IDIOTYPIC NETWORK INTERACTIONS, AUTOIMMUNITY AND THE PATHOGENESIS OF AIDS

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Abstract.

The functional disruption of the immune system in acquired immunodeficiency syndrome (AIDS) is incompletely understood. Many clinical features are common to AIDS and *Systemic lupus erythematosus* (SLE), and it is plausible that autoimmunity is involved in the pathogenesis of AIDS. The immune response directed against human immunodeficiency virus (HIV) and concomitant anti-idiotypic response may cross-react with host cell antigens and result in the depletion of certain T cell populations.

A model of AIDS pathogenesis has emerged from a version of idiotypic network theory with sharply defined molecular and cellular mechanisms. In this model, the network is postulated to be able to select clones that have variable (V) regions that are complementary to MHC molecules and clones that have V regions that are images of MHC molecules. A central part of the regulatory T cell repertoire consists of anti-class II MHC helper T cells and class II MHC-image suppressor T cells. The interactions between these two T cell populations are expected to be mutually stabilizing. The model includes a role for allogeneic stimuli as cofactors. The immune response to allogeneic lymphocytes includes a MHC-image response, which is directed against the idiotypes on the foreign receptors that recognize self. The immune response to the MHC-mimicking portions of HIV includes an anti-MHC-image response. MHC-image and anti-MHC-image responses are directed against each other (by definition), and may synergize with each other in such a way to lead to the eventual collapse of the normally self-stabilizing immune system network.

In this thesis, several findings that can be understood in the context of that model are described. These findings would otherwise have not been expected. Alloimmune mice make antibodies that bind gp120 and p24 of HIV, and autoimmune MRL-*lpr/lpr* and MRL-+/+ mice spontaneously make anti-gp120 antibodies. Anti-gp120 antibodies are interpreted as being anti-MHC-image since gp120 is considered to be an image of class II MHC antigens (based on its complementary to the CD4 antigen of T lymphocytes). MHC-image antibodies are typically produced during alloimmunization, and MRL mice were found to spontaneously make MHC-image antibodies. MHC-image antibodies can be detected by the inhibition of alloantibody (and complement) mediated cytotoxicity, and by enzyme-linked immunosorbant assay (ELISA) with polyclonal antisera and monoclonal antibodies. Injection of young MRL-*lpr/lpr* mice with low doses of gp120 or p24 of HIV suppressed the production of autoantibodies and enhanced the survival of these mice. These results support the idea that MHC-image and anti-MHC-image antibodies may be involved in the pathogenesis of lupus in these mice. The presence of both MHC-image and anti-MHC-image antibodies in alloimmunity suggests that synergy between immune responses to allogeneic cells and HIV is important in some groups that are at risk for AIDS.

Table of Contents

List of Figures.

List of Tables

List of Abbreviations

Abbreviations	Meaning
A cells	accessory cells (eg. macrophage)
Abs	antibodies
AIA	alloimmune, immunogen-absorbed
AIDS	acquired immunodeficiency syndrome
BMRC	bromolein-treated syngeneic mouse erythrocytes
BSA	bovine serum albumin
CTL	cytotoxic T lymphocytes
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EAE	experimental allergic encephalitis
ELISA	enzyme linked immunosorbant assay
Fab	antigen binding fragment of immunoglobulin
GVH	graft versus host
Hb	hemoglobulin
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HVG	host versus graft
Ig	immunoglobulin
IL	interleukin
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
MBP	myelin basic protein
MHC	major histocompatibility complex
$PBST_{20}$	phosphate buffered saline with 0.05% Tween 20
PC	phosphorylcholine
RA	rheumatoid arthritis
RF	rheumatoid factor
SLE	*Systemic Lupus Erythematosus*
SRBC	sheep red blood cells

Abbreviations	Meaning
ssDNA	single-stranded DNA
TG	thyroglobulin
V	variable

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Chapter 1. Network Models.

1.1. Introduction.

The immune system of the vertebrate organism is composed of many organs and tissues. Lymphocytes and monocytes, the cells which are involved in immune responses, move through these organs and tissues by way of blood and lymphatic vessels. The immune system functions to protect the host from pathogenic microorganisms, cancerous cells and cells perceived as altered. The immune system can respond to a diverse set of antigens, and generate effector mechanisms to elicit elimination of antigens. These effector mechanisms include nonadaptive responses such as phagocytosis by macrophages and cell lysis by natural killer cells. Adaptive immunity includes both antibody-mediated and cell-mediated responses which display specificity and memory. The immune system does not normally develop these types of immune responses to self components, which indicates the immune system is able to discriminate self from non-self. Burnet's clonal selection theory of self-tolerance suggested that self-reactive clones are purged from the body during ontogeny (ref. 1, p. 59). This part of Burnet's theory is known to be incorrect.

Although foreign antigens usually induce the proliferation and differentiation of effector B and T lymphocytes, and self antigens usually induce tolerance, this distinction in not absolute. Tolerance to foreign antigens can be induced in certain circumstances, and lymphocytes and antibodies to self-antigens can be demonstrated in normal individuals and in many autoimmune diseases.

The immune system must be able to regulate self-reactivity and immune responses to foreign antigens. A dysfunction in immunoregulation can result in the development of autoimmune diseases, cancers, or the failure to remove disease-causing microorganisms. The mechanisms of immunoregulation are not fully understood. Several models of immunoregulation have been proposed, but none have been universally accepted. Network theories of immunoregulation are based on the interactions between the variable (V) regions (idiotypes) of the antigen receptors of B and T lymphocytes and of secreted molecules. The V regions recognize both foreign antigens and each other, and therefore may be involved in the regulation of immune responses.

The V regions of immunoglobulins have two different structures that are important in immunoregulation. The site on the V region that is involved in the binding of antigen is called the paratope. The unique antigenic determinants generated by the hypervariable regions on the heavy and light chains are the idiotopes. Idiotopic determinants can be located within or adjacent to the antigen binding site or may be located on the framework residues of the V region. The set of idiotopes on a particular V region is known as its idiotype.

Kunkel et al.[2], and Oudin and Michel[3] described the set of unique antigenic determinants on the V regions of antibodies produced during the immune response to an antigen. Oudin used the word "idiotype" (*idios*, one's own) to describe the peculiarities of the antigenic determinants[4]. These antigenic determinants were not found on antibodies to other antigens or on antibodies from different animals[2,3,5,6]. Experiments with enzyme digested antibodies and anti-idiotypic antiserum further localized the antigenic

differences (idiotypes) to the V regions of the antibodies[2]. The experiments demonstrated that the individual subject produces only a fraction of all possible idiotypes that can be produced by a given species. Oudin and Cazenave later showed that antibodies to different determinants on an antigen, and different classes of antibodies can share idiotypic specificities as defined by reactivity with an anti-idiotypic antiserum[7]. The former phenomenon became known as the "Oudin-Cazenave enigma", since idiotypes had been defined as the antigenic determinants which were unique to antibodies of a particular specificity[2,3,5,6]. In order to account for this phenomenon, Jerne postulated that there are both paratopic and idiotopic repertoires, and different antibodies may overlap with respect to the idiotopic specificities without overlapping with respect to the paratopic specificities (antigen binding sites), and therefore, be recognized by the same anti-idiotypic antiserum[8].

1.2. Network Theory.

1.2.1. Jerne's model.

Jerne's network theory of regulation describes the immune system as a network of interconnecting V regions distributed on lymphocytes as antigen receptors and on soluble antibody molecules[8]. Jerne postulated that the V regions of antibodies and antigen binding receptors on lymphocytes can both recognize antigen and be recognized as an antigen with varying degrees of precision[8]. Antibodies that are directed against an antigenic determinant of an external antigen have idiotopes that are recognized by an

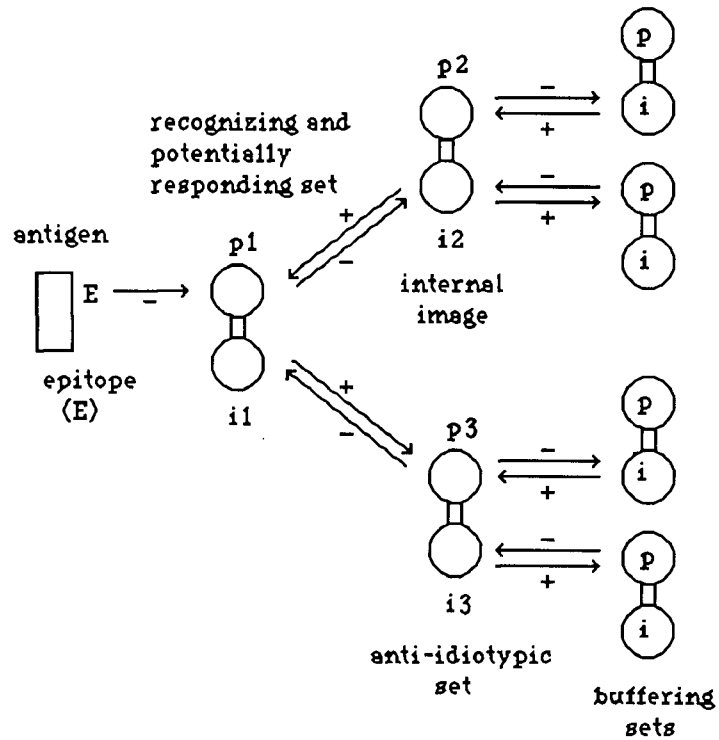
anti-idiotypic set of antibodies and have paratopes (antigen binding sites) that recognize an internal image of the antigen (also an antibody) (Figure 1). The idiotopes of the internal image may mimic the epitopes on the antigen, since both interact with the antigen binding site of the antibody. These ideas were supported by the experiments of Sirisinha and Eisen[9], and Rodkey[10] who demonstrated that an individual can make antibodies directed against the V regions of isologous or autologous antibodies.

