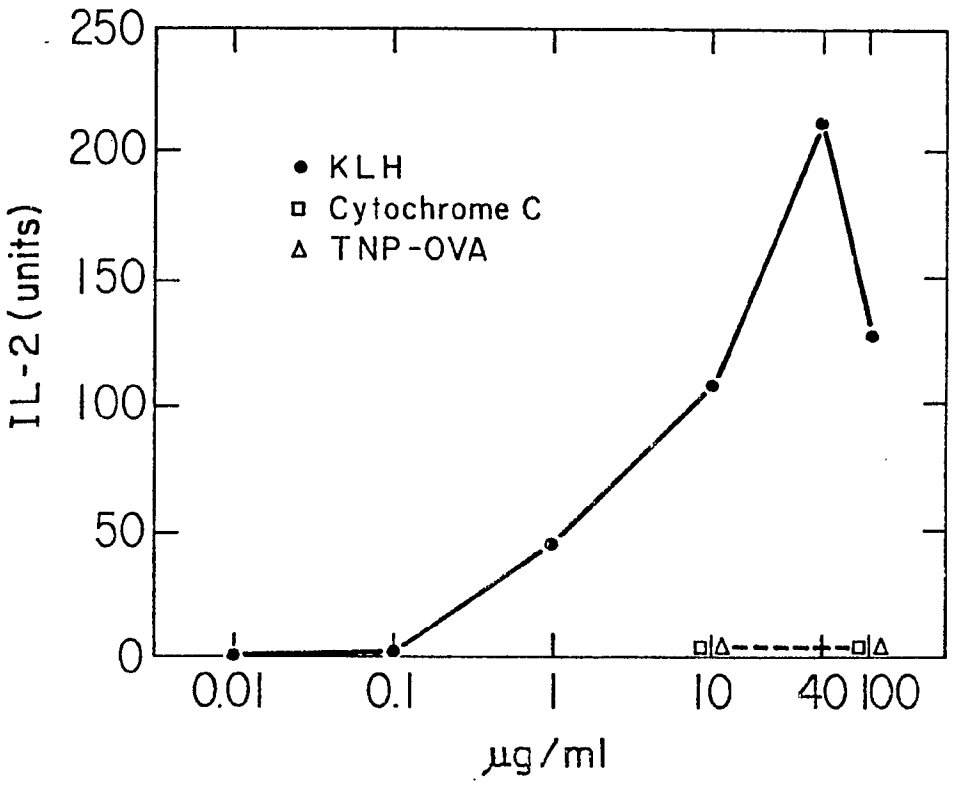


TABLE XIV

INHIBITION OF KLH INDUCED IL-2 PRODUCTION BY MURINE ANTI-I-E ANTIBODIED AND A HUMAN ANTI-Ia ANTIBODY

Monoclonal antibodies	Specificity	% Inhibition of IL-2 production	
		$CB6/F_1$*	$C3H/F_1$*
UPC10	β2-6 fructosan	0	0
25-9-3s	$I\text{-}A^b$	0	10
MK-D6	$I\text{-}A^d$	0	0
10-2.16	$I\text{-}A^k$	0	5
17-3-3s	Ia.22	75	98
Y-17	Ia.m44	50	88

*Source of antigen presenting cells

was mapped to genes encoding I-E^k determinants. The data depicted in this table show that IL-2 production was stimulated in response to KLH presented by cells from A/J, B10.A, B10A(2R), and B10(5R) strains of mice. The results with B10.A(5) strains were especially significant because this strain expresses a complementation product of E^b_β and E^k_α genes. All strains capable of presenting KLH to the hybridoma exhibited the cell surface epitope identified by anti-I-E antibody 17-3-3s; however, two strains, RIII and B10.S(9R), that bear the 17-3-3s determinant did not present KLH antigen to FN1-18. No significant IL-2 production was observed in cultures containing KLH cells from B10.A(4R), AFTR5 and (BALB/c x SJL)F_1 mice.

Further evidence bearing on the nature of the restriction elements was obtained by studying the effects of monoclonal anti-Ia antibodies. The results of experiments summarized in Table XIV show that the addition of anti-I-E antibodies (17-3-3s or Y-17) inhibited the IL-2 response of FN1-18 to KLH presented by cells of either CB6/F_1 or C3H/HeJ strains. In contrast, monoclonal antibodies specific for the I-A region gave no significant inhibition.

There are numerous studies demonstrating that certain epitopes on human Ia molecules are recognized by antibodies specific for murine I-A and I-E gene products(54). Furthermore, the study of amino acid sequences of murine I-Eβ molecules shows homology with human DRβ chain molecules(55). Based on this information, we examined whether this murine T cell hybridoma FN1-18 was capable of recognizing KLH in the context of human Ia molecules. This question was formally explored because our laboratory had demonstrated the anti-I-E antibody 17-3-3s identified a polymorphic determinant on T cell depleted human leukocytes.

Table XV includes HLA typing for 25 humans. It demonstrates that the epitope recognized by the anti-human Ia monoclonal antibody 109d6 is closely related but not identical to the epitope recognized on the human cells by

TABLE XV

ASSOCIATION OF ABILITY OF HUMAN MONONUCLEAR CELLS TO PRESENT KLH TO A MURINE T CELL HYBRIDOMA WITH THE PRESENCE OF ALLOANTIGENS DETECTED BY 17-3-3s MONOCLONAL ANTIBODY

	MHC Antigens					Immuno-fluorescence		KLH induced IL-2 production
	Class I			Class II				
Cells	A	B	C	DR	MT	109d6	17-3-3s	(units/ml)
C3H/HeJ						+	+	325
D.P	1,w30	8,18		3	3	-	+	241
K.K.	1,10	8	w7	3,4	2,3	+	+	194
R.B.	w24	18	w4	7	3	+	+	159
N.R.	3,29	7,w44		7,10	2,3	+	+	79
J.R.	1,24	w35,17	w4	5,7	2,3	+	+	109
D.O.	w24,w28	w51,w44	w4,w5	4,7	2,3	+	+	162
T.M.	2,11	w22	w1	4	2,3	+	+	130
B.S.	2,w24	w35,14	w4	5,7	2,3	+	+	295
1.S	1,w24	17,w38		4,7	3	+	+	89
J.K.	1	w35		5	2	+	+	85
P.M.	2,w24	w5,27	2,3	w4	3	+	+	<10
E.M.	w24	w35,w38	w4	4	3	+	+	<10
N.S.	32	w35,w44	w4	1	1	+	-	<10
M.R.	28	w35	w4	4	3	+	-	<10
K.D.	2,28	w44,w55	w3	6	1,2	-	-	<10
S.W.	2	27,28		2,3	2	-	-	<10
R.W.	2,26	8,w55	w9	5	2	-	-	<10
P.L.	1,2	14,w35	w4	w6	1,2	-	-	<10
P.G.	3,w24	w35,w39	w4	1	1	-	-	<10
S.L.	w24	17	w1,w3	5,6	1,2	-	-	<10
S.S.	11,24	18		2,5	1,2	-	-	<10
D.G.	3,29	7,w35	w7,w4	2,w6	1,2	-	-	<10
S.G.	11	40,w44	w3,s7	5	1,2	-	-	<10

17-3-3s. Eleven individuals expressed determinants identified by antibodies 109d6 and 17-3-3s and each was positive for the MT3 specificity as defined by allosera. Two individuals were reproducibly positive for the 109d6 determinant but lacked reactivity with the 17-3-3s antibody. This pattern of reactivity was confirmed by studies of other family members (data not shown). In one instance, an MT3 positive individual lacked reactivity with the 109d6 antibody but expressed the 17-3-3s antigen. This pattern of reactivity was found to be encoded by a haplotype in a family study (data not shown). An additional 5 individuals lacked reactivity to 109d6 and 17-3-3s (data not shown).

The capacity of human T-depleted mononuclear cells to present KLH to murine FN1-18 hybridoma cells is illustrated in Table XV. T-cell depleted peripheral blood mononuclear cells from 10 of 30 humans presented KLH to FN1-18 but elicited no response in the absence of KLH. The IL-2 production ranged from 79 to 295 IL-2 units for human compared to 250-325 units for murine antigen presenting cells. Cells from donors exhibiting the ability to present KLH were repeated at least three times in separate experiments and gave reproducible results. In preliminary experiments, it was determined that T cell depleted mononuclear cells should be used for antigen presentation because whole mononuclear cell preparations containing T cells from some individuals yielded up to 15 units of IL-2 in the absence of KLH. This response was eliminted by E rosette depletion of T lymphocytes from the mononuclear cells without significantly altering the antigen specific IL-2 response (data now shown).

Statistical analysis of the data presented in Table XV showed a correlation between the ability of human cells to present KLH to FN1-18 and the expression of a cross-reactive determinant recognized by the monoclonal antibody 17-3-3s ($r=0.768$, $p=0.00002$). In contrast, the correlation between antigen presentation and serologically defined human DR4

TABLE XVI

ANTIGEN PRESENTATION BY DR HOMOZYGOUS CELLS

Source of presenting cells	MHC haplotype	Antigen in culture	
		nil	KLH
$CB6F_1$	H-2 d/b	<10	254*
#2892	DR4 DW4 MT3,4	<10	154
#2046	DR4 DW10 MT3,4	<10	115
#7863	DR1 DW1 MT1	<10	<10
#2847	DR5 DW5 MT2,4	<10	<10
#4135	DR7 DW7 MT3	<10	60
#4035	DR7 DW11 MT3	<10	62

*IL-2 production (units). The human lymphocytes used in this experiment were kindly donated by Dr. N. Suciu-Foca (Columbia University, New York)

and/or DR7 (r=0.509, p=0.0048), MT3 (r=0.519, p=0.0009), or 109d6, (r=0.539, p=0.0027) specificities are lower. Further evidence supporting this are data obtained using presenting cells from homozygous Dw typing cells (Table XVI). Although the two alleles for DR4 linked to Dw4 or Dw10 and the two alleles for DR7 linked to Dw7 and Dw11 differ significantly at the level of amino acid sequence in both DR and DS products, both allelic forms have conserved a critical epitope on their cell surface which functions in antigen presentation to hybridoma FN1-18.

Family studies were undertaken to investigate whether the restricting element required for antigen presentation by human cells was inherited in association with a given HLA type. A representative family, Table XVII, illustrates the inability of the mother's cells to present KLH to FN1-18, while the father effectively presented this antigen. The data demonstrate that the capacity of human cells to function in antigen presentation segregates within this family as a trait linked to genes of the major histocompatibility complex inherited from the father. The paternal haplotype A1, Bw57, DR7, MT3, 17-3-3s$^+$ conferred this trait on both siblings. The ability of 17-3-3s to block the antigen presentation of the father and both daughters paralleled 17-3-3s cell surface staining of their antigen presenting cells (Table XVII). This pattern of inheritance indicates the restriction is related to determinants encoded within the MHC and wasconsistent with that found in three other families that are not illustrated.

The results in Table XVIII show that the monoclonal anti-I-E antibody, 17-3-3s, blocked antigen presentation not only in cultures containing C3H/He antigen presenting cells, but also with all human cells tested. In the same set of experiments, the monoclonal antibody 109d6, specific for a human MT3-like determinant, was used for comparison. Although it exhibited a similar staining pattern on human cells and shares the same isotype

TABLE XVII

SEGREGATION OF ABILITY TO PRESENT KLH ANTIGENS WITH THE HAPLOTYPE ENCODING THE 17-3-3s ANTIGENS: SELECTIVE INHIBITION BY ANTIBODY 17-3-3s BUT NOT ANTIOBDY 109d6

Relationship of donor	HLA haplotype assignment	Ia antigen expression detected by antibody 109d6	17-3-3s	IL-2 production(units) nil	KLH with: 17-3-3s	109d6
Parent ( )	a/b*	+	+	136**	<10	105
Parent ( )	c/d	-	-	<10	<10	<10
Sib 1	a/d	+	+	96	<10	88
Sib 2	a/d	+	+	102	<10	113

*Haplotype a = A1, Bw57, DR7, MT3; b = A3, Bw38, DR4, MT3;
c = A1, Bw35, DR2 MT1; d = AW24, BW35, DR5, MT2.