Jerne's model stressed the occurrence of numerous dualisms within the immune system; for example, the dichotomy of B and T lymphocytes, the idea that an antibody can recognize an antigen and be recognized as an antigen, and the ability of antigen-sensitive lymphocytes to respond either positively or negatively to a recognition signal[8]. Jerne's model also stressed the importance of suppression. He postulated that the influence of an anti-idiotype on an idiotype tends to be suppressive[8]. He suggested the introduction of antigen into the system removes the naturally occuring antibody (idiotype) directed against it from the system. The elimination of the idiotype removes the stimulatory effect that idiotype has on the anti-idiotypic set. The cells that produce the antibody (idiotype) are then no longer suppressed by the anti-idiotypic set and are able to respond to the antigen (Figure 2). Furthermore, the elimination of the idiotype (after the introduction of antigen) removes the suppressive effect that the idiotype has on the internal image set. The internal image set can then stimulate the idiotypic set. The production of the idiotype eventually stimulates the production of the anti-idiotypic set, and the response of the idiotypic set can be down-regulated by the anti-idiotypic set. Jerne's model is unique among network models in that the introduction of antigen into the system initally

Figure 1. Interactions between antigens, idiotypes, internal images and anti-idiotypes in Jerne's network model.

The immunoglobulin molecule or antigen receptor has a combining site (paratope, p) which recognizes an epitope (E) on an antigen. The same immunoglobulin molecule or antigen receptor also displays a set of idiotopes (idiotype, i) which are recognized by the paratopes on another immunoglobulin molecule or antigen receptor known as the "anti-idiotypic" set. The "internal image" set displays a set of idiotopes which are recognized by the paratopes on the original immunoglobulin molecule or antigen receptor, and therefore, mimics the epitope on the antigen. The interactions between the idiotypic and anti-idiotypic set are asymmetric. For example, the anti-idiotypic set suppresses the idiotypic set, whereas the idiotypic set stimulates the anti-idiotypic set.

Adapted from Jerne, N. K. "Towards a Network Theory of the Immune System", *Ann. Immunol. (Inst. Pasteur)*, 125 C, 373-389, 1974 (reference 8).



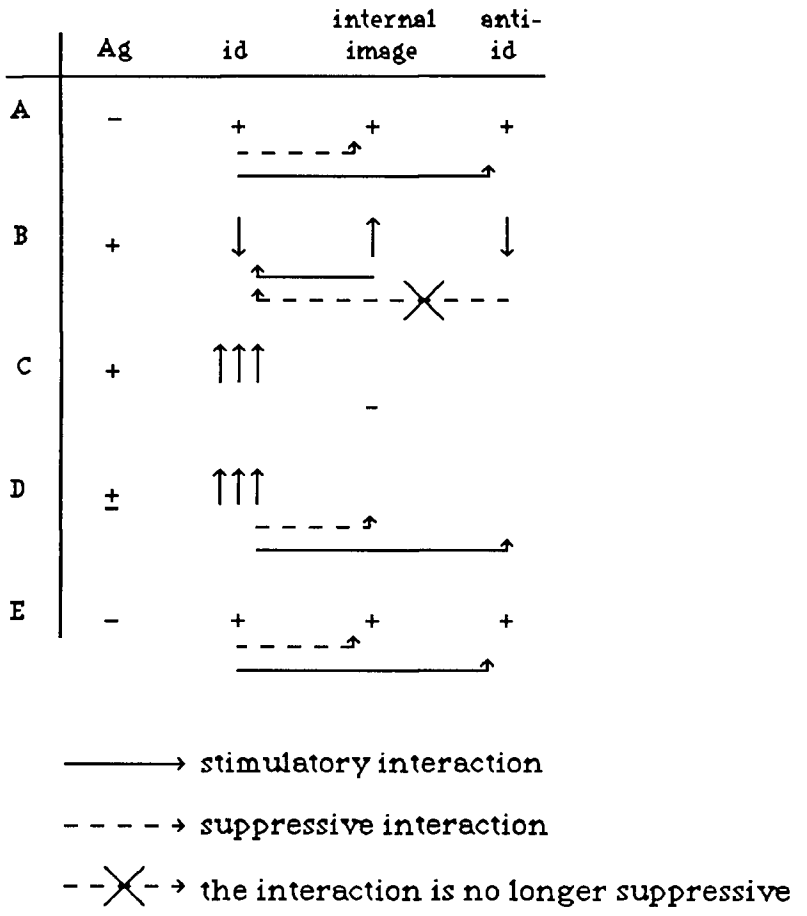
has a negative effect on the immune system (the removal of idiotype) rather than a direct stimulatory effect.

1.2.2. Richter's model.

Richter's network theory is based on the interactions between the antigenic determinants of antibodies and antigen receptors on lymphocytes as suggested by Jerne. As in Jerne's model, Richter's model features asymmetric interactions[11]. Richter's intent was to show that a small number of interactions within the immune system could be used to explain some basic dynamical phenonmena. He did not consider immunoglobulin classes nor the dichotomy of B and T lymphocytes in his network model. He postulated a chain of interactions in which antigen stimulation of one class of lymphocytes [idiotypes, "antibody-1" (Ab-1)] stimulates a second class of lymphocytes (anti-idiotypes, "Ab-2") which can stimulate a third set (anti-anti-idiotypes, "Ab-3") and so on (Figure 3). Anti-idiotypic clones can suppress the idiotypic clone and inhibit stimulation by antigen by blocking the receptors with antibody. A negative feedback mechanism was postulated to control the extent of the chain reaction and provide some stability for the immune system[11]. He had stimulation in the forward direction (Ab-1 → Ab-2 → Ab-3) and killing in the reverse direction (Ab-3 → Ab-2 → Ab-1). The state of immunity or tolerance is dependent on how far the antigen stimulates the chain of clones. Richter's model also introduces the concept of thresholds. For example, if the antigen stimulates only the first and second levels of clones, then low zone tolerance would result in which Ab-2 has reached the threshold level at which it suppresses Ab-1, but has not reached

Figure 2. The response of the idiotypic network after the introduction of antigen according to Jerne (8). (These ideas have not been supported by mathematical modelling).

When the immune system is in the virgin state (A), the idiotype is suppressing the internal image set and stimulating the anti-idiotypic set. When antigen is introduced into the system (B), the idiotype is removed from the system. The internal image set is no longer suppressed by the idiotype and the anti-idiotypic set is no longer stimulated by the idiotype. The production of the idiotype (C) is no longer suppressed by the anti-idiotypic set. As the antigen is being cleared from the system (D), the production of the idiotype suppresses the internal image set and stimulates the anti-idiotypic set. The immune response is complete (E), and the immune system returns to a resting state, similar to the initial state.



the threshold level at which it stimulates Ab-3[11]. Stimulation of the third level of clones results in immunity (due to Ab-3 suppressing Ab-2 and Ab-1 then "escaping"). Stimulation of the fourth level of clones results in high zone tolerance (Figure 4).

1.2.3. Hoffmann's model.

Hoffmann's symmetrical (plus-minus) network theory is also based on Jerne's postulate that the V regions of antibody molecules and antigen receptors can function as antigens, and that immunoregulation involves the interaction of V regions on antibodies and antigen receptors on lymphocytes, together with some more explicit postulates about the system. This model was developed after extensive reviews of both the experimental and the theoretical literature (90, 75 and 230 references in his 1975, 1978 and 1980 papers respectively).

Hoffmann postulated that, as a first approximation, there are two sets of lymphocytes involved in the immune response to any given antigen. Both sets of lymphocytes are derived from the same populations or classes of lymphocytes; they differ only in their specificities with respect to antigen. Lymphocytes with receptors that are complementary to the antigen are referred to as positive cells (idiotype), whereas lymphocytes with receptors that are complementary to the positive cells are referred to as negative cells (anti-idiotype)[12-14]. The antigen binding site of several antibody molecules or receptors (to a particular epitope on the antigen) may interact with a restricted set of anti-idiotypes, whereas the rest of the V region of the same antibody molecules or receptors may interact with a more diverse set of anti-

Figure 3. The idiotypic network chain reaction according to Richter (11).

Antigen stimulates clone 1, which in turn stimulates clones 2 and so on. The extent of the chain reaction is limited by the thresholds of stimulation (Figure 4) and by the suppressive interactions that clone 3 has on clone 2, and so on.

Adapted from Richter, P. H., "A Network Theory of the Immune System", *Eur. J. Immunol.*, 5, 350 - 354, 1975 (reference 11).

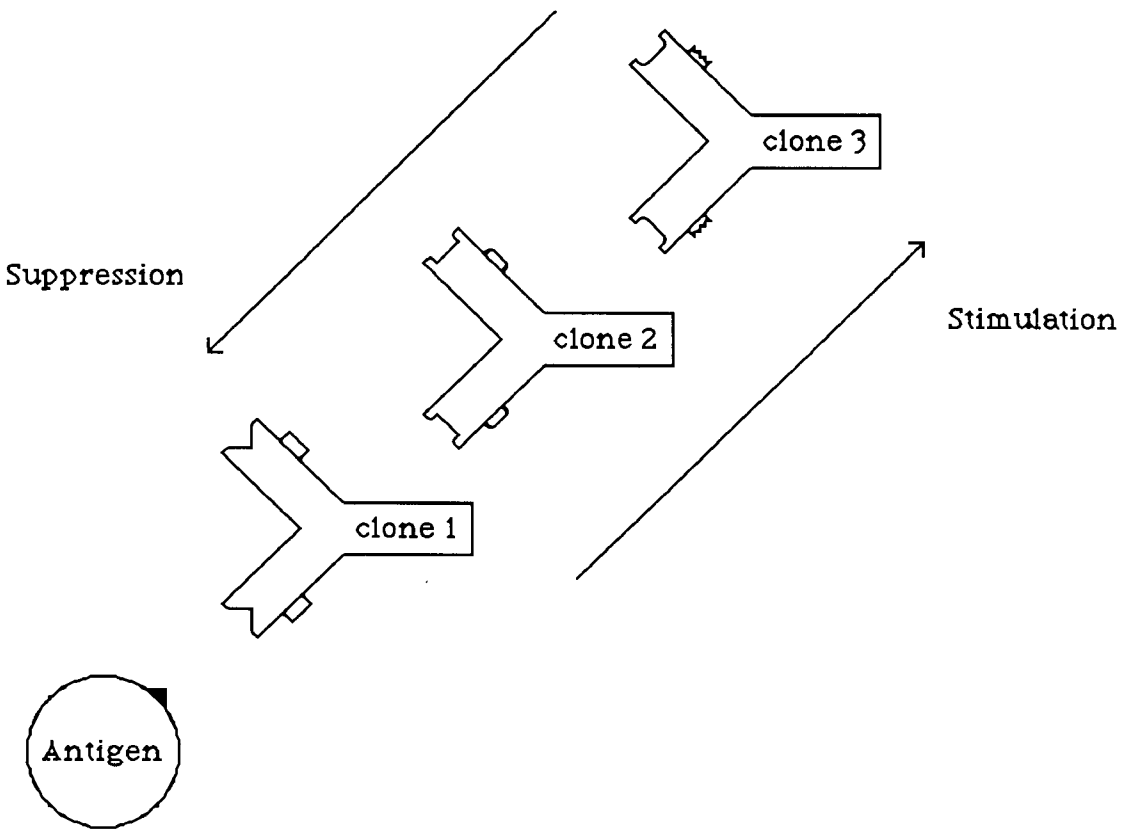


Figure 4. The concept of threshold stimulation in determining the state of the immune system in Richter's model (11).

The state of the immune system is determined by the extent of the chain reactions which result after antigen perturbation. For each level of clones, the stimulation by the antigen or the preceding clone must surpass a threshold level (ie. the concentration of the antigen or the preceding clone must be above a threshold). When the immune system is in the virgin state, the interactions between idiotypes and anti-idiotypes are below the threshold for stimulation and suppression. Low zone tolerance results when the stimulation reaches the second level of clones. The immune response occurs when the stimulation reaches the third level of clones, and high zone tolerance is the result of the stimulation of the fourth level of clones.

State of the immune system	Ag stimulation of clones (level)			
	1	2	3	4
Virgin	–	–	–	–
Low zone tolerance	↓	↑		
Immune response	↑	↓	↑	
High zone tolerance	↓	↑	↓	↑

– level below the threshold for stimulation and suppression

↑ elevated level compared with virgin state

↓ suppressed level compared with virgin state

idiotypes (Figure 5). The restricted anti-idiotypic set could be stimulated more by many different idiotypic clones that recognize the same epitope (on the antigen), whereas, the diverse set of anti-idiotypic clones could each be stimulated by fewer clones. Therefore, the antigen binding site of an antibody molecule or receptor (with respect to a particular epitope) may be more important than the rest of the V region in the regulation of the immune response to antigen[12]. Apart from this, the interactions between idiotypes and anti-idiotypes are assumed to be symmetrical, so that no fundamental distinction is made between the paratope (antigen binding site) and the idiotope (antigenic determinants). The anti-idiotypic and internal image sets of lymphocytes of Jerne's network theory are then functionally identical[12].

The cross-linking of receptors is assumed to be the mechanism of specific stimulation of both B and T lymphocytes. Lymphocytes are also assumed to be effective antigens, and can stimulate other lymphocytes by cross-linking complementary receptors. The interactions between positive and negative cells are symmetrical in the sense that whatever influence a positive cell has on a negative cell is potentially reciprocal. The interactions that could occur between complementary lymphocyte populations include stimulation (cross-linking of receptors), inhibition (blocking of receptors) and elimination (killing)[12-14]. It was further postulated that T cells are more readily triggered by antigen than B cells, and that T cells proliferate more readily in response to antigen. Although many experiments support the concept that T cell receptors recognize antigens as processed peptides within the cleft of MHC molecules (reviewed in ref. 165), Hoffmann and Grant suggest that this interpretation may not apply to the T cell repertoire

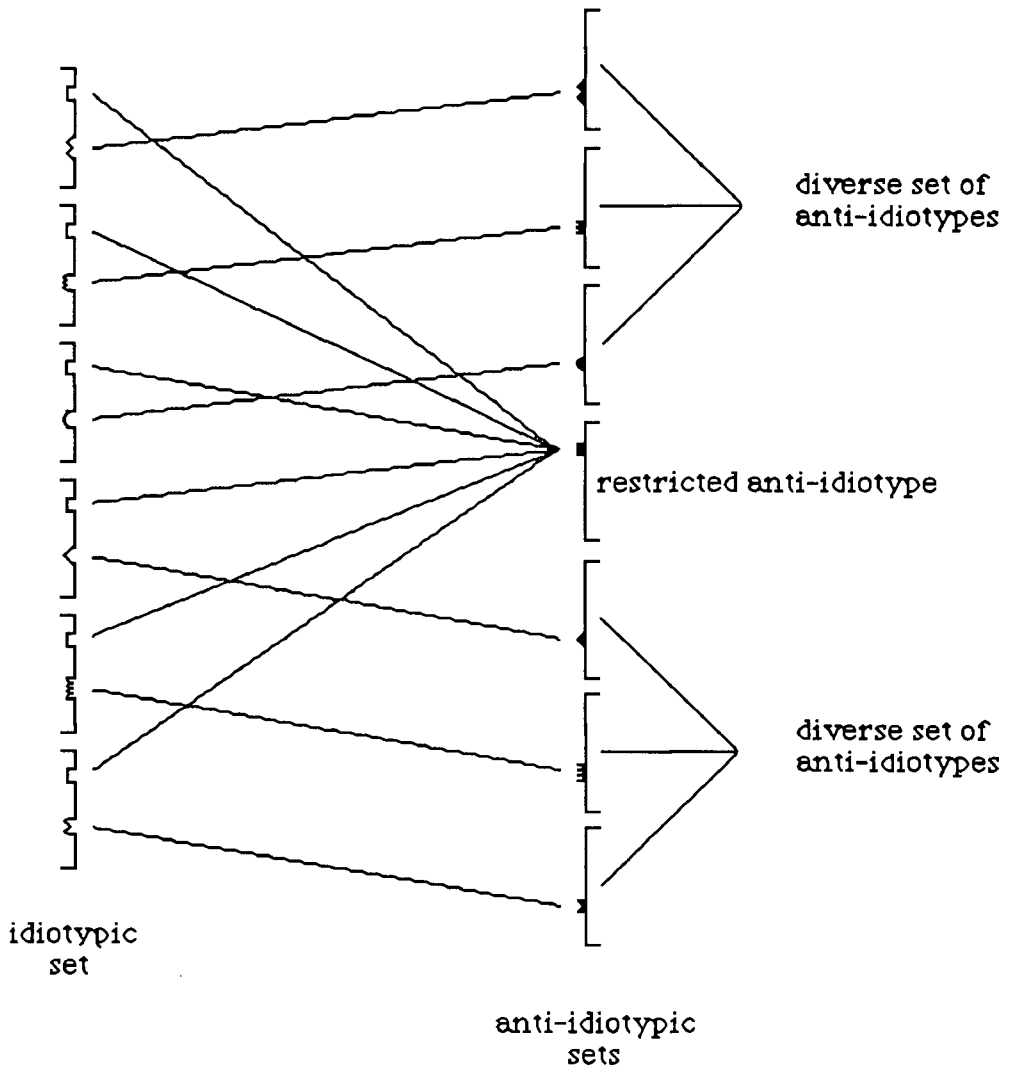
in general[42]. They suggest that these experiments typically involve secondary immune response and involve various selective processes[42]. This would include the selection of T cells in the thymus prior to the introduction of antigen, and during the primary immune response, as well as a selective process for the antigen fragments (eg. antigen fragments that fit into the cleft of a MHC molecule are protected from degradation and are presumably stimulatory for a longer period of time than antigen fragments that are not protected)[42]. Several experiments have shown that T cells can be activated by receptor cross-linking in the absence of MHC molecules. For example, T cells can be stimulated by monoclonal antibodies[166], antigens on liposomes (without MHC molecules)[167], antigens bound to polyacrylamide beads[168] and by multivalent antigens[169]. Hoffmann and Grant's theory suggests that T cells that recognize an antigen fragment in the cleft of the MHC molecule are stimulated more than T cells that recognize the antigen alone (i.e. without the involvement of MHC molecules), and that these T cells survive the selection processes during the course of the primary immune response[42].

It was also postulated that the mutual stimulation of two complementary sets could be stabilized, if at least one cell type, namely T cells, produced a specific monovalent molecule (after activation by antigen) that could block the receptors of cells of the complementary specificity[12]. These antigen-specific factors would consist of a constant region and a variable region that is identical to the V region of the T cell receptor[12].

It follows from these postulates that the immune system can have four stable states with respect to any given antigen. In the "virgin state", there is a sub-threshold amount of mutual stimulation of both positive and negative

Figure 5. Schematic drawing to show that the portion of a V region that binds the antigen is more important than the rest of the V regions in immunoregulation.

The restricted set of anti-idiotypes interacts with the antigen binding site of all the V regions that reconises a particular epitope of a given antigen, whereas the diverse set of anti-idiotypes interact with only a few of the V regions (at a site of the V region not involved in antigen binding site) in the same set. Therefore, the restricted set of anti-idiotypes likely plays a more important role in regulating these idiotypes during the immune response to the antigen.



cells. In the "immune state", there is an elevated level of positive B and T cells, and the negative B and T cells are suppressed. In the "anti-immune state", the converse is the case. In the "suppressed state", there are elevated levels of both positive and negative T cells, and the stimulated T cells produce factors continuously which can block complementary receptors on both B and T cells. (There may or may not be elevated levels of positive and negative B cells in the suppressed state)[12-14].

Specific T cell factors are assumed to be cytophilic for accessory cells (A cells) such as macrophages and bind to a receptor via the constant portion of the factor[12-14]. This leads to the idea that A cells are activated when antigen cross-links the V regions of these bound factors[12-14]. This model for A cell activation is analogous to the way that antigen and immunoglobulin E (IgE) stimulates mast cells and basophils[15]. Activated accessory cells such as macrophages secrete non-specific factors such as interleukin-1 (IL-1) which provide a second signal to allow the proliferation of T cells after stimulation by cross-linking of antigen receptors[13,14]. The activated accessory cells also secrete differentiation factors (e.g. IL-6)[16] which allow the B cells to secrete antibody after stimulation by cross-linking of antigen receptors.