TABLE XVIII

INHIBITION OF IL-2 PRODUCTION BY MONOCLONAL ANTIBODIES SPECIFIC FOR MURINE OR HUMAN MHC ENCODED ALLODETERMINANTS

Source of APC	KLH induced IL-2 production in presence of						
	nil	MK-D6[a]	17-3-3S[b]	Genox 3.53[c]	1LR-2[d]	109d6[e]	22c6[f]
$CB6F_1$	349*	306	27(+)	291(-)	329(-)	290(-)	340(-)
R.B.	157	205	5(+)	135(-)	167(-)	145(+)	29(+)
K.K.	113	95	8(+)	86(-)	42(+)	68(+)	10(+)
T.M.	97	104	13(+)	93(-)	75(+)	73(+)	10(+)
N.R.	193	142	21(+)	167(-)	136(+)	139(+)	24(+)
B.S.	167	196	31(+)	170(-)	70(+)	144(+)	25(+)

Monoclonal antibody specificity. a. $I\text{-}A^d$ b. I-E c. MT1-like d. MT2-like e. MT3-like and f. monomorphic Ia. cultures contained Iscove's serum free medium supplemented with 1% purified monoclonal antibodies.

*IL-2 units ( ) results of immunofluorescence staining with monoclonal antibodies negative <10% positive >80% cells stained.

with 17-3-3s, antibody 109d6 did not significantly inhibit KLH presentation by human cells except in the case of KK where partial inhibition was observed. The MT-2 specific antibody I-LR2 stained all four donors expressing an MT-2 phenotype. It gave a marginal inhibition in three out of four donors, only showing a significant inhibition in donor KK. A monoclonal antibody specific for MLR (Genox 3.53) which did not stain the human mononuclear cells of five donors was used as a negative control and did not alter IL-2 production. As expected, the monoclonal antibody 22c6 specific for a monomorphic human Ia determinant strongly inhibited KLH presentation by human but not by mouse cells.

These results suggest that the restricting element, in both humans and mice, recognized by the receptor on the murine T cell hybridoma is a determinant closely related but not identical to the antigenic determinant to which antibody 17-3-3s binds. These determinants are not strongly influenced by the considerable species differences in the remainder of the Ia molecule.

H. Antigen Recognition by a T Cell Clone Outside the Context of MHC: A role for Mls gene products in antigen presentation

After subcloning by limiting dilution, one T cell clone A9.4 exhibited the unique property of responding to KLH in the presence of irradiated syngeneic CB6/F1 spleen cells, as well as spleen cells from either parental strain (Table IV). Antigen recognition by T lymphocytes usually involves the association of antigen with products of class I or class II MHC genes. In an F1 animal, helper T lymphocytes usually recognize antigen in the context of polymorphic Ia determinants expressed by one parent, but not both parents. The interest in this clone was to determine whether a nonpolymorphic determinant encoded by the MHC or whether molecules encoded outside the MHC could be used for antigen presentation.

TABLE XIX

PROLIFERATION OF A9.4 SUBCLONE IN CULTURE MEDIUM SUPPLEMENTED WITH SERUM FROM DIFFERENT ORIGIN

Origin of serum added to culture‡	medium	3H-Thymidine incorporation (cpm)					
		$CB6F_1$ cells**		BALB/c cells		C57BL/6 cells	
		nil	KLH	nil	KLH	nil	KLH
fetal calf (5%)	75±16*	4,073±22	20,644±846	3,970±410	19,826±589	3,944±66	19,714±68
horse (5%)	66±2	2,891±32	14,070±42	2,819±140	14,086±662	2,884±75	14,276±270
mouse (2%)	44±6	2,747±33	12,975±219	2,515±41	12,277±345	2,830±48	13,348±84
human (5%)	71±10	2,316±24	14,560±56	2,233±140	12,033±1058	2,189±28	13,254±517

*cpm, mean ± S.D. of triplicate culture.

**Subclone A9.4 ($1x10^5$ cells/culture) stimulated with syngeneic $CB6/F_1$ or parental BALB/c, C57BL/6 ($1x10^6$ cells/culture). 50 µg/ml KLH added to cultures.

‡RPM-1640 supplemented with heat inactivated pooled serum from various sources.

Subclone A9.4 was maintained in RPMI-1640 medium supplemented with fetal calf serum. Therefore, the proliferative response of this clone was studied to assess whether it could result from proteins contained in fetal calf serum which had become associated with MHC determinants on antigen presenting cells. Experiments designed to examine this possibility showed that A9.4 cells proliferate in vitro upon culture with KLH and irradiated syngeneic or parental spleen cells independently of the species difference in serum used to supplement the culture (Table XIX).

To exclude the possibility that non-polymorphic I-A determinants were involved in the genetic restriction to either parent, the effect of anti-Ia monoclonal antibodies on the proliferative response of this clone was studied. The results, presented in Table XX, show that anti-I-A^b and anti-I-A^d monoclonal antibodies inhibited the proliferative response of subclone A9.4 to both irradiated parental spleen cells. In order to determine whether these results were due to Ia molecules passively absorbed by A9.4 T cells from the irradiated filler cells, the following experiment was performed. A9.4 cells were cultured for six weeks with either CB6/F_1, BALB/c and C57BL/6, irradiated spleen cells. The cells harvested from each of these cultures were used to test the effect of anti-Ia monoclonal antibodies on the proliferative response to CB6/F_1, BALB/c and C57BL/6 irradiated spleen cells. The results presented in Table XIV show that the response of subclone A9.4 cultured on BALB/c filler cells was inhibited by anti-I-A^d antibodies, but not by anti-I-A^b antibodies. Conversely, the proliferative response of this clone cultured on C57BL/6 was inhibited only by anti-I-A^b and not by anti-I-A^d antibodies. These results suggest that the inhibition of proliferation of subclone A9.4 by monoclonal anti-I-A antibodies is due to the passive absorption of Ia molecules from the

TABLE XX

INHIBITION OF SUBCLONE A9.4 PROLIFERATIVE RESPONSE TO STIMULATOR CELLS WITH MONOCLONAL ANTI-Ia ANTIBODIES

Monoclonal antibodies added during the culture	A9.4 subclone cultured 6 weeks on								
	$CB6F_1$ filler cells			BALB/c filler cells			C57BL6 filler cells		
	$CB6F_1$*	BALB/c*	C57BL6*	$CB6F_1$	BALB/c*	C57BL6	$CB6F_1$*	BALB/c	C57BL6*
anti-Ia^d	69.4**	58.9	65.3	55.9	52.8	32.6	6.6	-6.7	12.1
(MK-D6)	71.4	69.9	60.8						
	69.5	69.9	60.8						
anti-Ia^b	49.7	57.7	56.4	6.8	6.6	1.1	68.8	71.1	77.1
	83.8	73.4	70.1						
	82.7	76.9	70.1						
anti-Ia^k	-0.4	6.8	-3.7	6.8	-4.2	-7.3	6.5	-2.2	-16.3
(10.2-16)	16	21.0	-0.9						
	9.9	2.4	-0.9						

*Stimulating cells

**% of inhibition

Subclone A9.4 was repeatedly cultured on syngeneic or parental filler cells for 6 weeks before assaying for inhibition of proliferation to stimulator splenic cells with monoclonal anti-Ia antibodies. All cultures contained Iscove's serum free medium supplemented with 1% affinity purified monoclonal antibodies. Results are presented as percent inhibition of proliferation. Three separate experiments were performed on cells grown as syngeneic $CB6/F_1$ filler cells.

TABLE XXI

ABSORPTION OF ANTI-Ia ANTIBODIES WITH SUBCLONE A9.4 CULTURED WITH SYNGENEIC FILLER CELLS

MnAb	C'	Abs†	%* Viable cells
	-	-	3+1
anti-Ia^b	+	-	87+5
	+	+	10+5
			2+1
anti-Ia^d	+	-	83+5
	+	+	12+5
nil	+	-	2+1

†Absorption experiments were carried out by incubation for 45 min at 37°C of 0.2ml of antibody with 10^6 A9.4 T cell clone cultured on CB6/F_1 filler cells.

CB6/F_1 T depleted splenic lymphocytes were incubated for 45 min at 37°C with ascitic fluid of monoclonal antibodies specific for I-A^b (1:12,000 dilution) and I-A^d (1:20,000 dilution) and rabbit complement.

*Cytotoxic activity of anti-Ia antibodies was measured by trypan blue dye exclusion on duplicate wells.

filler cells onto the T cell clone.

The presence of Ia molecules bound to subclone A9.4 was further substantiated in absorption experiments. The results illustrated in Table XXI show that the complement mediated cytotoxic activity of monoclonal anti-I-A^b and anti-I-A^d antibodies was drastically reduced after incubation with subclone A9.4 cultured on CB6/F1. The cytotoxic activity of both monoclonal antibodies could not be absorbed by the T cell clone after it had been cultured for seven days in IL-2 without any irradiated filler cells. This data further supports the suggestion that anti-I-A inhibition is due to the passive absorption of class II molecules onto this clone. Indeed, there are recent reports which demonstrate that highly purified antigen specific T blasts (56) or T cell clones (57) can passively bind Ia determinants from allogeneic or syngeneic feeder cells.

An alternative explanation of this paradoxical proliferative response is that the genetic restriction is due to molecules encoded outside the MHC. The proliferative response of this clone was therefore tested in the presence of irradiated spleen cells derived from animals of different Mls haplotypes. The rationale of this investigation was based on information indicating that BALB/c and C57BL/6 share the same silent Mls^b determinants, and that Mls gene products induce strong T cell proliferative responses, similar to I-A gene product. Data illustrated in Table XXII shows that only stimulator strains which express Mls^b determinants were able to induce the proliferative response of subclone A9.4 with KLH. In particular, BALB.K ($H\text{-}2^k$, Mls^b) induced proliferation; whereas, Ab.Y ($H\text{-}2^b$, Mls^c) did not. This indicates that subclone A9.4 can recognize KLH in association with Mls determinants. In addition to the antigen specific proliferation, this clone showed significant proliferation in the absence of antigen to syngeneic (CB6/F1) cells, as well as to both parental cells (BALB/c and

TABLE XXII

MLS REACTIVITY OF SUBCLONE A9.4

Stimulator Strain	H-2	MLS	Exp. 1 nil	Exp. 1 KLH	Exp. 2 nil	Exp. 2 KLH
nil	-	-	266±91*	266±97	209±19	130±30
$CB6F_1$	b/d	b	1,954±109	4,829±841	3,668±630	13,820±533
C57BL/6	b	b	1,107±77	3,457±137	3,765±805	10,955±777
BALB/c	d	b	2,173±128	4,632±416	4,768±106	12,607±627
BALB/B	b	b	1,770±197	3,457±61	ND	ND
BALB/K	k	b	1,157±119	3,055±364	4,481±272	10,313±980
BXD:1	d	b	2,021±148	4,568±302	4,902±197	11,742±1,564
2	b	a	158±23	209±140	511±116	781±123
6	d	b	2,267±340	5,070±138	4,413±328	7,541±786
8	b	a	108±16	148±50	593±419	378±126
12	d	b	1,757±128	3,990±97	2,941±786	10,521±1,445
24	d	a	127±49	381±106	158±67	150±72
29	b	a	158±12	86±45	248±121	421±55
B10.D2	d	b	925±71	3,736±341	ND	ND
AB.Y	b	c	245±145	218±196	ND	ND
RIII/J	r	a	188±50	145±93	ND	ND
CBA/J	k	d	ND**	ND	455±55	530±54

*cpm ± mean ± SD of triplicate culture.

**ND

Proliferation of subclone A9.4(10^4 cells/culture) to syngeneic and allogeneic splenic stimulator cells was carried out with $5x10^5$ in Exp.1 and with $1x10^6$ stimulator cells in Exp. 2. In both experiments the antigen proliferation was carried out with 50 µg/ml KLH.

C57BL/6). This clone did not proliferate, however, in the presence of irradiated spleen cells from mice bearing H-2^k, H-2^r, H-2^s, H-2^a, H-2^u, or H-2^q haplotypes (Table VI).

The genetic restriction of subclone A9.4 to Mls^b determinants is further supported by the results obtained with RI BXD mice. Only the recombinant strains which possess chromosome 1 from BALB/c and bear Mls^b determinants were able to induce a proliferative response with or without KLH. Interestingly, the RI BXD 6 strain exhibiting a recombinant in chromosome 1 and bearing Mls^b determinants was unable to stimulate the proliferation of A9.4 cells. Therefore, these results suggest that this T cell clone recognizes autologous determinants encoded by the Mls^b allele as well as the foreign antigen associated with such determinants.