Evidence for the potential symmetrical killing by idiotypic and anti-idiotypic antibodies has been obtained *in vitro* using monoclonal antibodies[17]. In non-symmetrical theories (e.g. Jerne's original version of the network theory and Richter's model), an anti-idiotypic antibody can kill an idiotype bearing target, but not vice versa[8,11]. In symmetrical network theory, an anti-idiotypic antibody can kill an idiotype-bearing cells and an idiotypic antibody can kill an anti-idiotype-bearing cells. This has been

called "first symmetry". Sheep red blood cells (SRBC) coated with phosphorylcholine (PC) or Fab fragments of an anti-T15 monoclonal antibody could be lysed by an anti-PC monoclonal antibody bearing the T15 idiotype. Likewise, SRBC coated with the Fab fragment of the anti-PC monoclonal antibody (bearing the T15 idiotype) could be lysed by the anti-T15 monoclonal antibody. In both directions, the lysis of the SRBC was dependent on the presence of complement[17]. In symmetrical network theory, the V region does not have functionally distinct parts; it is a shape that can interact with any antigen with a shape that is sufficiently complementary to any part of itself (sites on the V region). Since there are many potential contact sites on the V region, it follows that there is some degree of multispecificity for any one V region[17]. One antigen may interact with one site of the V region, and a second antigen may interact with a different site of the same V region. The multispecificity of the anti-PC monoclonal antibody could be demonstrated in the T15 system. PC-hapten could inhibit the lysis of PC-coated SRBC by the anti-PC monoclonal antibody (T15 bearing), but could not inhibit the lysis of anti-T15 Fab-coated SRBC by the same anti-PC monoclonal antibody[17]. Hence, there are different sites in the T15 idiotype for interacting with PC and anti-idiotypic antibody. The idea of multispecificity is important in symmetrical network theory, and is used to account for the phenomena of MHC-restriction and alloreactivity.

The V region of the T cell receptor is considered to be a large "sticky end" with various surface areas that are functionally equivalent. T cell receptors typically bind self MHC antigens and foreign antigens at different sites on the V region[14]. T cell receptors with V regions that are precisely complementary to self MHC molecules are not selected in the thymus, nor are

T cell receptors that lack any complementarity to self MHC molecules. Since the T cell receptor is considered to be multispecific, the T cell receptors that bind self MHC antigens and foreign antigens may also bind to allogeneic MHC antigens.

In a later section, I will discuss additional features of this model, including the role of T cells with low connectivity being helper T cells and T cells with high connectivity being suppressor T cells. An autoimmunity model of AIDS pathogenesis has also emerged from the symmetrical network theory and will likewise be described in a later section.

1.2.4. Coutinho's model.

Coutinho has suggested that the there are both "clonal" (non-network) and network organizations within the immune system[18-21]. He hypothesized that the idiotypic interactions of a putative network should be evident prior to the provocation of an immune response by (external) antigen[19]. Such autonomous activity would be inconsistent with simple clonal selection theories in which self-reactivity is forbidden; however, network theories would predict the recognition and interactions between the idiotopes and paratopes expressed on the antigen receptors of lymphocytes within the immune system[19].

In order to investigate the extent of network interactions, Coutinho and his colleagues have produced hybridomas from the spleens of normal neonatal mice and consider these hybridomas to be representative of "natural" antibodies. The monoclonal IgM antibodies produced by these hybridomas were found to display high levels of connectivity; that is, they

each interacted with several other monoclonal antibodies from the same set[18,22]. 28% of the interactions were scored as positive by an ELISA which detected the binding of one monoclonal antibody to a second monoclonal antibody immobilized on the plate[22]. Furthermore, several of these "natural" antibodies were found to be reactive to self-antigens such as cytoskeleton proteins[23]. However, hybridomas from LPS-activated spleen cells of normal adult mice did not have this high level of connectivity when screened in the same assay systems as the neonatal hybridomas[18].

"Naturally" activated B lymphocytes (i.e. large cells) from normal mice had a higher frequency of autoreactive cells to mouse thyroglobulin (TG) and bromolein-treated syngeneic mouse erythrocytes (BMRC) than did the resting B lymphocyte population (i.e. small cells). The frequency of B cells reactive to external antigens such as keyhole limpet hemocyanin (KLH), beef hemoglobin (beef Hb) and SRBC was similar in the small and large cell fractions of normal spleen[24]. "Naturally" activated T lymphocytes were found to include both T helper and T suppressor cells, but lacked T lymphocytes with cytotoxic activity (i.e. CTLs)[24].

Coutinho's model divides the normal immune system into two components, namely the "actual" and the "available" repertoires[18-21]. The "actual" repertoire consists of approximately 10% of all the lymphocytes and is postulated to embody the part that is regulated by idiotypic network interactions[18-20]. These lymphocytes are large cells that have been activated by antigens within the internal environment and display the autonomous activity of the immune system[24]. The "available" repertoire consists of the remaining 90% of lymphocytes, and are small resting cells that can be activated by external antigens. Coutinho postulates that these

cells represent a part of the clonal repertoire which is not functionally connected to the idiotypic network[18-20]. The frequency of internally activated lymphocytes has been found to be similar in antigen-free mice maintained on low molecular weight, chemically-defined diets. This suggests that the occurrence of internally activated lymphocytes in normal healthy mice is not the result of stimulation by environmental antigens[21]. Coutinho suggests that although the results do not formally prove the existance of a network, "they set the upper limit for the operation of a putative functional network"[19].

Coutinho postulated that the developing immune system of the neonatal animal starts out as a highly connected (but not completely connected) network. As the immune system matures, newly produced immunocompetent lymphocytes (from the bone marrow) that find complementary patterns (either on antibody molecules, other lymphocyte receptors or the internal milieu) are recruited into the network[25]. This set has high connectivity, and may exist throughout the lifespan of the individual as "background" activity[18]. The cells that do not find complementary partners remain as resting cells that are not functionally connected to the idiotypic network[25]. These cells are postulated to be the "available" or "clonal" repertoire that provide immune responses when stimulated by external antigens[18-20]. Coutinho suggests that "network experiments" done with conventional antigens, mitogens and anti-idiotypic reagents have involved this compartment of the immune system[18,20].

1.2.5. De Boer and Perelson's model.

De Boer and Perelson's network model of immunoregulation is based on experimental results reported in the literature and computer simulation. Their model does not yet include roles for T lymphocytes (and their specific factors) and accessory cells (e.g. macrophages) in immunoregulation, nor the role of antibody and complement mediated killing of complementary clones[26]. De Boer and Perelson's model is similar to that of Coutinho, in that they divide the immune system into two compartments which they call the "idiotypic network" and the "clonal repertoire". Novel clones that emerge from the bone marrow and are stimulated (via receptor cross-linking) above a threshold level can compete to enter the idiotypic network (i.e. repertoire selection). Clones that fail to expand are eventually removed from the network, and clones that are not stimulated or over-stimulated (suppressed) remain in the non-network "clonal repertoire". They characterize the V region of each clone using bit-strings, and a string matching algorithm is used to determine its complementarity (and affinity) to other clones. The size of the network is inversely related to the specificity of the receptors. Networks with clones that are highly specific tend to be larger than networks comprised of clones with multireactive receptors. The network becomes "complete" when it is large enough to recognize any bit pattern that occurs within the system[26].

1.2.6. Comparison of Network Models.

Richter, Hoffmann, Coutinho, De Boer and Perelson have developed idiotypic network models to describe the regulation of the immune system. In Richter's model, the idiotypic interactions are sub-threshold prior to perturbation by the antigen, whereas in the other models, the idiotypic interactions play an active role prior to the introduction of antigen.

Hoffmann's symmetrical network theory includes the main components of the immune system, namely B and T lymphocytes, accessory cells and molecules produced by these cells (e.g. antibodies, specific T cell factors, and two non-specific factors)[12-14]. Coutinho, De Boer and Perelson divide the immune system into clonal (non-network) and network repertoires.

Hoffmann's symmetrical model of the immune system postulates that whatever influence a negative lymphocyte (or antibody) has on a positive cell is potentially reciprocal[12-14]. De Boer and Perelson's model is also based on symmetrical interactions. In their model, clones with complementary patterns on their receptors can stimulate each other and compete to enter the network[26]. In Coutinho's model, only lymphocytes that find some sort of complementary pattern can become activated into the idiotypic network[20,21,25]. The complementary pattern is not restricted to the receptors on lymphocytes[25] and it is not clear whether symmetrical interactions between lymphocytes play a major role in Coutinho's model. Richter's model is clearly asymmetric since idiotypes can stimulate anti-idiotypes, but anti-idiotypes can only eliminate (and not stimulate) idiotypes[11].

The symmetrical network theory of Hoffmann has provided a foundation for interpreting the results observed in many experiments. These would include "network experiments" in which idiotypic reagents are used to modulate the immune response to antigen or the expression of particular idiotypes. Low and high zone tolerance, self-tolerance, I-J, MHC-restriction, and the role of CD4 and CD8 molecules in the determining functional phenotypes of T cells have all been described in the context of this theory. Since its formulation in 1975, the model has received only minor revisions to include non-specific factors, one of which is interleukin-1.

1.2.7. Network Connectivity.

A quantitative parameter, namely "connectivity" plays a role in the symmetrical network theory of Hoffmann. Connectivity as defined by Hoffmann, is a measure of the extent that a V region of one lymphocyte (or clone) interacts with the V regions of other lymphocytes within the immune system. Other authors have called this the "field" of the given clone[26]. The immune state is one of low connectivity, the virgin state is one of intermediate connectivity and the suppressed state is one of high connectivity[12-14]. This makes it possible to relate the presence of CD4 on helper T cells to the class II MHC-restriction of CD4 cells, and similarly, to relate the class I MHC-restriction of CD8 cells to their suppressive capabilities[27]. T cells bearing the CD8 marker (Ly-2,3 in the mouse) have affinity for class I MHC molecules[28] which are expressed on the surface of almost all cells[29]. We can therefore expect T cells that recognize class I MHC antigens to be stimulated and secrete more "anti-class I MHC" T cell factors

than the factors of other specificities secreted by other T cells. The "anti-class I MHC" factors are picked up by the A cells, which then stimulate and select T cells that are "anti-anti-class I MHC", since these cells could also pick up the second signal for proliferation (IL-1) from the A cells[27]. The selection of "anti-anti-class I MHC" T cells means that the "anti-class I MHC" T cells ($CD8^+$ cells) would have high network connectivity, which is required of T suppressor cells in the theory[14,27]. T helper cells bearing the CD4 marker (L3T4 in the mouse) have affinity for class II MHC molecules, which are expressed on the surface of A cells and B cells[28]. T cells that have the CD4 antigen can interact directly with class II MHC molecules on the A cells and independently of other V regions in the system (i.e. they may not need a T cell factor bridge to get the first specific proliferation signal). Therefore, "anti-class II MHC" T cells ($CD4^+$ cells) can have a low network connectivity, which is required of T helper cells in the theory[14,27]. The interactions of helper T cells and suppressor T cells in the regulatory T cell compartment of the immune system network is discussed in the context of symmetrical network theory in Chapter 2.

Chapter 2. Anti-idiotypes in alloimmunity.

2.1. Introduction: Idiotypic interactions in alloimmunity.

The production of auto-anti-idiotypic antibodies and lymphocytes has been shown to occur in many systems that use conventional antigens such as haptens, proteins and viruses[153]. The immune response to foreign cells may also include the production of auto-anti-idiotypic antibodies. Hyperimmunization of rats with alloantigens on fibrosarcoma cells induced the production of an anti-idiotypic antibody to an idiotype expressed on the alloantibody produced early in the immune response[30]. Serum taken from late bleedings reacted with the serum taken from an earlier bleeding of the same animal (idiotype-anti-idiotype interaction). The anti-idiotypic antibody produced later in the immune response may be responsible for the clearance or suppression of the idiotype expressed earlier.

The immune response to foreign lymphocytes also contains other classes of anti-idiotypic antibodies. The immune response of the host to foreign lymphocytes includes a host-versus-graft (HVG) response in which the host's immune system recognizes the foreign cells and responds to eliminate the antigen. However, the injected immunocompetent lymphocytes may respond (transiently) to the alloantigens of the host and produce a graft-versus-host (GVH) response. Thus, anti-idiotypic responses made by the immunocompetent host may include the conventional anti-idiotypic reponse to the host's primary response (ie. anti-anti-foreign), and also an anti-idiotypic reponse to the idiotypic receptors on the foreign immunocompetent lymphocytes that recognize the alloantigens on the host.

The latter anti-idiotypic response is an "anti-anti-self" response and has been observed by Ramseier[31], Ramseier and Lindemann[32,33], Binz and Wigzell[34] (Figure 6) and by Hoffmann et al.[35] (Figure 7).

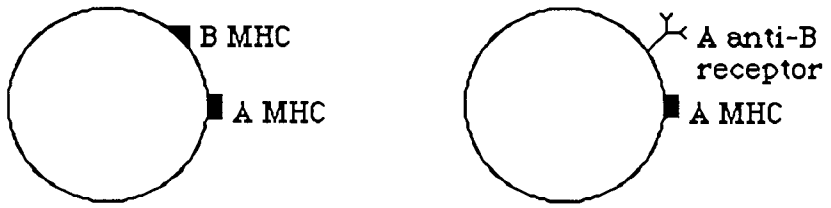
Immunization of (A x B)F_1 animals with normal T and B cells[32-34] or normal T cells[31,34] from parental strain A could induce the production of anti-idiotypic antibodies to the alloantigen receptors on the parental lymphocytes. For example, strain (A x B)F_1 animals immunized with lymphoid cells from strain A parent produced "F_1 anti-(A anti-B)" anti-idiotypic antibodies (Figure 6). Such anti-idiotypic antibodies could inhibit the recognition of strain B alloantigens by strain A lymphocytes *in vitro*, but not the recognition of strain A alloantigens by strain B lymphocytes[33].

Furthermore, T lymphocytes from strain A animals treated with anti-(A anti-B) antibodies and complement failed to react to strain B alloantigens, but were still reactive to third party alloantigens in a GVH assay. The anti-idiotypic antibodies [anti-(A anti-B)] could also agglutinate SRBC coated with A anti-B immunoglobulin. These results suggested T and B lymphocytes directed against the same alloantigen shared idiotypic determinants on their antigen receptors as detected by reactivity with an anti-idiotypic antiserum[34].

Anti-anti-self responses induced by alloimmunization of F_1 animals could also be detected in the T cell compartment. (A x B)F_1 rats immunized with parental strain A T lymphocytes induces a T cell response by the host to the anti-MHC^B receptors on the parental T lymphocytes. That is, the F_1 rats immunized with low doses of parental strain A T lymphocytes (doses that did not induce GVH reactions) developed specific resistance to local GVH responses when they were later immunized with larger doses of parental

Figure 6. The immunization of F_1 animals with parental lymphocytes induces the production of anti-idiotypic antibodies to the anti-alloantigen receptor on the parental lymphocytes.

(A x B)F_1 animals express both sets of alloantigens inherited from the parental strains and do not have lymphocytes with antigen receptors against these structures. The injection of strain A parental lymphocytes will not induce the production of alloantibody against A MHC, but could induce the production of antibodies against the antigen receptor on the parental lymphocytes that recognizes the B alloantigen. This is an "F_1 anti-(A anti-B)" or an anti-anti-self response.



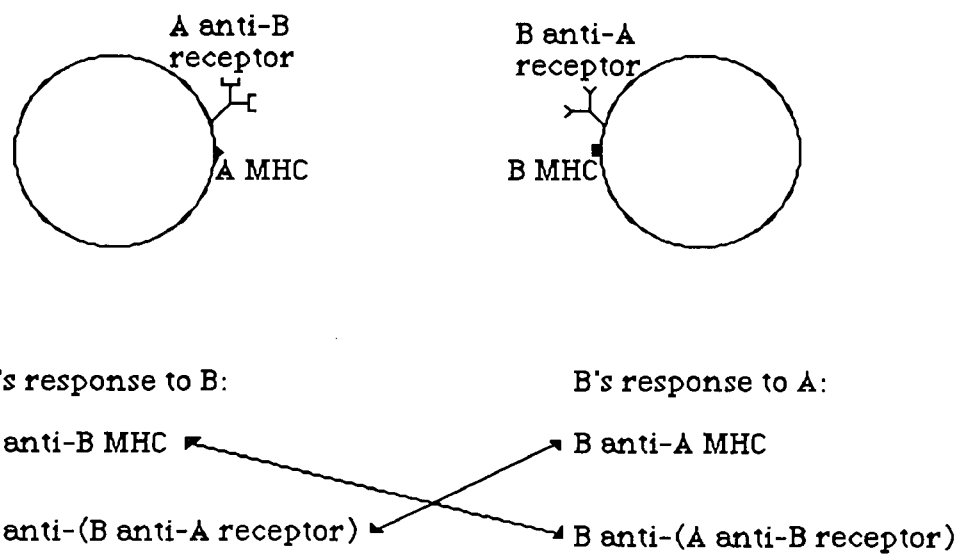
(A x B)F1's response to A: A's response to (A x B)F1:

(A x B)F1 anti-(A anti-B) ⟷ A anti-B

Figure 7. The immunization of mice with allogeneic lymphocytes produces anti-MHC and MHC-image responses.

An immune response in an animal of strain A to allogeneic cells from an animal of strain B consists of two components: the normal anti-foreign (A anti-B) response, and the response of A to the receptors on B strain cells that recognize A (that is, "A anti-(B anti-A)", which is MHC-image). The converse immunization results in the production of "B anti-A" and "B anti-(A anti-B)" responses. The anti-anti-self antibodies made in the "A anti-B" immunization are able to specifically inhibit cytotoxicity of the "B anti-A" anti-foreign antibodies against A strain cells, and vice versa. The total (anti-foreign plus anti-anti-self) "A anti-B" response is complementary to (has V regions that fit) the total "B anti-A" response.

Adapted from Hoffmann, G. W., Cooper-Willis, A., and Chow, M., "A New Symmetry: A anti-B is Anti-(B anti-A), and Reverse Enhancement", *J. Immunol.*, 137, 61 - 68, 1986 (reference 35).



strain A T lymphocytes. The resistance to GVH responses was specific since immunization with strain B lymphocytes still resulted in a GVH response[37]. Adoptive transfer of spleen cells or purified T cells [with anti-(A anti-B) specificity] from such rats could protect naive F_1 rats from GVH-disease when they were immunized with parental strain A T lymphocytes[36]. The F_1 rats also resisted GVH responses by "anti-B" T cells of third party strains, which was interpreted as meaning that there is little or no polymorphism of the idiotypes on anti-MHC receptors of T cells[38]. An alternative explanation is that the V regions of anti-(A anti-B) clones that are selected sufficiently resemble B alloantigens such that all anti-B responses are inhibited. The T cells involved in GVH resistance were shown to have cytotoxic T cell activity. Such anti-idiotypic cytotoxic T cells derived from the (A x B)F_1 rat were able to kill anti-MHC^B blasts derived from an "A anti-B" response and anti-MHC^B blasts derived from third party strains[39].

The Binz and Wigzell work was controversial, in that the experiments were difficult to reproduce in other laboratories. For example, although Wilson and his collegues were able to demonstrate T lymphocytes with anti-idiotypic activity to anti-MHC T cell receptors, they were unable to produce an anti-idiotypic antiserum to the anti-MHC T cell receptors[38]. The anti-idiotypic antiserum used by Binz and Wigzell in most of their experiments (serum pool 1003) had exceptionally high anti-idiotypic activity.

Hoffmann et al. extended the studies of Ramseier and Lindemann, and Binz and Wigzell with a different assay and using alloantisera from fully allogeneic strains of mice. A new symmetry relationship was derived by considering the interactions that occur when lymphocytes from two different immune system networks confront each other. The relationship of

the responses between two immune systems has been called "second symmetry"[35]. When mice of strain A are immunized with fully allogeneic lymphocytes from mice of strain B, the immune response includes responses to both conventional histocompatibility antigens (A anti-B, anti-foreign) and to receptors on the foreign lymphocytes that recognize the host. The receptors of the allogeneic lymphocytes include "anti-host MHC" specificity, and the response to them is an "anti-anti-(self MHC)" or "self MHC-image" response. The anti-foreign response of strain A (A anti-B) is complementary to the anti-anti-self response of strain B [B anti-(A anti-B)], and vice versa. Anti-anti-self antibodies were specifically able to inhibit the cytotoxicity of alloantibody[35]. For example, "A anti-(B anti-A)" inhibited the cytotoxicity of "B anti-A" antibodies against strain A target cells. Furthermore, anti-anti-self antibodies were able to enhance the survival of skin allografts. Strain A mice injected with "B anti-(A anti-B)" antibodies specifically retained B skin grafts longer than third party skin grafts[35]. This phenomenon has been called "reverse enhancement" because the alloantiserum used to treat the recipient is made in the donor, in contrast to conventional enhancement which uses anti-graft serum made in the recipient (reviewed in ref. 40). These experiments demonstrated that the anti-anti-self antibodies recognized idiotypic determinants common to both B cells (as measured by the inhibition of alloantibody in a serological assay) and T lymphocytes (as measured by the delay in skin graft rejection).