I. Discussion

This study was designed in order to analyze the genetic restriction of monoclonal T cell populations isolated from an F_1 strain of mice. The specific aim was to generate a panel of T lymphocyte clones and T cell hybridomas specific for KLH antigen. Two techniques were used to characterize the various MHC determinants involved in the KLH induced response of these monoclonal T cells. The first involved mapping T cell recognition to a particular Ia molecule using syngeneic, parental, allogeneic, and H-2 recombinant strains of mice as a source of antigen presenting cells. Second, inhibition of T cell recognition of antigen by monoclonal and anti-Ia antibodies directed toward determinants on the antigen presenting cells. In this study, the specificity of antibody blocking correlated with haplotype specificity and I-region mapping of the T cell antigen induced response. The combination of monoclonal T cells and monoclonal anti-Ia antibodies have been essential in demonstrating that recognition of Ia molecules on

presenting cells by T lymphocytes is directly responsible for certain Ir gene effects. These reagents have also proven useful for developing the concept of epitope restriction on a single Ia molecule. Our study with monoclonal T cells parallels the serological studies indicating that Ia molecules have multiple distinct epitope clusters (58).

Spleen cell preparations from nine different H-2 haplotype strains of mice, including syngeneic and parental strains, were used to study both the MLR and antigen specific response of five selected T cell clones and three T hybridomas. Based on either a proliferative response or IL-2 response, the KLH specific monoclonal T cells can be classified into five groups. (1). Clones C1.3, D18 and SW2.3 are genetically restricted to I-A molecules encoded by one parental haplotype. Clone C1.3 proliferates to KLH in the presence of A_α^b A_β^b, molecules, clone D18 in the presence of A_α^b A_β^b molecules, clone A12 in the presence of A^d_α A^d_β, and hybridoma SW2.3 secretes IL-2 in the presence of KLH and A^d_α A^d_β molecules. (2) Clones which are KLH specific in the presence of I-A encoded gene products and also display alloreactivity. Clone A12.11 proliferates subsequent to stimulation with H-2^k strains tested, and clone F_6 proliferates with H-2^q strains. (3) T cell clones that recognize F_1 hybrid Ia molecules (A_α^b A_β^d and A_α^d A_β^b), or F_1 transcomplementation Ia molecules (E_α^d E_β^d). Hybridoma FN1-18 secretes IL-2 in response to KLH in the presence of an I-E transcomplementation gene product. (4) T lymphocytes with a KLH restricted response to a parental Ia molecule and autoreactive to the other parental Ia molecule. Hybridoma FN13-21 recognizes KLH in association with I-A^d antigen presenting cells, and also responds to I-A^b in the absence of antigen. (5) Antigen recognition by clone A9.4 was shown to be restricted to determinants encoded by the Mls^b allele.

Analysis of the antigen specific T lymphocytes which also exhibits an allospecificity, represents one of the most interesting models to study the receptor of T cells. The clonal nature of T cell clones exhibiting double specificity was demonstrated by subcloning under stringent limiting dilution (0.5 cells/well) and BUdR experiments. The subclone exhibited the same reactivity pattern as parental clones. Furthermore, in BUdr-suicide experiments, both KLH and allogeneic induced reciprocal proliferative responses were ablated upon BUdR and light exposure.

Two major hypothesis can be entertained to explain the ability of a single T cell to recognize foreign antigens in association with syngeneic MHC encoded determinants and to proliferate in response to allogeneic Ia antigens: a) a certain degree of mimicry between the alloantigens and the neoantigens created by the association of foreign antigens with the syngeneic MHC determinants and b) that T cells carry two receptors with different recognition specificities. These two hypothesis will be examined in greater detail in the next two chapters dealing with their functional activity and inhibition using anti-clonotype reagents.

T cells exhibiting a similar double specificity are those like hybridoma FN13-21 which is antigen specific and autoreactive. It specifically recognizes KLH in association with the I-A^d gene product but can also respond with IL-2 production to I-A^b bearing cells in the absence of antigen. Subcloning of FN13-21 shows this to be a stable property, present in all subclones derived from the original cloned hybridoma. Moreover, the autoreactivity of FN13-21 to I-A^b can be shown in the absence of fetal calf serum. Such autoreactive hybridomas have been desdribed before as some antigen-specific hybridomas respond to syngeneic APC alone as well as to APC in association with antigen (53). However, FN13-21 is unusual because it does not react to I-A^d alone, but requires I-A^d for presentation

of antigen and is inhibited from responding to antigen by anti-I-A^d, but not by anti-I-A^b. On the other hand, anti-I-A^b inhibits the autoreactivity to C57BL/6 spleen cells, whereas, anti-I-A^d has no effect on this response. Thus, the T cell hybridoma's cross-reactivity between antigen in the context of I-A^d gene products, and I-A^b gene products alone, represent an epitope which the monoclonal antibodies to I-A antigens fail to perceive.

The only comparable reports in the literature concern cross-reactivity at the T cell receptor level between allogeneic histocompatibility antigens and antigen in association with self (59). These have been interpreted as demonstrating that the preferential of T cells recognizing allogeneic histocompatibility antigens in the thymus gives rise to cells which recognize antigen in association with self Ia, but not self Ia alone. It is clear, however, that autoreactive T cells do exist, since T cell lines responding to syngeneic antigens have been described not only for class II (41) but also for class I (60) and for Mls antigens (61). The physiological role of these autoreactive cells in vivo is unknown.

One of the central findings in this study is that a murine T cell hybridoma FN1-18 exhibited a specific response to KLH antigen when the antigen was presented by accessory cells which were either of human or murine origin; and that the T cell recognition event was genetically restricted by an element closely related to a polymorphic epitope common to the class II MHC molecules of both species. This epitope was associated with, but is not identical, to the determinant recognized by monoclonal antibody 17-3-3s.

The use of H-2 congenic and recombinant strains of mice resulted in the delineation of the restriction of interaction between T cell and accessory cell to products encoded by genes of the I-E locus. Strains expressing gene products of the I-A locus E^b_β or E^k_β together with the I-E

locus products E_{α}^{d} or E_{α}^{k} produced complementation molecules that bear the restricting determinants needed for KLH presentation to FN1-18 hybridoma. The molecular analysis of the gene products for the I-E_{β} chain suggests that the region around the E_{β} gene is a hot spot for recombination (62).

Two lines of evidence support the close relationship of this restriction element to an Ia antigenic determinant recognized by monoclonal antibody 17-3-3s: First, all strains of mice capable of presenting KLH to the T cell hybridoma bear the polymorphic epitope recognized by the antibody. Secondly, presentation of the KLH antigen was specifically blocked by the addition of the 17-3-3s and Y-17 antibodies but not by other antibodies directed to I-A antigenic determinants. Antibody 17-3-3s is specific for H-2 k and r haplotypes (63) but can also interact with transcomplementation product of E_{β}^{b}, ^{k,r,} or ^{s} with $E_{\alpha}^{d,k}$ or ^{r} (64), whereas Y-17 reacts with a conformational or combinatorial determinant formed by the association of $E_{B}^{k,r,b}$ or ^{s} and E_{α}^{k} or ^{r} gene products designated as specificity Ia.m.44 (65). The failure of RIII and B10.S(9R) cells to present KLH to hybridoma FN1-18 suggests that the I-E epitope identified by the hybridoma is not identical to the epitope defined by anti-I-E antibodies available at this time.

There are several reports indicating that antibodies specific for murine I-E antigens can interact with MHC encoded molecules from various species, including human DR structures (66). At the level of amino acid sequences, there is approximately 70% homology of murine I-E molecules with human DR chain molecules (67). However, there is no previous evidence that these shared interspecies epitopes can function as genetic restriction elements.

The most intriguing observation of this study was that T-depleted mononuclear cells from human subjects present KLH to a murine hybridoma,

and that this ability shows a strong correlation (r=0.768) with immunofluorescence staining by the murine anti-I-E reagent 17-3-3s. Although the amount of IL-2 production by FN1-18 in response to KLH is lower in the context of human than mouse cells, the response is significant. The differences in the magnitude of response may be related to a number of factors such as differences in the ability to process KLH by cells from murine spleen versus human peripheral blood, differences attributable to other structural features of murine and human Ia determinants, or to the possible influence on the hybridoma of species specific soluble factors derived from the antigen presenting cells.

It clearly appears that the Ia epitope recognized by 17-3-3s is needed for KLH presentation to hybridoma FN1-18 as illustrated by the inhibition studies. In contrast to 17-3-3s, another antibody, 109d6, exhibiting a related but non-identical pattern of reactivity with an MT3-like antigen, produced no inhibition, or was only marginally inhibitory in antigen presentation. The human MT3 specificity as defined by alloantisera is composed of a number of separate epitopes, some of which are recognized by different monoclonal reagents. Antibodies 17-3-3s and 109d6 probably identify two of these separate epitopes (68). The differences exhibited by these two antibodies in the inhibition studies emphasize the distinction of the epitopes and supports the hypothesis that the epitope closely linked to that identified by 17-3-3s is preferentially utilized in antigen presentation to hybridoma FN1-18. Since neither the primary structure nor the conformation of the Ia molecules bearing the epitopes defined by the antibodies 17-3-3s and 109d6 are known, it is not possible to interpret these data in this respect. However, in view of the extremely close but non-identical relationship of the epitope identified by antibody 17-3-3s with the restriction element recognized by the T cell receptor of the hybridoma,

it is possible that the antibody and receptor recognize slightly different conformational determinants on the Ia molecules shared by both species. This hypothesis is supported by the inability of T-depleted mononuclear cells from two individuals to present KLH despite the expression of a determinant identified by antibody 17-3-3s on their cell surface. These exceptions could be accounted for by the effect of other amino acid substitutions in neighboring areas of the Ia molecule that slightly alter the conformation in this region. Taken together with the known differences between murine and human Ia, the data of this study argue in favor of the view that only a small region of the Ia molecule is involved in antigen presentation. It also raises the question of whether these analogous structures are products of conserved portions of polymorphic genes, or whether they are the result of convergent evolution.

In conclusion, our results demonstrate for the first time that a serologically defined Ia epitope can function as a genetic restriction element, on both human and mouse cells, in antigen presentation to a murine T cell hybridoma. These results also demonstrate that the syngeneic nature of the MHC encoded determinants is not critical for the recognition of foreign antigens by the T cells of a given species. The T cell recognition of a particular foreign antigen depends on the association with a structurally similar Ia epitope which is encoded by the MHC complex of various species.

Another T cell clone of great interest recognized KLH in the context of determinants encoded by the Mls^b allele. The frequency of precursor T cells specific for syngeneic Mls^b determinants appears to be a rare event; only one clone out of our panel of KLH specific clones exhibited this pattern of proliferation. While class I and class II MHC determinants play a major role in antigen recognition by T lymphocytes, non-MHC

determinants may also be involved in regulating this important mechanism of immunological responsiveness.

Various congenic and recombinant inbred strains were used to characterize the autologous histocompatibility determinants recognized by subclone A9.4. The KLH induced proliferation is independent of classic MHC determinants as demonstrated by the lack of T cell activation by AB.Y ($H\text{-}2^b$, Mls^c). The results using the RI BXD strains demonstrate that non-MHC determinants were responsible for the proliferation of this clone. Four of the strains tested contain recombinations within chromosome one. Two strains, RI BXD 2 and 8, containing a recombination between the dipeptidase-1 gene of C57BL/6 and the Mls gene of DBA/2, did not induce proliferation of this clone; whereas, strain RI BXD 6, which contains a reciprocal recombination between the dipeptidase-1 gene of DNA/2 and the Mls locus of C57BL/6, did induce proliferation of the T cell clone. The implication of these findings is that T cell clone A9.4 recognizes KLH in the context of syngeneic Mls gene products.