In summary, immunization of animals with allogeneic cells may result in the production of auto-anti-idiotypic antibodies to idiotypes produced early in the immune response[30]. However, when the inoculum contains immunocompetent lymphocytes (allogeneic or semi-allogeneic), anti-

idiotypic responses to the foreign idiotypes that recognize the host may also be produced[31-35]. The anti-idiotypic antibodies have anti-anti-(self MHC) (self MHC-image) activity. Anti-idiotypic antibodies can block allorecognition by T lymphocytes *in vitro*[31-33],and *in vivo*[34,35], and inhibit cytotoxicity of alloantibody *in vitro*[35]. Anti-idiotypic T lymphocytes to alloreactive T lymphocytes can provide resistance to GVH disease[36,38,39].

2.2. Results.

2.2.1. MHC-image antibodies in alloimmune serum detected using inhibition of antibody-mediated cytotoxicity.

Repeated immunization of mice with allogeneic lymphocytes produces highly cytotoxic antisera. Such antisera typically contain antibodies to the MHC alloantigens and to the foreign idiotypic receptors that recognize the host MHC[35]. For example, B6 anti-Balb/c antiserum raised by repeated immunization of B6 mice with large doses of Balb/c lymphocytes (described in detail in Chapter 7) contains two classes of specific antibodies: B6 anti-Balb/c (conventional anti-foreign) and B6 anti-(Balb/c anti-B6) which is B6 MHC-image. Similarily, the two classes of specific antibodies in Balb/c anti-B6 antiserum are Balb/c anti-B6 and Balb/c anti-(B6 anti-Balb/c) which is Balb/c MHC-image. The MHC-image antibodies in the B6 anti-Balb/c antiserum are complementary to the anti-foreign antibodies in Balb/c anti-B6 antiserum, and vice versa. The specific classes of antibodies that are produced during alloimmunization and the assays used to detect such antibodies are described in Figure 12.

As shown in Figure 8A, the killing of B6 lymph node cells by Balb/c anti-B6 antiserum is inhibited by B6 anti-Balb/c antiserum but neither by normal B6 serum nor normal Balb/c serum. As seen in a previous study[35], the inhibition of cytotoxicity of an alloantiserum by the reciprocal alloantiserum is close to 100%, indicating that the idiotypes on most anti-MHC antibodies in a polyclonal antiserum are recognized by the idiotypes on the MHC-image antibodies. Absorption of an alloantiserum against the immunogen removed the anti-MHC antibodies (Figure 9), but not the MHC-image antibodies (Figure 8). We refer to such absorbed sera as "alloimmune, immunogen-absorbed" sera or "AIA" sera. Thus, B6 anti-Balb/c antiserum absorbed against Balb/c lymphoid cells (ie. B6 anti-Balb/c AIA) retained the MHC-image antibodies as measured by the inhibition of the cytotoxicity of the Balb/c anti-B6 antiserum against B6 target cells (Figure 8A). Similar results are obtained in the converse direction, in which the killing of Balb/c lymph node cells by B6 anti-Balb/c antiserum is inhibited by Balb/c anti-B6 antiserum or Balb/c anti-B6 AIA serum, but not by normal Balb/c serum nor normal B6 serum (Figure 8B).

If we attempt to use an alloimmune serum, say A anti-B, to inhibit the killing by A anti-B (that is, by itself) as a negative control for this type of experiment, we see "negative inhibition". The anti-MHC antibodies in the "inhibitory serum" increase the amount of killing of the target lymph node cells. Therefore, the best negative control for this experiment is the AIA serum, with the same specificity as the cytotoxic antiserum used in the experiment, but lacking the anti-MHC antibodies. Thus Balb/c anti-B6 AIA serum (with Balb/c MHC-image antibodies) inhibited anti-Balb/c cytotoxicity (Figure 8B) but not anti-B6 cytotoxicity (Figure 8A), while B6 anti-Balb/c AIA

Figure 8. MHC-image antibodies in alloimmune and alloimmune, immunogen-absorbed (AIA) sera detected by the inhibition of cytotoxicity assay.

^{51}Cr-labelled lymph node cells were incubated with the inhibitory and lytic antisera for 20 minutes, washed, and incubated with complement for 60 minutes. Cell lysis was determined by measuring the amount of ^{51}Cr released into the supernatant. Each point represents the mean and standard deviation of triplicates. Panel A. Inhibition of killing of B6 lymph node cells by Balb/c anti-B6 antiserum using B6 anti-Balb/c antiserum (❑), and B6 anti-Balb/c AIA serum (○), but not normal B6 serum (△) nor Balb/c serum (▲). Additional controls are Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA serum (●). Panel B. Inhibition of killing of Balb/c lymph node cells by B6 anti-Balb/c antiserum using Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA serum (●), but not normal B6 serum (△) nor Balb/c serum (▲). Additional controls are B6 anti-Balb/c antiserum (❑) and B6 anti-Balb/c AIA serum (○). Balb/c anti-B6 antiserum was diluted 1/400 and killed 51.9% of B6 target cells in the absence of inhibitors. B6 anti-Balb/c antiserum was diluted 1/1200 and killed 52.9% of Balb/c target cells in the absence of inhibitors.
These results indicate that anti-anti-self antibodies made during an alloimmune response have MHC-image activity, since they inhibit cytotoxicity mediated by polyclonal alloantisera.

Statistical analysis of the data (at the 1/10 dilution) by Analysis-of-variance (ANOVA)[164].
Panel A. B6 anti-Balb/c and B6 anti-Balb/c AIA sera are significantly different than normal B6 serum with p values of $\leq$ 0.005. B6 anti-Balb/c and B6 anti-Balb/c AIA sera are different with a p value of $\leq$ 0.25.
Panel B. Balb/c anti-B6 and Balb/c anti-B6 AIA sera are significantly different than normal Balb/c serum with p values of $\leq$ 0.005. Balb/c anti-B6 and Balb/c anti-B6 AIA sera are different with a p value of $\leq$ 0.05.

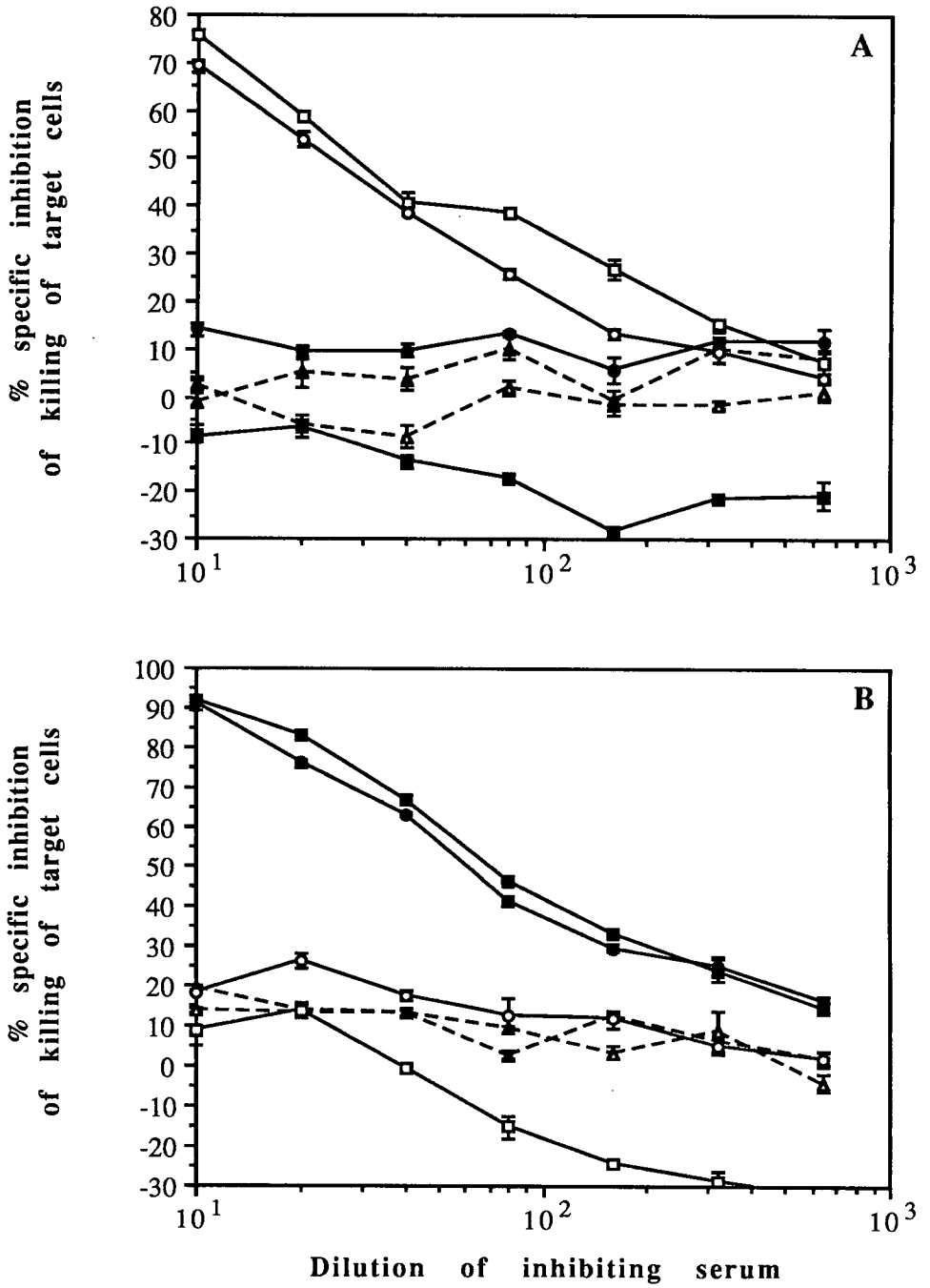
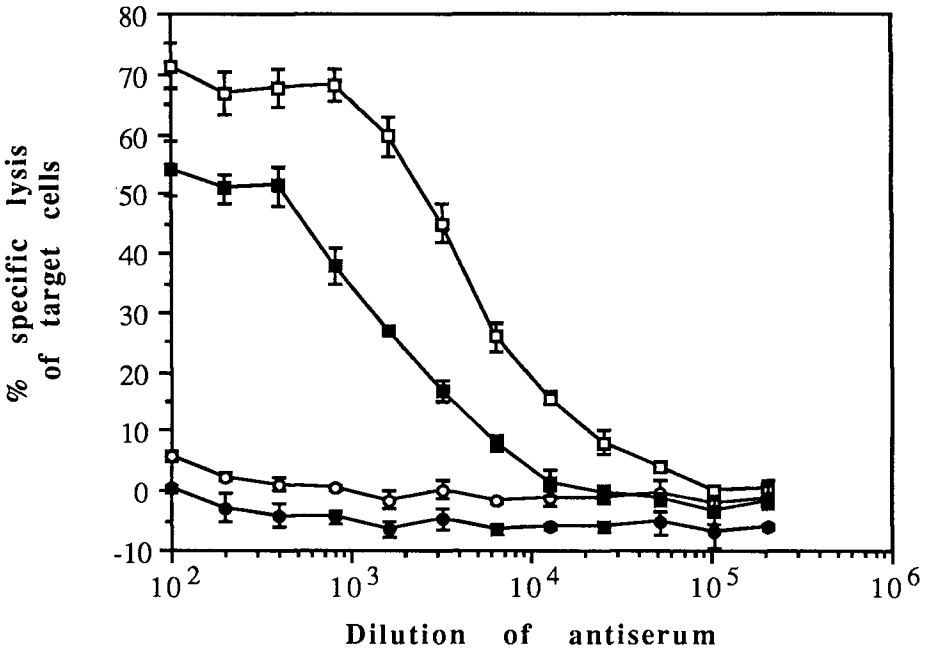

A
B
% specific inhibition of killing of target cells
% specific inhibition of killing of target cells
80
70
60
50
40
30
20
10
0
-10
-20
-30
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
10^1
10^2
10^3
Dilution of inhibiting serum

Figure 9. Cytotoxicity of Balb/c anti-B6 and B6 anti-Balb/c alloantisera before and after absorption with the immunogen.

^{51}Cr-labelled lymph node cells were incubated with the antisera for 20 minutes, washed, and incubated with complement for 60 minutes. Cell lysis was determined by measuring the amount of ^{51}Cr released into the supernatant. After 5 serial absorptions with glutaraldehyde-fixed cells, no anti-MHC antibodies can be detected by cytotoxicity of target cells. Each point represents the mean and standard deviation of triplicates. B6 anti-Balb/c antiserum (❑) and B6 anti-Balb/c alloimmune, immunogen-absorbed (AIA) serum (○) killing Balb/c lymph node targets. Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA antiserum (●) killing B6 lymph node targets.



serum (with B6 MHC-image antibodies) inhibited anti-B6 cytotoxicity (Figure 8A) but not anti-Balb/c cytotoxicity (Figure 8B).

2.2.2. An ELISA for detecting MHC-image antibodies.

ELISA technology offers many advantages for detecting antibodies in hyperimmune serum. ELISA requires considerably less serum than other assays (eg. the inhibition of cytotoxicity assay), and the results can be obtained more quickly. Therefore, it was of interest to develop an ELISA to detect MHC-image antibodies in alloantisera. Normal sera, alloimmune sera (say "A anti-B") and AIA (A anti-B, absorbed with B) sera were used to coat the ELISA plates at the dilutions indicated. These sera would be analogous to the inhibitory sera in the inhibition of cytotoxicity assay. Biotinylated alloimmune serum ("B anti-A", analagous to the lytic serum in the inhibition of cytotoxicity assay) was allowed to react with the antibodies bound to the plates, and the bound biotinylated antibodies were detected with Strep. avidin-alkaline phosphatase conjugate (avidin isolated from *Streptomyces avidinii* and conjugated with alkaline phosphatase) and the appropriate substrate. As shown in Figure 10A, biotinylated Balb/c anti-B6 antiserum binds strongly to the reciprocal B6 anti-Balb/c antiserum and B6 anti-Balb/c AIA serum. Since the signal generated is about the same for both unabsorbed and absorbed sera on the plate, the main contribution is not from anti-MHC antibodies on the plate, but from the MHC-image antibodies on the plate. Furthermore, MHC-image antibodies could be detected in the serum coated on ELISA plates when it is diluted in carbonate buffer 10^5-fold. In contrast to the ELISA, MHC-image antibodies could not be detected in the

inhibition of cytotoxicity assay when the inhibitory serum dilution was greater than 10^3. Thus, the ELISA was a more sensitive assay for detecting MHC-image antibodies. The biotinylated Balb/c anti-B6 antiserum does not bind strongly to normal B6 serum, normal Balb/c serum or Balb/c anti-B6 antiserum nor Balb/c anti-B6 AIA serum. Figure 10B shows the reciprocal experiment to that of Figure 10A, in which biotinylated B6 anti-Balb/c antiserum binds strongly to Balb/c anti-B6 antiserum and Balb/c anti-B6 AIA serum, and less strongly to normal Balb/c serum, normal B6 serum, B6 anti-Balb/c antiserum and B6 anti-Balb/c AIA serum.

We found that normal mouse serum often contained low levels of MHC-image antibodies that can be detected by both the inhibition of cytotoxicity assay and ELISA. Antibodies that are images of MHC may be part of the normal repertoire as previously suggested by Rossi et al[41]. In their experiments, culture supernatants from LPS-stimulated splenic lymphocytes were tested for reactivity to ELISA plates coated with anti-I-E and anti-I-A monoclonal antibodies. Their results indicate that the frequency of B cells in normal mice with antibody V regions reactive with anti-class II monoclonal antibodies may be as high as 0.9%.

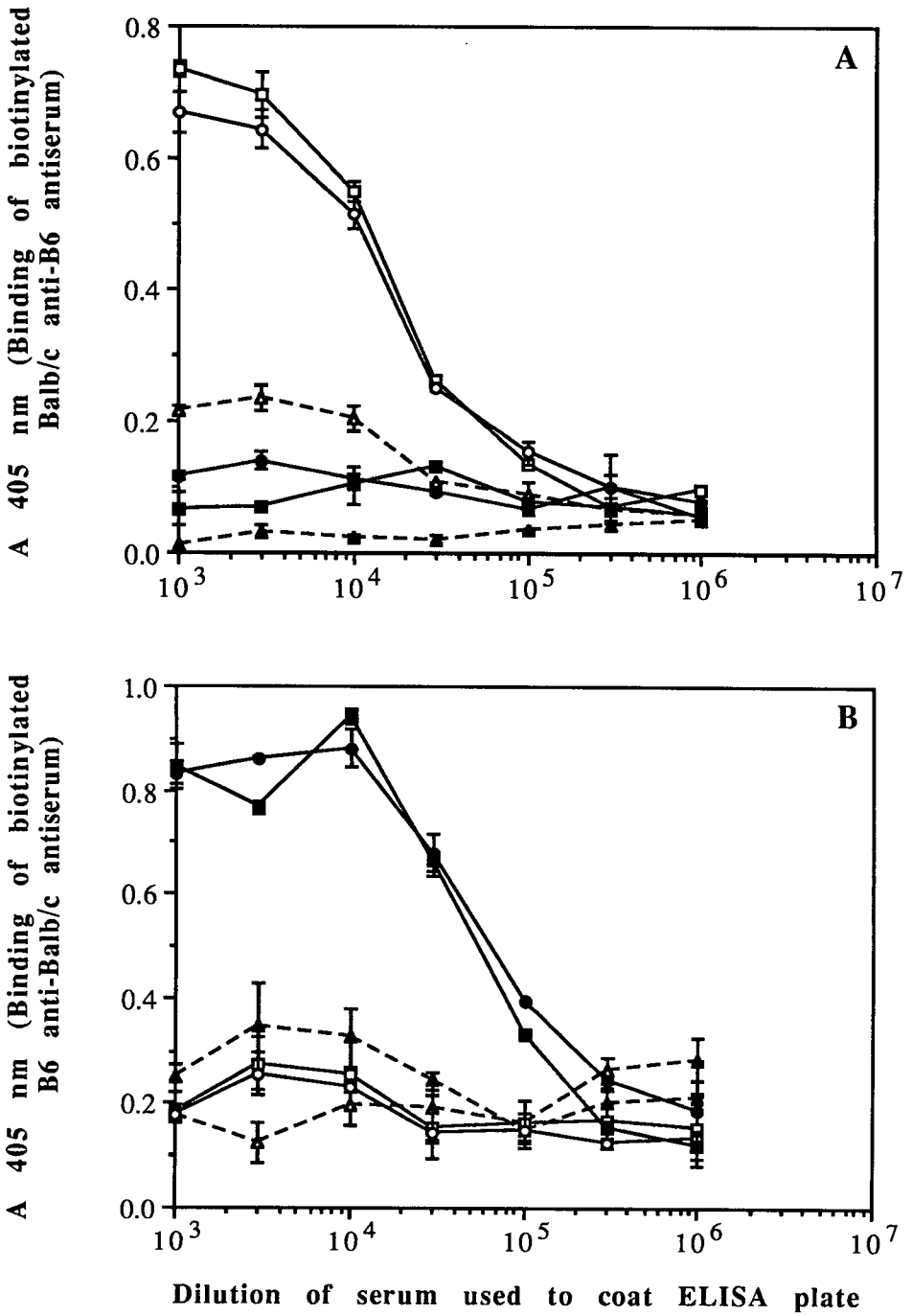
2.2.3. AIA serum can be used to enhance allograft survival.

AIA sera have been shown to be effective in specifically enhancing the survival of skin allografts in mice[35]. Those results have been confirmed with our B6 anti-Balb/c and Balb/c anti-B6 alloantisera. B6 mice were injected intraperitoneally (i.p.) with 20 µl of normal Balb/c serum or Balb/c anti-B6 AIA serum on days -7, -4, -1 and 0 relative to grafting with Balb/c

Figure 10. MHC-image antibodies in alloimmune and alloimmune, immunogen-absorbed (AIA) sera detected by the MHC-image ELISA.

Normal, alloimmune or AIA sera were coated directly to ELISA plates at the dilutions indicated. After incubation with biotinylated alloantiserum, the bound antibodies were detected with Strep. avidin-alkaline phosphatase conjugate and substrate (t = 40 minutes). The resulting signal is interpreted as resulting from the binding of biotinylated anti-MHC antibodies to the MHC-image antibodies on the plate. Each point represents the mean and standard deviation of duplicates. Panel A. Biotinylated Balb/c anti-B6 alloantiserum binds to B6 anti-Balb/c antiserum (❑), and B6 anti-Balb/c AIA serum (○), but neither normal B6 serum (△) nor Balb/c serum (▲). Additional controls are Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA serum (●). Biotinylated Balb/c anti-B6 antiserum was diluted to a final concentration of 1/500. Panel B. Biotinylated B6 anti-Balb/c alloantiserum binds to Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA serum (●), but neither normal B6 serum (△) nor Balb/c serum (▲). Additional controls are B6 anti-Balb/c antiserum (❑) and B6 anti-Balb/c AIA serum (○). Biotinylated B6 anti-Balb/c antiserum was diluted to a final concentration of 1/500.