Critical differences between our KLH-Mls and KLH-MHC restricted T cell clones were observed. First, the clone which recognizes KLH in the context of non-MHC gene products does not provide help to TNP-Ova primed B cells in the presence of TNP-KLH in the generation of an anti-TNP-response; whereas, T clones which demonstrate associative recognition of KLH and MHC encoded determinants exhibited helper activity for both IgM and IgG responses. We cannot rule out the possibility that other Mls restricted T cell clones will be able to provide such help. Second, the A9.4 T cell subclone passively binds to Ia antigens derived from irradiated filler cells since its proliferative response to $CB6F_1$, BALB/c and C57BL/6 stimulatory cells was inhibited with anti-Ia antibodies corresponding to filler cell haplotypes. This inhibition may be related to a

steric hindrance mechanism by which anti-Ia antibodies bound to passively absorbed Ia alters the ability of the receptor of A9.4 clones to interact with Mls antigens expressed on stimulatory cells.

The mechanisms by which Ia binds to this T cell clone is unclear. There are at least three possible mechanisms for this type of binding: 1) The binding of Ia molecules may be due to specific anti-Ia receptors. This is unlikely since a single clone is binding to both parental haplotypes as well as the F_1 Ia antigen. 2) Ia molecules may bind to a non-specific receptor for these molecules by mechanisms similar to the binding of the Fc fragments of immunoglobulin to the Fc receptor. 3) Ia binding may occur through complimentary binding of these molecules by the Ia molecules which are expressed on activated T cell clones. These hypotheses are currently under investigation.

MHC determinants play a major role in the differentiation and maturation of T lymphocytes. They are also involved in genetically restricted antigen recognition during the effector phases of cell mediated immune responses. The existence of a T cell clone restricted to syngeneic Mls determinants suggests that this locus may also play a role during the developmental stages of T lymphocyte differentiation.

CHARACTERIZATION OF THE FUNCTIONAL PROPERTIES OF LYT 1^+ T CELL CLONES: ANALYSIS OF MHC RESTRICTION BETWEEN T AND B LYMPHOCYTES

A. Introduction

The first section of this thesis deals with the role of MHC gene products in the genetic restriction between T cells and antigen presenting cells. MHC restriction at this level represents one of the earliest recognition events in the immune response process, and it does not explore genetic restriction at subsequent steps in the immune pathway. The present study was undertaken in an effort to characterize the ability of cloned T cells to function as helper cells in a T cell dependent antibody response, and to study the rules governing the genetic restriction at this level of response. One intriguing approach has been to use Lyt 1^+ T cell clones which recognize foreign antigen in association with self MHC encoded antigens and display alloreactivity. Information regarding the genetic restriction of B cells with T cell clones possessing a double specificity is limited. However, this issue is of theoretical interest with regard to the relationship between the receptor recognizing the foreign or alloantigens, and the products of T clones critical for the activation of B cells.

The demonstration of helper activity in monoclonal T cells has provided insight into the mechanisms by which T cell-help is effective in antibody responses. Hodes et al. (69) studying cloned T helper cell activation of B cell subpopulations, have demonstrated normal activity of these clones in a hapten-carrier plaque forming assay. The *in vitro* response to the soluble antigen TNP-(T,G)-A-L had previously been shown to be under the control of I-A encoded Ir genes (70); however, cloned antigen specific and H-2 restricted T cells in this *in vitro* system,

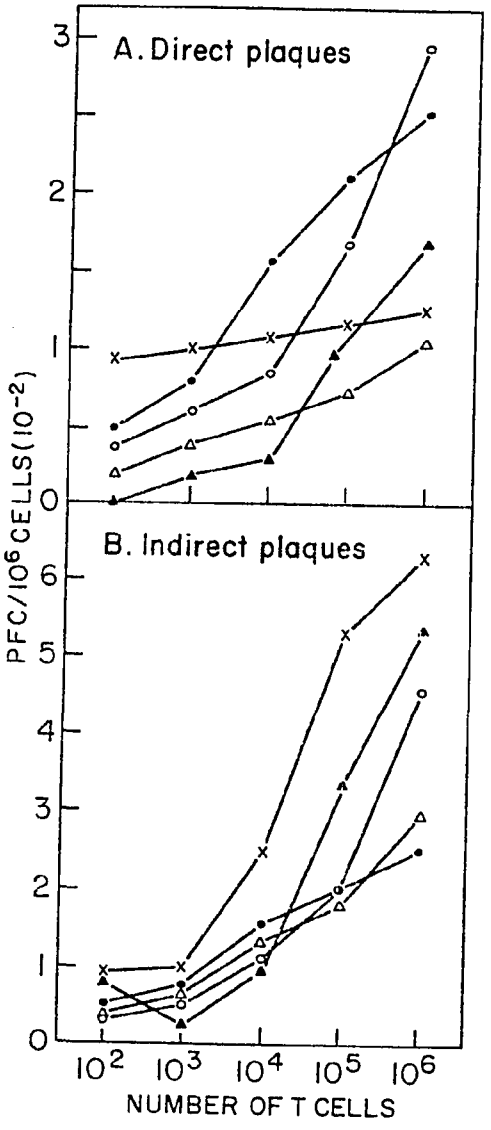
could provide help in a non-H-2 restricted manner, once they had been activated.

The reports by Hodes and Singer have suggested that cloned T helper cells may exhibit different mechanisms by which they function. In particular, certain cloned T helper cells have been reported to be H-2 restricted for recognition of B cells as well as accessory cells (71), whereas, others have been shown to be restricted for recognition of accessory cells alone (69). Similarly, responses mediated by cloned T helper cells have either demonstrated a requirement for carrier-hapten linkage (69), or not (72). Recent work by Asano et al. has characterized two distinct pathways for B cell activation in T dependent antibody responses (73). The difference between the two pathways residues not in the T cell populations, but lies in the B cell populations, which contains two subpopulations that can be activated by T lymphocytes through different mechanisms. One B cell population which is absent in CBA/N mice, bears the Lyb^5 surface marker, requires only soluble lymphokines from T cells, and high doses of antigen to generate an IgM antibody response. The other B cell population lacks the Lyb5 surface marker present in CBA/N mice, requires H-2 restricted T helper cells in addition to low doses of antigen, and the hapten must be chemically linked to the carrier molecule in order to elicit an IgG response. Using antigen specific, H-2 restricted monoclonal T helper cells, Asano et al. demonstrated that the same monoclonal T cell was capable of activating each B cell population, but through distinct mechanisms.

The function of T suppressor cells in collaboration with B cells has been shown to be genetically restricted. It has been demonstrated that two distinct T suppressor cell pathways function to regulate the MHC-restricted and carrier-hapten linked IgG responses of $Lyb5^-$ B cells (74).

Figure 2

Dose-effect relationship between the number of T cells and anti-TNP PFC response. Various numbers of (o—o) lymph node cells from KLH primed CB6/F1 mice, (●—●) clone D18, (Δ—Δ) subclone A12.11, (▲—▲) subclone C1.3, and (X—X) clone F^6 cultured with $21x10^6$ TNP-OVA primed B cells in the presence of (.001 μg/ml) TNP-KLH. The response was measured on day 4.



The in vitro activation of both Lyt 1^+ antigen non-specific T suppressor cells and Lyt 2^+ antigen specific T suppressor cells has been shown to require the interaction of accessory cells and antigen-primed T cells (75). This interaction was MHC restricted in that T cell recognition of I-A encoded determinants or accessory cells was required for T suppressor cell activation.

The availability in our laboratory of monoclonal T helper cells with a double specificity for alloantigens provided us with an excellent tool with which to examine the requirements of B cell activation by T cell with various alloantigen specificity (76). The ability of alloantigen to induce the production of T cell lymphokines required by $Lyb5^+$ B cell to generate an anti-TNP response was examined.

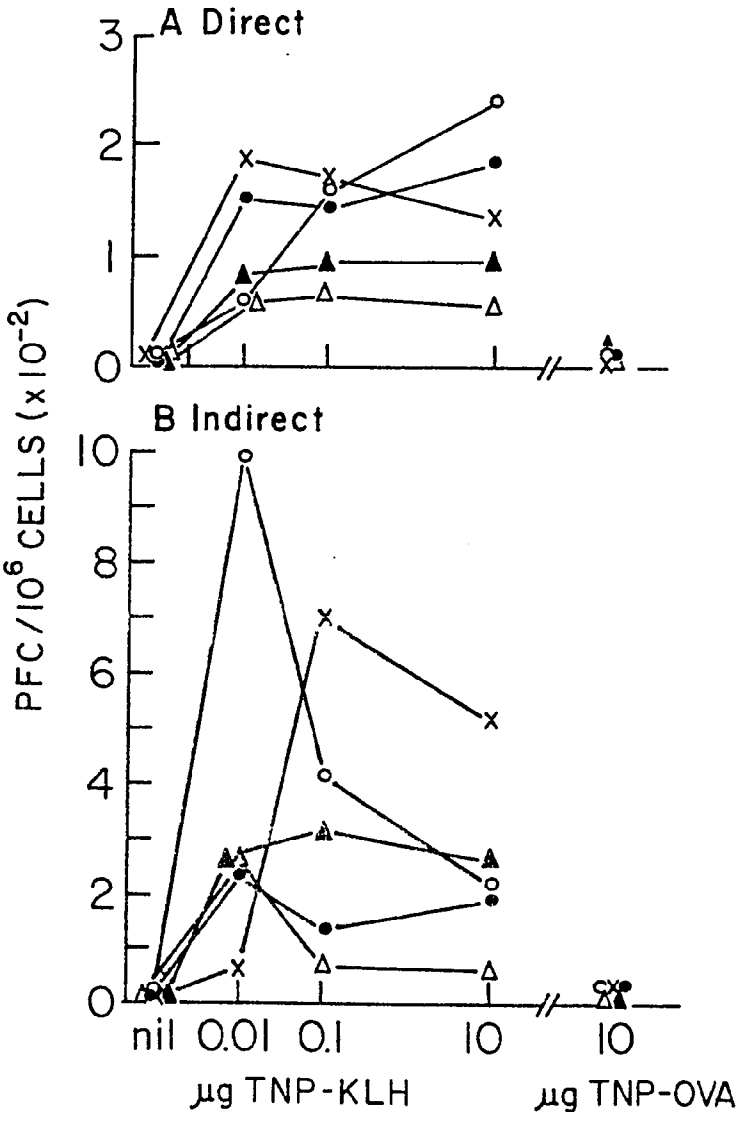
B. Helper Function of T Cell Clones: Antigen concentration and T cell titration effect on anti-TNP response.

The helper activity of clones expressing Lyt 1^+, 2^- surface markers was tested in an in vitro antibody synthesis assay by the incubation of KLH specific T clones or lymph node cells with highly purified B cells from TNP-Ova primed $CB6/F_1$ mice in the presence of TNP-KLH. Similarly, cultures were incubated with TNP-Ova as a specificity control. The results depicted in Fig 2 show the effect of antigen dose on the generation of an anti-TNP response. All the clones tested provided a KLH carrier specific helper activity at various antigen doses for IgM and IgG response. While with KLH, primed lymph node T cells a higher IgG PFC response was observed with low antigen dose, no significant variations in the magnitude of IgM and IgG PFC response were noted upon incubation of T cell clones with various doses of antigen (0.001-10μg).

The experiments presented in Fig 3 compares the helper capacity of increasing numbers of T clones with KLH primed lymph node cells in the

Figure 3

Dose-effect relationship between antigen concentration and anti-TNP-PFC response. $3x10^5$ cells from (o—o) lymph node cells from KLH primed $CB6F_1$ mice (●—●) clone D18, (Δ—Δ) subclone C1.3 (X-X) clone F_6 and (▲—▲) subclone A12.11 were cultured with $2x10^6$ TNP-OVA primed B cells. The response was measured on day 4.



generation of an anti-TNP response. In this experiment, $4x10^6$ B cells were incubated with various concentrations of T cell clones per culture. In this series of experiments, we observed that clone F_6 and subclone Cl.3 provided stronger helper activity than a heterogeneous lymph node population of KLH primed cells. The T cell clone A9.4 which recognizes KLH in the context of syngeneic Mls^b gene products was tested in a separate experiment. The results presented in Table III show that the magnitude of direct and indirect anti-TNP-PFC response of B cells incubated with clone F_6 was consistently higher in comparison to lymph node cells derived from KLH primed CB6/F_1 mice. However, no significant helper effect was detected with subclone A9.4 tested four times during an 18-month period. Furthermore, no helper effect was obtained at the various cell densities and doses of antigens (0.001-10 µg/ml TNP-KLH) that have been tested. These results indicate that subclone A9.4, despite its ability to proliferate to KLH, is unable to exhibit helper function.

C. Genetic restriction of anti-TNP response with clones exhibiting double antigen specificity

The genetic restriction of helper function was further studied with the two T clones displaying proliferative responses to both KLH plus self-antigens and alloantigens. The data depicted in Table XXIII show that clone F_6 and subclone A12.11 cooperated only with CB6/F_1 B cell to provide carrier specific help. In contrast, no significant helper activity was observed in C3H/He primed B cells cultured with clone F_6, A12.11, or KLH primed CB6/F_1 lymph node cells. In this experiment, only C3H/He primed lymph node cells provide help for the C3H/He B cell anti-TNP response. Similarly, clone F_6, as well as CB6/F_1 lymph nodes,did not provide help for B10.Q TNP primed B cells, whereas, KLH primed B10.Q lymph

Figure 4

Dose-effect relationship between number of T cells and anti-TNP PFC response developed by TNP-OVA primed syngeneic and allogeneic B cells. The cultures were incubated with 10 μg TNP-KLH and the direct PFC response was measured on day 4.

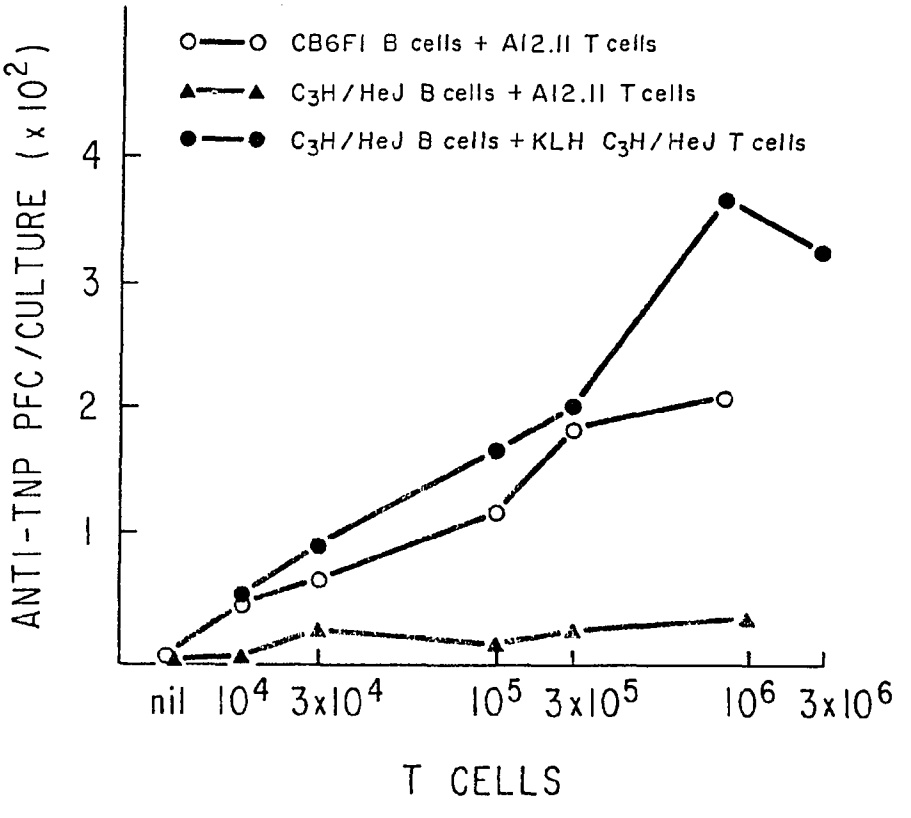


TABLE XXIII

GENETIC RESTRICTION OF HELPER FUNCTION OF T CELL CLONES

TNP-primed B cells	KLH specific T cells	Anti-TNP-PFC response/culture					
		Exp. 1 TNP-OVA***		TNP-KLH***		Exp.2 TNP-KLH	
		Direct	Indirect	Direct	Indirect	Direct	Indirect
$CB6F_1$	-	0	0	18±11**	0	6±3	3±1
"	$CB6F_1$*	0	0	354±12	756±33	435±21	944±42
"	A12.11	0	0	174±31	213±21	54±7	218±12
"	F^6	3±2	12±3	16±2	138±12	543±3	1,032±6
C3H/HeJ	-	1±1	0	13±3	2±1	9±6	21±12
"	$CB6F_1$*	14±8	11±7	33±9	55±20	30±3	48±3
"	C3H/HeJ*	27±12	0	386±42	633±26	321±12	846±6
"	A12.11	0	0	126±83	0	9±3	36±3
B10.q	-	2±1	6±2	3±1	0	3±3	15±3
"	$CB6F_1$*	0	8±2	14±7	83±6	3±3	21±9
"	B10.q	0	0	178±10	625±30	183±12	503±3
"	F_6	0	0	0	0	9±9	12±12

*Lymph node lymphocytes (10^6 cells/culture).

**PFC mean response ± SEM of duplicate cultures.

***10 μg/ml.

TABLE XXIV

IN VITRO ANTI-SRBC PFC RESPONSE SUBSEQUENT CULTURING B CELLS WITH A12.11 T CELL CLONE IN PRESENCE OF SRBC AND KLH

Origin of B cells ($2x10^6$)*	T cells ($3x10^5$)	Irradiated Spleen cells	Antigen SRBC10^7	Antigen KLH40µg	Anti-SRBC PFC/culture
$CB6F_1$	-	-	-		0
	-	-	SRBC	KLH	26±4
C3H/HeJ	-	-	-		2±2
	-	-	SRBC	KLH	19±4
CBA/N	-	-	-		0
	-	-	SRBC	KLH	8±3
$CB6F_1$	A12.11	-	-		2±2
	A12.11	-	SRBC	KLH	36±5
C3H/HeJ	A12.11	-	-		12±3
	A12.11	-	SRBC	KLH	4±2
CBA/N	A12.11	-	-		0
	A12.11	-	SRBC	KLH	10±3
$CB6F_1$	A12.11	$CB6F_1$	-		6±3
	A12.11	$CB6F_1$	SRBC	KLH	31±5
C3H/HeJ	A12.11	$CB6F_1$	-		0
	A12.11	$CB6F_1$	SRBC	KLH	20±3
CBA/N	A12.11	$CB6F_1$	-		2±2
	A12.11	$CB6F_1$	SRBC	KLH	18±4

Control for in vitro SRBC PFC response

Spleen cells from SRBC primed mice

$CB6F_1$	-		204±111
	SRBC	KLH	1,432±13
C3H/HeJ	-		192±10
	SRBC	KLH	1,072±14
CBA/N	-		91±6
	SRBC	KLH	839±20

Control for helper function of A12.11 cells

B cells from TNP-OVA primed ($2x10^6$)	T cells ($3x10^5$)	Antigen (0.01 µg/ml	Anti-TNP PFC/culture
$CB6F_1$	nil	TNP-KLH	8±4
$CB6F_1$	A12.11	TNP-KLH	184±42

*B cells were prepared as described in Material and Methods

nodes provide excellent help for both direct and indirect anti-TNP plaque responses. It is important to note that cultures containing B10.Q or C3H/He TNP primed B cells and CB6/F1 KLH primed lymph node cells exhibited weak but significant allogeneic effect. This effect was also observed in cultures containing C3H/He TNP-Ova primed B cells with subclone A12.11 or B10.Q TNP-Ova primed B cells with clone F_6.

The results illustrated in Fig 4 show that the incubation of $2x10^6$ C3H/HeJ TNP primed B cells with various concentrations of (10^4-10^6) A12.11 T cells does not lead to a significant increase of anti-TNP PFC above background. In contrast, a dose dependent increase of the anti-TNP PFC response was observed when B cells were incubated with various concentrations of T cells (10^4-$3x10^5$), i.e., C3H/HeJ TNP primed B cells with C3H/HeJ KLH primed T cells or CB6F1 primed B cells with A12.11 cells. Furthermore, we investigated whether A12.11 cells were capable of providing help in a non-specific manner to syngeneic B cells (CB6/F1) or to the allogeneic B cells (C3H/HeJ and CBA/N) expressing H-2^k haplotype. This was particularly important since Asano et al. reported data indicating that the same T cell can help in a MHC restricted manner, the $Lyb5^-$ B cells, and in a MHC non-restricted fashion, the $Lyb5^+$ B cells. The Marrack and Kappler assay system (77) was employed to investigate whether clone A12.11 stimulated by KLH in the presence of syngeneic APC, can help normal B cells to mount a SRBC PFC response upon the culture in the presence of SRBC.

The data depicted in Table 24 show that spleen cells from SRBC primed CB6F1, C3H/HeJ and CBA/N develop an excellent anti-SRBC PFC response upon in vitro culture with SRBC. It is important to note that a very weak anti-SRBC-PFC response was observed in the cultures where A12.11 clone was incubated in the presence of KLH and SRBC with B cells from CB6F1, C3H/HeJ or CBA/N. Furthermore, the addition to the culture of CB6F1 spleen cells did not augment the anti-SRBC PFC response.

TABLE XXV

RESPONSE STIMULATED BY A12.11 T CELL CLONE REQUIRES LINKED CARRIER-HAPTEN RECOGNITION

TNP-OVA primed B cells**	KLH Specific T cells	anti-TNP PFC response/culture							
		nil		TNP-KLH 0.001 µg/ml		TNP-OVA 0.001 µg/ml		TNP-OVA 0.001µg + KLH 40 µg/ml	
		direct	indirect	direct	indirect	direct	indirect	direct	indirect
CB6/F_1	-	0	0*	2+1	0	6+2	16+4	4+2	4+4
C3H/He	-	0	0	0	4+2	4+2	12+4	2+1	0
CB6/F_1	CB6/F_1 L.N.	5+5	6+3	88+24	948+111	11+7	30+20	33+8	132+34
C3H/He	C3H/He L.N.	2+2	4+4	27+27	382+82	6+2	12+4	45+10	128+23
C3H/He	CB6/F_1 L.N.	2+0	2+2	5+2	24+11	2+1	8+4	2+2	8+4
CB6/F_1	A12.11	2+1	0	38+5	344+40	2+2	0	4+2	6+2
C3H/He	A12.11	0	0	4+2	10+2	1+1	4+2	3+1	8+4

*PFC mean response ± SEM, day 5 response

**$4x10^6$ TNP-OVA primed B cells per culture

D. Requirement for Hapten-Carrier Linkage

Recently, Asano et al., (73) have demonstrated that while the activation of Lyb^{5-} B cell subset occurs in the presence of MHC restricted T cells, the T-dependent activation of $Lyb5^{+}$ B cells can occur in the absence of MHC-restricted T_H-cell B cell interaction and in the absence of covalent carrier-hapten linkage. Because of their results, the requirement for carrier-hapten linkage in the activation of B cells with clone A12.11 which exhibits double specificity was studied. The results depicted in Table XXV showed that T cell help provided by A12.11 clones required carrier-hapten linkage because no anti-TNP-PFC response was obtained when A12.11 clone was cultured with TNP-Ova primed B cells in the presence of TNP-Ova and KLH. In contrast, a significant PFC response was observed when TNP-Ova primed B cells were cultured in similar conditions with KLH primed lymph node T cells.

These results taken collectively showed that the two T cell clones with double specificity can only provide help in a genetic restricted manner and that the helper function requires covalent carrier-hapten linkage.

E. Discussion

The set of findings reported in this section of the thesis deals with the helper function of the KLH specific clones. All four clones were able to provide help for $CB6F_1$ TNP-Ova primed B cells to mount an IgM and IgG PFC response in the presence of 0.001-10 µg/ml TNP-KLH conjugate. The helper function was dependent of carrier-hapten linkage since no significant anti-TNP-PFC response was observed upon the culture of $CB6F_1$ TNP-Ova primed B cells and A12.11 T clone in the presence of TNP-Ova alone or TNP-Ova and there is a critical difference between our KLH-MIs and KLH-MHC restricted T cell clones were observed. The clone which recognizes

KLH in the context of non-MHC gene products does not provide help to TNP-Ova primed B cells in the presence of TNP-KLH in the generation of an anti-TNP response, whereas, T clones which demonstrate associative recognition of KLH and MHC encoded determinants exhibited helper activity for both IgM and IgG responses. We cannot rule out the possibility that other Mls restricted T-cell clones will be able to provide such help.

It appears that the properties of our clones depending on hapten-carrier linkage, or their ability to provide help for IgM and IgG with both low and high antigen doses, are very different from the properties of clones described by Asano et al. (73). However, our data using KLH-heterogeneous T cells clearly show that the clones described by Asano et al (73) exist in the repertoire since we observed indeed a significant high IgG response with lymph node T cells in the presence of low dose of antigens. The ability of our clones to provide help with low dose of antigen as 0.001 μg/ml suggests that the clones described in this paper have a high affinity for the carrier determinant(s). The study of helper T cells recognizing carrier determinants in association with self-encoded MHC determinants and exhibiting alloreactivity provided an excellent tool to investigate the rules which govern the genetic restriction of T-B cell cooperation.

There are numerous reports suggesting the existence of two antigen specific helper T cell subsets. The first subset recognizes carrier determinants in association with self-MHC encoded determinants and cooperate with B cells via an antigen-carrier bridge (69) in a MHC restricted manner (71). The existence of this subset of helper T cells was confirmed by cloning experiments (see data reviewed in 78). A second subset, first, clearly demonstrated by the experiments of Marrack and Kappler, can induce the differentiation of unprimed B cells in response to a par-

ticulate antigen (77) or even a polyclonal activation of B cells (79) through the releasing of lymphokines. Asano et al. (73) described an antigen specific T cell clone exhibiting the properties of both subsets which were MHC restricted in their interaction with Lyb5$^-$ B cells and helped Lyb5$^+$ B cells in MHC unrestricted fashion. Défranco et al (80) has also shown that antigen specific T cell clones can induce the activation and differentiation of resting B cells in a MHC restricted manner in the presence of antigen for which T cells are specific as well as polyclonal activation of both Lyb5$^+$ and Lyb5$^-$ subsets in the presence of antigen and APC of proper MHC haplotype. These reports suggest that the same antigen specific T cells can cooperate with B cells in a MHC restricted and unrestricted manner. However, it clearly appears from our results that at various T:B cell ratio (10^4-10^6T:2x10^6B), the A12.11 KLH specific helper T clone was able to activate B cells only via a carrier-antigen bridge and in a MHC restricted fashion. No polyclonal activation of IgM secretion, or specific activation of SRBC precursors was observed with syngeneic B cells or allogeneic Lyb5$^-$ and Lyb5$^+$ B cells. These data are particularly important since other investigators showed that allospecific T cells can provide help to B cells (81,82,83).

The ability of T cell clones specific for a foreign antigen and exhibiting a secondary alloreactive to provide help through only MHC restricted T-B cell interaction could be explained: a) By differences in the affinity of the receptor of T cell clones for the carrier determinants versus alloantigen or alternatively. b) By the inability of T cells recognizing foreign antigens in association with self-MHC encoded determinants to recognize antigen in context of alloantigens since determinant selection (84) requires different association of the same antigen with various Ia determinants due to the allelic polymorphism of genes encoding for

these determinants. Whether or not this is a general property of antigen specific helper cells exhibiting alloreactivity remains to be elucidated.

CLONOTYPIC MARKERS EXPRESSED ON KLH SPECIFIC T CELL CLONES AND HYBRIDOMAS

A. Introduction

Structural and molecular analysis of the T cell antigen receptor is critical in understanding the mechanism surrounding the genetic restriction of T cell recognition. The receptor found on T cells exhibit a high degree of antigen diversity and specificity as is found in the immunoglobulin repertoire. In contrast to the immunoglobulin receptor of B cells which can bind soluble foreign antigen, the receptor of T cells recognize antigens associated with self-MHC molecules on the surface membrane of cells (24). One major difficulty in the study of the T cell receptor is the heterogeneity of the T cell population. Unlike the discovery of myelomas, B cell neoplasms, and hybridomas which facilitated the isolation of the structure of immunoglobulin receptors on B cells, previous T cell technology has lacked a similar homogeneous population of cells. Only recently has T cell cloning and T cell hybridoma methodology allowed for a more rationale approach to study the receptor. Some of the different strategies used to define the T cell receptor will be discussed in this section.

Initial approaches to the isolation of the T cell receptor were based on the assumption that T cells and B cells recognize a wide variety of foreign Ag with a fine degree of specificity, and therefore must share similar variable (V) regions of their receptors. It was believed to be unlikely that two separate gene complexes had evolved to encode proteins specific for the same Ags in both T and B cells (85). Experiments demon-

strating that antibodies against the variable regions of Ig molecules could bind to the surface of Ag-specific T cells (86) encouraged attempts to show an involvement of Ig V region genes in the formation of the T cell receptor. T cells stained with fluorescence labelled anti-Id or rosetted with SRBC coated with anti-Id antibodies have provided evidence that T cell receptors carry determinants which are similar to immunoglobulin variable region determinants (87). In addition, both the idiotype and V_H framework specificities were identified on isolated receptors from Ig^- cells (88).

Anti-idiotype antibodies reacting with the functional T cell receptor for antigen would be expected to be effective either in blocking T cell function or alternatively, substitute for antigen in the induction of the proliferation of effector and regulating T cells. Lonai et al. (89) using autoradiography was able to demonstrate binding of anti-Id antisera to T cell subsets. This anti-Id antisera were inhibitory for the antigen binding of both Lyt 1^+ and Lyt 2^+3^+ subsets. Anti-Id antiserum specific for anti-MHC antibodies has been used to examine the anti-self receptor of T cells. Nagy et al. (90) has been able to show that anti-Id can compete with soluble I-A^k antigens for the binding site of T cells. This antiserum, in the presence of complement, eliminates T cells responding to different antigens in an I-A^k restricted fashion. Binz et al. (86) using similar anti-Id antibodies to allo-MHC antigens, was able to mimic in vitro the allo-MHC antigens in their T cell triggering capacity.

The soluble products of ant gen specific T cells bear the same shared idiotype with immunoglobulins (91). Idiotypes expressed on antibodies to 4-hydroxy-3-nitrophenyl acetyl (92) and L-glutamic acid 60-L-alanine 30-L-trosine 10 (GAT) (93) have also been found on the specifically activated T cells and suppressor factors (94).

The early antiidiotype studies used heterologous antibodies. More recently, Cerny et al. (95) used a panel of monoclonalantiidiotype antibodies against TEPC-15 and HOPC-8 myeloma proteins to study the idiotype repertoire of suppressor T cells specific for phosphocholine, a cell/well antigen of Streptococcus pneumoniae. His findings indicate that the antigen receptor on T suppressor contain shared determinants with the immunoglobulin V-region. Idiotypic determinants recognized by anti(anti-Id) antibodies were identified on idiotype specific suppressor T cells in MOPC-460 T cell system (96). Evidence of this kind which is based on serological cross-reactivity between antiidiotype antibodies against immunoglobulins and activated T cells will not become convincing unless the molecules bound by these antibodies are characterized to specifically react with antigens.

Two T cell specific loci containing several genes coding for T cell markers have recently been reported by Owen et al. (97) to lie downstream of CH gene complex. Owen (97) has proposed a model that these determinants represent the constant region of T cell receptors. A panel of four monoclonal antibodies have evolved from this work, all of which define surface alloantigens on T lymphocytes linked to the Igh-1 locus. These monoclonal antibodies were generated in mice against activated T cell blasts from allotype congenic mice. The antigenic determinants of T cells described by these monoclonal antibodies may be allotypic determinants in the constant region of the antigen specific receptor. In vivo treatment of mice with MnAb T_{su} at day 4 suppresses the primary immune response to sheep red blood cells (SRBC). It has been inferred that the activation of regulatory cells in the SRBC response occurs through interaction of the monoclonal antibody with constant region determinants of the T cell receptor.

Tokuhisa and Tangiguchi (98) have reported an alloantiserum similar to Owen's produced by Igh allotype congenic pairs which bind the soluble factors of antigen specific suppressor T cells. The allotypic determinants detected by the antiserum appear to be expressed on the constant region of the antigen binding molecule of an antigen specific suppressor T cell factor (99). However, there is no direct evidence that this locus is involved in the antigen specific T cell receptor.

Several lines of independent study have failed to show evidence of immunoglobulin variable region gene products on T cells. These include analysis of T cell DNA that did not show rearrangement of immunoglobulin heavy chain genes as has been shown to occur in B cells. In B cells, the constructions of a functional H chain gene involves splicing of a diversity (D) segment to a joining (J) segment and then of a V_H segment to DJ recombinant (D→J), V→DJ). The T cell lines and T lymphomas that that have been studied showed only a DJ non-functional rearrangement (100). Moreover, cDNA probes for V-region of both light (L) and heavy chain could not detect any mRNA in either T cell lines or T cell hybridomas (101). In summary, experiments aimed at identifying immunoglobulin related T-lymphocyte antigen receptors have not produced convincing results. None have shown conclusive results concerning the biochemical structure, phenotypic markers, or genes encoding for the T cell antigen receptor.

A second approach to the problem has been to raise monoclonal Abs that specifically recognize Ag-specific T cell clones or T cell hybrids. The ability of these monoclonal Abs to inhibit or stimulate the Ag-specific response of these T cell clones has suggested that their binding is specific for the T cell receptor. All the reported studies describe situations where the monoclonal antibodies were clonotypic in their specificity.

Lancki et al. described an antibody which bound an alloreactive murine cytotoxic clone (102), and Meuer et al. (103) described monoclonals that bound an alloreactive human T cell clone. Haskin et al. (104) characterized a monoclonal directed against an IL-2 secreting Ova specific hybridoma, which in addition to blocking IL-2 release, the monoclonal antibody inhibited binding of the T cell to antigen-pulsed presenting cells.

Recently, another monoclonal antibody, L3T4 which is not clonotype in nature, but is generally associated with a subpopulation of T cells, has been shown to block the function of class II MHC restricted T cell hybridomas (105). Greenstein et al. has shown a D^d-specific T cell hybridoma uses a receptor molecule, bound by L3T4, to directly interact with non-polymorphic Ia determinants (106). The physiological role of non-polymorphic L3T4-mediated interactions between T cells and antigen-presenting cells remains unclear. Interactions of the L3T4 receptor with Ia may be unrelated to the antigen receptor of T lymphocytes, or alternatively, it may be plays a secondary role in cell-cell communication.

In addition to defining the molecular nature of the T cell receptor, clonotypic antibodies have helped define the mechanism of activation of T lymphocyte. Two laboratories have described monoclonal antibodies directed against clonotypic molecules on cloned helper or cytotoxic T cells which inhibit antigen-specific function in soluble form but induce IL-2 secretion or proliferation when polymerized on a solid support (107). This implies that cross-linking of antigen receptors, and not receptor occupancy, provides the relevant signal for activation of T lymphocytes.

The finding that monoclonal sources of T cells can be activated by antigen and self-MHC or by allogeneic MHC alone, strengthens the hypothesis that alloreactivity is indeed a cross-reactive by product of the molecular mechanism of antigen and self-recognition. Kaye et al. (108)

has described a monoclonal antibody which can inhibit activation of a monoclonal T cell by both allogeneic Ia and antigen plus self-Ia, but not by the non-specific stimulator Con A (108). The use of this anti-clonotypic antibody strongly supports the hypothesis that a single molecule mediates both self-Ia plus antigen and non-self Ia recognition.

The structure of the molecule bound by the anti-clonotype antibodies have been characterized by immunoprecipitation and polyacrylamide gel electrophoresis. The receptor has an apparent molecular weight of 85-95 kilodaltons. When the molecule is chemically reduced, it has been shown to be composed of two chains, an α subunit with a molecular weight 40-45 kilodaltons and a β subunit, molecular weight 42-44 kilodaltons (109). Differences in the distance of migration in the gel was consistently greater for the β chain than the α chain. This distinction is probably due to the existence of only one intramolecular disulfide bond in the α chain, and two in the β chain.

Isoelectric focussing of the murine T cell receptor under equilibrium and non-equilbrium conditons has shown the α chain to have an acidic isoelectric point, and the β chains to be slightly basic (110). Peptide fingerprint analysis has shown the two subunits are composed of variable and constant regions, similar to immunoglobulin heavy and light chains (111). These studies could not provide a primary amino acid structure because of difficulty in preparing sufficient amounts of purified receptor proteins.

The overall molecular weight and charge properties of the murine receptor are similar to those described for the polypeptides which make up the receptor on human T cell lines and tumors (112). The α chain in man, however, appears to have a slightly higher molecular weight than the β chain.

Structural analysis of the molecules identified by these anti-clonotype antibodies could have led to the cloning of the T cell receptor genes. DNA segments with sequences corresponding to T cell receptor protein sequences could have been synthesized and used as probes for identifying the genes. However, another approach to isolating the T cell receptor was made possible with the advent of DNA technology. The most successful of these approaches to isolating the receptor was devised by Davis et al.(113), where T cell mRNA was isolated and hybridized with the cDNA of B cell mRNA, to yield unique mRNA from T cells. It is important to note that this mRNA would have included in its pool, along with the T cell receptor message, mRNA encoding other unique T cell differentiation Ags.

A series of assumptions were made which led to the development of their strategy. The first of these assumptions is that the mRNA of question will be expressed only in T cells and not in B cells. This could have been false if T cells share variable regions of their receptor molecule with those of B cells, and provided sufficient mRNA homology to allow hybridization to occur. A second assumption is that the genes encoding the T cell receptor exhibit a high degree of diversity, such as B cells use to increase their Ag recognition repertoire. The possibility exists that some other mechanism is used to generate the diversity seen in T cells. They further reasoned that the mRNAs for the T cell receptor protein should be found on membrane-bound polysomes, as one would expect a nascent receptor polypeptide to attach to the endoplasmic reticulum by a leader peptide.

Membrane-bound polysomal RNA of antigen-specific, MHC-restricted T helper hybrydomas was used to synthesize ^{32}P-labelled cDNA. This cDNA was "subtracted" with B cell mRNA and the resulting probes were used to screen a T helper cDNA library. Positive clones were then used to identify somatic gene rearrangements in T cells. Labelled probes, prepared from

the clones, were hybridized to Southern blots of restriction digests of genomic DNA from a thymoma, a T cell hybridoma, and parental strain liver cells. One clone, TM86, was found to produce different patterns of hybridization to various digested T cell DNA preparations as compared to those DNA preparations from liver cells.

These data support the investigators' hypothesis that genomic rearrangements of a T cell receptor gene should be different for T cells of different Ag specificity or phenotype. It is also significant that hybridization of TM86 with B cell or liver cell genomic DNA exhibited no pattern of somatic rearrangement, as is seen with T cells. The investigators suggest that clone TM86 represents the β chain of the T cell receptor.

In an additional article by these authors (114), they report a close similarity of the amino acid sequences predicted by TM86 cDNA sequences to immunoglobulin variable, constant, and joining regions. This work, which was performed using a computer search of known immunoglobulin sequences, showed this similarity is much closer than that of the postulated superfamily of immunoglobulin-like molecules, which includes a wide variety of very distantly related cell-surface glycoproteins.

Tonegawa and his colleagues have reported what appears to be an α-chain gene from a murine alloreactive CTL clone, 2C (115). They used the subtraction cloning method utilized by Mark Davis to isolate the β chain. Determinantion of the nucleotide sequences of the α and β clones has shown them to be approximately 70% different; however, both the α and β chain sequences exhibit homology to immunoglobulins. Both chains have cysteine residues just outside a transmembrane region that might form a disulfide bond that holds them together. Tonegawa suggests the α chain binds the MHC antigens, and the β chain binds foreign antigen. The proteins corresponding to the cDNA clones have a molecular weight of approximately 32,000

for the peptides only. This is consistent with those determined for the membrane proteins, which include a carbohydrate portion in addition to their peptide backbones.

In comparing the different strategies to isolate the receptor, it appears that determinantion of the primary structure of the T cell receptor has proceeded more rapidly by isolating and sequencing nucleic acids produced in Ag-specific T cells. Eventually, it should be possible to study the function of the T cell receptor proteins by transferring the cloned genes into cells and seeing how this affects their specificity for antigen and other T cell activities. However, the actual binding activity, subunit structure of the receptor protein, as well as its assembly attachment to the membrane, and the rate of turnover during different states of activation of the T cell, can only be determined at the protein level. For this purpose, the combination of strategies utilizing anti-clonotypic monoclonal Abs will be very important in answering many of these questions.

B. Generation of Anti-clonotype Antibodies

Two series of immunizations were tried in order to generate B cell hybridomas secreting antibodies specific for one of the panel of monoclonal T cells. One group of CB6/F_1 mice were immunized with the syngeneic KLH specific T cell line from which all the T cell clones were derived. The hybridomas derived from this fusion were designated as either S or L to indicate whether they were the product of a fusion of spleen cells or lymph node cells from the immunized animal. The second group of CB6/F_1 mice were immunized with a semisyngeneic T cell hybridoma FN1-18 (AKRxCB6/F_1). The initial screening for antibodies in the culture supernatants was determined by indirect immunofluorescence staining techniques and indirect radioimmunoassay. T cell clones were incubated for 45 min with culture supernatant,

TABLE XXVI

BINDING SPECIFICITY OF SYNGENEIC MONOCLONAL ANTI-ID ANTIBODIES

Monoclonal antibodies	KLH Specific Hybridomas			KLH Specific Clones					Miscellaneous		
	FN1-18	FN13-21	SW2.3	A12.11	C1.3	D18	F6	BW5147	$CB6F_1$ OVA-Specific T Line	$CB6/F_1$ Thymocytes	SP2/0
nil	226±92*	312±72	417±52	689±168	414±28	588±61	433±93	387±68	704±139	1,215±352	306±18
3D 11.12 (Thy1.2)	33,954±815	27,087±731	21,822±269	13,489±957	10,046±218	16,802±133	9,585±202	1,177±390	8,886±178	7,780±64	978±154
NEI.017 (Lyt1.2)	17,948±43	14,402±453	10,655±235	21,312±391	17,948±20	19,578±391	16,747±520	888±186	10,738±203	3,263±105	788±302
NEI.006 (Lyt2.3)	1,293±471	1,539±338	933±32	879±36	647±38	470±212	919±67	678±218	3,230±133	3,028±94	568±100
10.2-16 ($I\alpha^k$)	353±76	776±194	594±139	643±191	843±77	827±129	732±90	639±25	460±31	1,278±57	508±92
S3a.6-18 (KLH T Line)	10,303±868	1,011±344	947±68	9,544±241	544±140	831±123	831±241	897±11	976±77	1,482±126	970±136
2050.21 (FN1-18)	7,944±391	968±50	427±168	499±19	899±270	579±70	406±162	939±56	783±136	1,669±81	338±194
L9.3	1,927±160	1,620±220	1,718±153	759±106	903±129	464±102	564±119	893±134	804±100	1,674±43	543±85

*cpm mean of duplicate ± SD

washed, and stained with fluoresceinated goat anti-mouse Ig. A fluorescence-activated cell sorter was used to determine the percent of positive stained cells. The hybridomas secreting monoclonal antibodies specific for a single T cell clone or to a few T cell clones or hybridoma were selected and cloned by limiting dilution cultures. The specificity of the B cell monoclonals obtained through cloning were studied in an RIA, in which their binding to T cell clones was measured using an ^{125}I labeled rat anti-murine Kappa Ig monoclonal antibody. Those clones secreting monoclonal antibodies specific for T cells were typed for immunoglobulin light and heavy chain isotypes and expanded in ascites.

C. Binding Specificity of Anti-clonotype Antibodies

Both immunization protocols yielded B cell fusion products secreting antibodies specific for T cells. The binding specificity of the various monoclonal antibodies was tested against T cell clones or hybridoma from our panel which recognize KLH in association with various MHC encoded class II determinants. The binding of these antibodies was studied in an RIA using a ^{125}I-labeled rat anti-murine Kappa monoclonal antibody for the second stage reagent. The data illustrated in Table XXVI show the binding of two antibodies S3a.6-18 and L9-3 derived from the syngeneic immunization, and one antibody SW2050.21 derived from the semisyngeneic immunization. The monoclonal antibody s3a.6-18 binds only to the T cell clone A12.11 and T hybridoma FN1-18. Antibody L9-3 displays a low level to all the T cell hybridoma tested, and no binding to the T cell clones. Antibody SW2050.21 is specific for only hybridoma FN1-18. The lack of binding by any of these antibodies to the BW5147 (AKR) lymphoma line, the CB6/F_1 derived Ova specific T cell line, or CB6/F_1, thymocytes, suggest that these antibodies interact with a receptor protein. These findings exclude the possibility that

TABLE XXVII

INHIBITION OF BINDING OF ^{125}I-LABELED MONOCLONAL ANTI-CLONOTYPE ANTIBODIES TO F1-18 HYBRIDOMA BY COLD ANTIBODIES

Incubation with unlabeled antibodies	^{125}I-antibody	cpm*
nil	S3a-6-18	3,590±168
S3a-6-18	S3a-6-18	897±226
SW2050-21	S3a-6-18	4,030±378
nil	SW2050-21	4,960±320
S3a-6-18	SW2050-21	4,110±440
SW2050.21	SW2050-21	1,310±292

*cpm = mean ± SD of triplicate.

10^5 FN1-18 cells were incubated for 2 hrs at 26° with cold MAB at 100/µg/ml, washed, and then incubated additional 2 hrs with ^{125}I-labeled antibody.

these antibodies react with other T cell antigens such as T300, LAF-1, L3T4, L3T4, T4 or gp54 protein (data not shown). Therefore, these data suggested that the syngeneic s3a.6-18 monoclonal antibody obtained from a CB6F1 mouse immunized with a KLH specific line, recognizes antigenic determinants shared by only A12.11 and FN1-18 T cell clones. This data also indicates that antibodies s3a.6-18 and SW2050.21 recognize two different epitopes, and that the KLH specific T cell clone A12.11 expresses both of the epitopes.

This interpretation of binding results was supported by cross-inhibition experiments. The data presented in Table XXVII show that the preincubation of cells with cold 2050.21 inhibited only the binding of labeled 2050.21.

D. Effect of Anti-clonotypic Antibodies on the KLH Induced Proliferative Response of T Cell Clones

The ability of anti-receptor antibodies to block the binding of ligand have been described in a number of hormonal systems (116). To demonstrate that the monoclonal antibodies in our system interact with the T cell receptor, we test the ability of these antibodies to inhibit the activation of three T cell clones in the presence of KLH. The experiment shown in Fig 5 illustrates that antibody s3a.6-18 can inhibit the antigen induced proliferation of T cell clone A12.11. In addition, the specificity of this inhibition was shown by the lack of inhibition with clone D18 and C1.3. This data correlated with the binding data illustrated in Table XXVI. The inhibitory effect was dependent on the concentration of antibody added to the culture. Concentration as low as 1.0 µg/ml showed partial inhibition of proliferation to antigen.

Since the T cell clone A12.11 exhibited an allospecificity for H-2^k, in addition to its KLH specificity, antibody S3a.6-18 was tested for its ability to inhibit the proliferation of A12.11 in the presence of C3H/He (H-2^k) stimulator cells. 50 µg/ml of antibody inhibited greater than 80%

Figure 5

The effect of an anti-clonotype on the KLH induced proliferative response of three T cell clones. $2x10^4$ monoclonal T cells were cultured with 10^6 syngeneic spleen cells in the presence of KLH (40 μg/ml) and various concentrations of monoclonal antibodies. (Δ-Δ) S3a.2-18, isotype γ2a, (o—o) L9-3, isotype γ2a, and (□-□) MOPC104E, isotype μ. Panel a=clone D18; b=clone C1.3; c=clone A12.11.

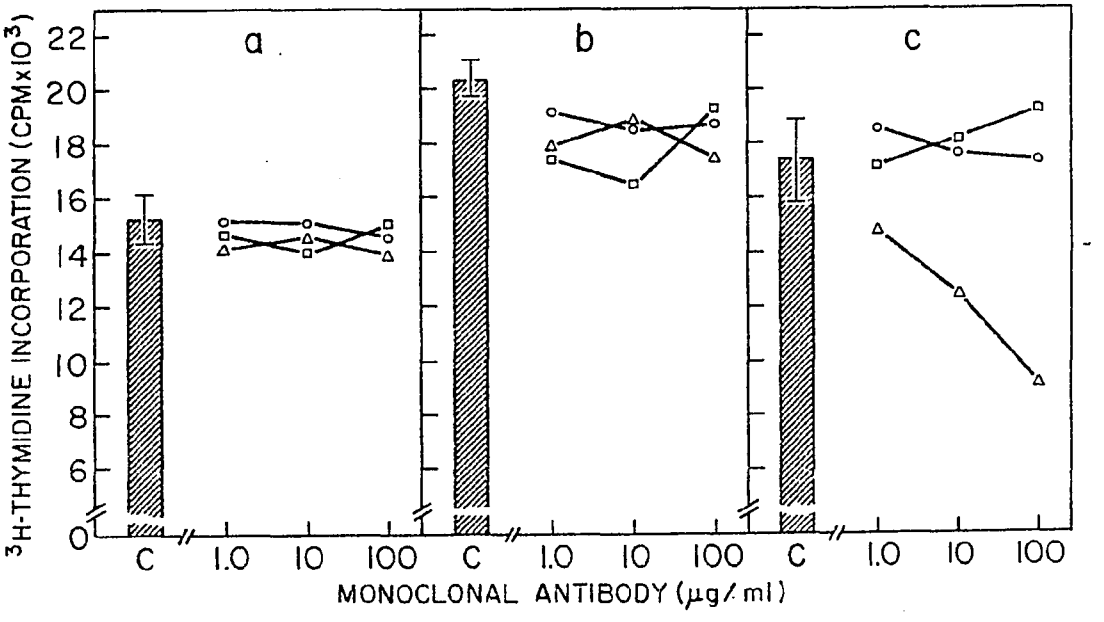


TABLE XXVIII

EFFECT OF ANTI-Id ANTIBODIES ON THE PROLIFERATIVE RESPONSE OF A12.11 T CELL CLONE

Antibody added to the culture	Specificity	A12.11	CB6/F_1+KLH*	C3H/HeJ
nil	-	-	130±34**	90±8
nil	-	+	19,588±2,699	24,636±386
S3a.6-18	KLH T clone	+	3,343±1,367	3,450±824
L9-3	KLH T clone	+	20,207±351	22,611±487
UPC-10	β2-1 fructosan	+	17,261±1,517	22,399±780
76-42	anti(anti-A48Id)	+	19,275±2,110	18,912±319

*Cultures contained Iscove's serum free medium supplemented with 1% purified monoclonal antibodies.

**Tritiated thymidine incorporation of triplicate cultures presented in cpm ± S.D.

of the allospecific T cell proliferation (Table XXVIII). This data supports the hypothesis that antibody s3a.6-18 is specific for the antigen receptor on clone A12.11. Also, the fact that antibody s3a.6-18 can block the activation both by antigen and self-Ia, and by allogeneic Ia, strongly suggests that both these determinants can be recognized by a single receptor molecule on clone A12.11.

E. Effect of Anti-clonotype Antibodies on the Anti-TNP Response

In this series of experiments, different concentrations of antibody s3a.6-18 were added to $2x10^5$ T cell clones cultured with $2x10^6$ TNP-Ova primed B cells in the presence of TNP-KLH antigen. Concentration of s2a.6-18 as low as 0.01 μg/ml could inhibit 90% of the plaques as compared with the controls (Fig 6).

This inhibition of T cell-B cell cooperation, was specific for T cell clone A12.11, and did not inhibit clones D18 or C1.3. The addition of antibodies to the cultures with the same ($\gamma 2\alpha$) or different (μ) isotypes did not effect the generation of anti-TNP plaques. This data support at the level of T cell function, that the specificity of antibody s3a.6-18 is for the antigen T cell receptor of clone AR2.11.

F. Effect of Antiidiotype Antibodies on IL-2 Production of Hybridoma FN1-18

In further experiments, we have investigated the effect of these two monoclonal antibodies on the ability to FN1-18 cells to produce IL-2 subsequent to in vitro stimulation with KLH in the presence of $CB6/F_1$ cells. The data depicted in Table XXIX shows that among anti-Id, and anti-I-E^k or anti-I-A monoclonal antibodies, only s3a.6-18 and 17-3-3s caused the inhibition of IL-2 production. 2050.21 which bind to FN1-18 lacked to inhibit IL-2 production, while s3a.6-18 is an IgG2a, 2050.21 is an IgM. Because of lack of the inhibition of 2050.21 compared to s3a.6-18, we

Figure 6

Effect of anti-clonotype antibodies on the anti-TNP response. $2x10^5$ monoclonal T cells were cultured with $2x10^6$ TNP-OVA primed B cells in the presence of TNP-KLH and various concentrations of monoclonal antibodies. (Δ–Δ) S3a.2-18, isotype γ2a, (o—o) L9-3, isotype γ2a, and (□—□) MOPC104E, isotype μ. Panel a=clone D18; b=clone C1.3; c=clone A12.11.

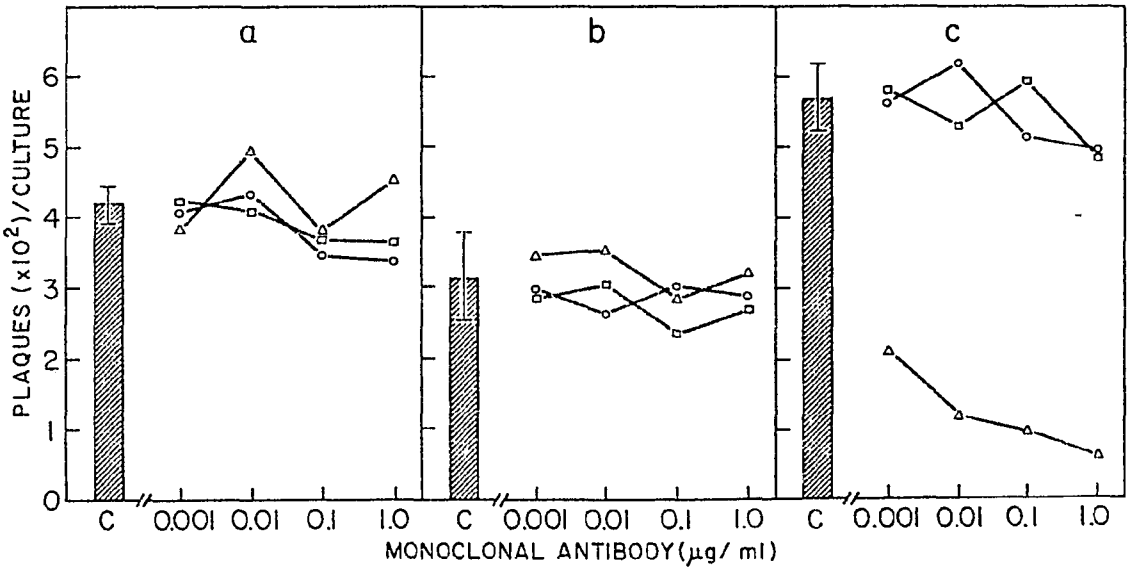


TABLE XXIX

EFFECT OF MONOCLONAL ANTIBODIES ON IL-2 PRODUCTION BY FN1-18 CELLS SUBSEQUENT TO KLH STIMULATION

Monoclonal antibodies	Concentration (μg/ml) 10	1	0.1	0.01	0.001
anti-I-E^k	37*	30	24	20	30
anti-I-A^d	163	172	224	154	152
anti-I-A^b	204	192	193	170	184
MOPC104E	194	156	198	189	169
S3a-6-18	54	66	48	84	126
S3a-2-18	169	166	142	207	174
SW2011.3	231	173	168	203	183
SW2050.21	149	218	220	191	140
SW2056.13	228	236	210	201	237

*IL-2 units

addressed the question of whether or not the APC cells can play a role in the presentation or focussing of anti-Id to T cells. We treated the presenting cells for two hours with an antibody specific for the Fc receptor, and used these treated cells in a second group of IL-2 inhibition experiments with SW2050.21 and s3a.6-18. The treatment with anti-Fc antibodies did not alter the inhibition results. This experiment was attempted in order to test whether antibody bound to cell surfaces through its Fc portion could alter its inhibition activity.

G. Discussion

This series of experiments characterize two monoclonal antibodies directed against monoclonal T cell populations. Antibody SW2050.21 exhibits a clonotypic binding to T cell hybridoma FN1-18; however, this antibody does not inhibit the secretion of IL-2 by FN1-18 in the presence of KLH and antigen presenting cells. Antibody s3a.6-18 inhibits both the KLH specific and allospecific proliferation of T clone A12.11. This antibody also inhibits the ability of A12.11 to cooperate with TNP-primed B cells to generate anti-TNP plaques. Taken together, these results suggest that antibody s3a.6-18 recognizes the antigen receptor on clone A12.11.

Antibody s3a.6-18 has played an important role in helping to define the molecular nature of the T cell receptor on clones exhibiting a double specificity. Two major hypothesis can be entertained to explain the ability of a single T cell to recognize foreign antigens in association with syngeneic MHC encoded determinants and to proliferate in response to allogeneic Ia antigens: a) a certain degree of mimicry between the alloantigens and the neoantigens created by the association of foreign antigen with the syngeneic MHC determinants and b) that T cells carry two receptors with different recognition specificities.

The first hypothesis is supported by a recent finding demonstrating the inhibition of an alloreactive (H-2^d) T cell hybridoma by a synthetic polypeptide antigen (41). Our results are also congruent to the first hypothesis: the incubation of A12.11 subclone with anti-I-A^d inhibited the KLH proliferative response in the presence of BALB/c APC and the anti-I-A^k antibody inhibited the proliferative response induced by C3H/HeJ stimulating cells. The anti-clonotype antibody s3a.6-18 inhibited both responses. Thus, when anti-I-A antibodies were added to the cultures, they masked I-A^d determinants crucial for KLH induced proliferative response or I-A^k determinants required for alloproliferative response. In contrast, the anti-clonotypic antibody s3a.6-18 subsequent to the binding to T cell receptor prevented the recognition and triggering of the proliferation by KLH plus I-A^d or I-A^k allogeneic cells. Therefore, our data strongly favor a single receptor hypothesis and a mimicry between antigen plus self and alloantigens. However, the inability of anti-I-A^k antibody to inhibit KLH induced proliferation and of anti-I-A^d antibody to inhibit allogeneic proliferation suggest only a certain degree of mimicry since the alloantigens are only "infidele" copies or "internal image" of neoantigens created by association of foreign antigens with self MHC encoded determinants. It should be mentioned that our experiments do not formally rule out the two receptors hypothesis since it is possible to imagine that V genes encoding for recognition of KLH plus I-A^d and I-A^k share common clonotypic determinant or that our anti-clonotypic antibody was specific for the product of DNA segment encoding for constant region of T cell receptor (113).

The study using the two monoclonal antibodies which bind hybridoma FN1-18 suggests that the T cell receptor of these cells bears several

antigenic determinants: One determinant is unique for the FN1-18 hybridoma and another determinant recognized by antibody s3a.6-18 is shared with the KLH specific T cell clone A12.11. The molecular data on the organization of genes encoding for the α and β chain of the T cell receptor has revealed a striking similarity to genes encoding for immunoglobulins. From this data, it can be predicted that the idiotypes of the T cell receptor will be very similar to the idiotypic system of immunoglobulin molecules.

Utilization of monoclonal T cell populations as immunogenes to produce syngeneic monoclonal antiidiotype antibodies will allow the analysis of domains on the T cell receptor molecule involved in foreign antigen binding and MHC binding. These reagent can also provide insight into the rate of turnover of the receptor during different states of T cell activation. In addition, these reagents will be useful in studying the expression of cloned genes which are transferred into other lymphoid cell types.

X. APPENDIX: ABBREVIATIONS

MHC = major histocompatibility complex

T cell = Thymus derived lymphocyte

KLH = Keyhole limpet hemocyanin

Ova = Ovalbumin

TNP = Trinitrophenyl

BUdR = 5-bromo-deoxy uridine

HGPRT = Hypoxanthine quanine phosphoribosyltransferase

FACS = Fluorescence activated cell sorter

MLR = mixed lymphocyte response

PFC = Plaque forming cell

APC = Antigen presenting cells

SRBC = Sheep Red Blood Cells

RI = recombinant inbred

PEG = polyethylene glycol

HAT = medium containing hypoxanthine, aminopterin and thymidine

H-2 = major histocompatibility complex of the mouse

RIA = radioimmunoassay

PBS = phosphate buffered saline

SP 2/0 = a myeloma cell line of BALB/c origin

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