Statistical analysis of the data (at the 1/3000 dilution) by ANOVA.
Panel A. B6 anti-Balb/c and B6 anti-Balb/c AIA sera are significantly different than normal B6 serum with p values of ≤ 0.01 and ≤ 0.025 respectively. B6 anti-Balb/c and B6 anti-Balb/c AIA sera are different with a p value of ≤ 0.25.
Panel B. Balb/c anti-B6 and Balb/c anti-B6 AIA sera are different than normal Balb/c serum with p values of ≤ 0.25. Balb/c anti-B6 and Balb/c anti-B6 AIA sera are different with a p value of ≤ 0.25.


A
A 405 nm (Binding of biotinylated Balb/c anti-B6 antiserum)
0.8
0.6
0.4
0.2
0.0
10^3
10^4
10^5
10^6
10^7
B
A 405 nm (Binding of biotinylated B6 anti-Balb/c antiserum)
1.0
0.8
0.6
0.4
0.2
0.0
10^3
10^4
10^5
10^6
10^7
Dilution of serum used to coat ELISA plate

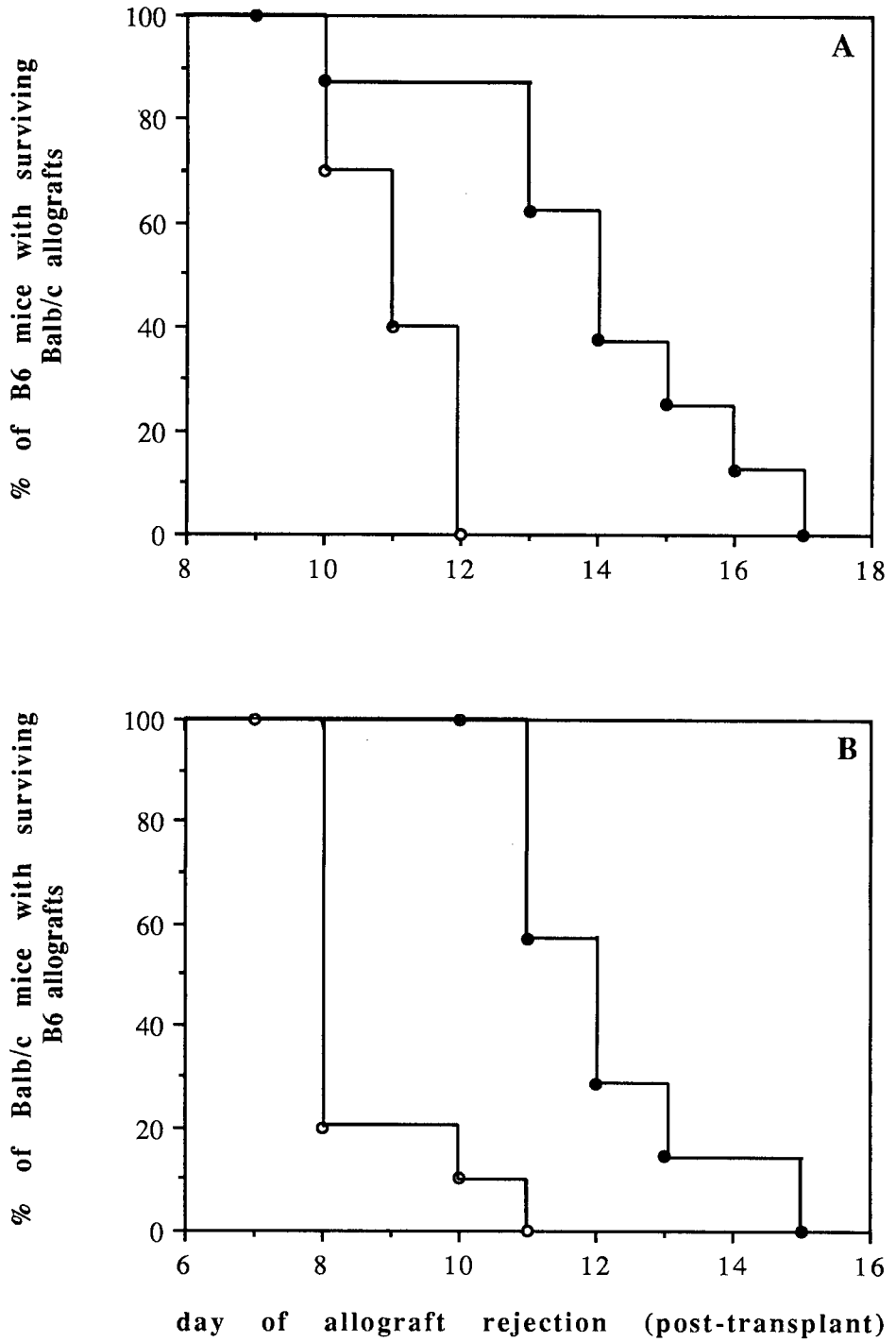
skin. As shown in Figure 11A, the Balb/c skin grafts survived longer in B6 mice treated with Balb/c anti-B6 AIA serum than in mice treated with normal Balb/c serum. In the reciprocal experiment, B6 skin allografts survived longer in Balb/c mice treated with B6 anti-Balb/c AIA serum than in mice treated with normal B6 serum (Figure 11B). Skin allograft survival was increased an average of 3 days in mice treated with AIA serum in both experiments. In previous published work, this effect was shown to be specific for the MHC of the graft, and it was suggested that it is due to an inhibiting effect of MHC-image antibodies in alloimmune serum[35].

2.2.4. Interactions between reciprocal "alloimmune, immunogen-absorbed" (AIA) sera.

Antibodies with anti-MHC-image activity may also be present in the serum of alloimmunized mice. The anti-MHC-image responses could be induced by the MHC-image responses [this would be an anti-(self MHC-image) response} or more directly by the foreign MHC-image epitopes on T cells in the inoculum acting as antigens [ie. an anti-(foreign MHC-image) response][42,43]. Such anti-MHC-image antibodies may be detected by their interaction with a serum containing biotinylated MHC-image antibodies. For this ELISA, biotinylated alloimmune, immunogen-absorbed (AIA) serum is the source of MHC-image antibodies to detect MHC-image/anti-MHC-image interactions since an unabsorbed antiserum would also detect anti-MHC/MHC-image interactions. Figure 12 is a schematic drawing of the interactions of the antibody populations in a reciprocal pair of AIA sera.

Figure 11. MHC-image antibodies enhance allograft survival in mice.

Mice were injected intraperitoneally (i.p.) with NMS or 20 µl alloimmune, immunogen absorbed (AIA) serum on days -7, -4, -1, and 0 prior to grafting with allogeneic skin. Panel **A.** Balb/c mice grafted with B6 skin. Balb/c mice injected with B6 NMS (10 mice, ○) had a graft survival time of 9.3 ± 0.7 (mean ± SEM). Balb/c mice injected with B6 anti-Balb/c AIA serum (7 mice, ●) had a graft survival time of 12.1 ± 1.5 (This is significantly different from the NMS group with $p \leq 0.0001$ by ANOVA). Panel B. B6 mice grafted with Balb/c skin. B6 mice injected with Balb/c NMS (10 mice, ○) had a graft survival time of 11.1 ± 0.9. B6 mice injected with Balb/c anti-B6 AIA serum (8 mice, ●) had a graft survival time of 14.0 ± 2.1 (This is significantly different from the NMS group with $p \leq 0.005$ by ANOVA).


A
% of B6 mice with surviving Balb/c allografts
100
80
60
40
20
0
8
10
12
14
16
18
B
% of Balb/c mice with surviving B6 allografts
100
80
60
40
20
0
6
8
10
12
14
16
day of allograft rejection (post-transplant)

For this experiment, normal sera, alloimmune sera and AIA sera (say A anti-B AIA) were used to coat the ELISA plates at the dilutions indicated. A biotinylated converse AIA serum (say B anti-A AIA serum) was then allowed to react with the antibodies bound to the plates, and the bound biotinylated antibodies were detected with Strep. avidin-alkaline phosphatase conjugate and the appropriate substrate. Both AIA sera were extensively absorbed with the immunogen to remove the anti-MHC antibodies (Figure 9). Figure 13A shows that biotinylated Balb/c anti-B6 AIA serum binds strongly to the reciprocal B6 anti-Balb/c antiserum and B6 anti-Balb/c AIA serum. The biotinylated Balb/c anti-B6 AIA serum does not bind strongly to normal Balb/c serum or Balb/c anti-B6 antiserum or Balb/c anti-B6 AIA serum. However, the amount of binding to normal B6 serum is almost 50% of the amount of binding to B6 anti-Balb/c antiserum and B6 anti-Balb/c AIA serum. Figure 13B shows the reciprocal experiment to that Figure 13A, in which biotinylated B6 anti-Balb/c AIA serum binds strongly to Balb/c anti-B6 antiserum and Balb/c anti-B6 AIA serum, and less strongly to normal Balb/c serum, normal B6 serum, B6 anti-Balb/c antiserum and B6 anti-Balb/c AIA serum. Interactions between MHC-image and anti-MHC-image antibodies could be best detected when the serum coated to the plate was diluted to 1/5 x 10^4. This may be due to weakly associated complexes present in alloimmune sera and AIA sera that dissociate at higher dilutions, making the bound antibodies available for interaction with the antibodies in the converse serum.

The binding of one AIA serum to the reciprocal AIA serum supports the idea that there are both MHC-image and anti-MHC-image antibodies present in alloimmune sera. Since AIA serum binds only to the reciprocal

Figure 12. Assays used to detect anti-MHC, MHC-image and anti-MHC-image antibodies; schematic. Results for each of these assays are presented in this thesis.

The anti-MHC antibodies in an alloimmune serum have V regions that are complementary to the MHC antigens in the inoculum. The anti-MHC-image antibodies are made in response to the foreign T cell idiotypes that recognize self. The V region shape of these antibodies are similar, but not identical to the MHC antigens in the host that makes them. These MHC-image antibodies are very similar to the MHC antigens in that they react with the alloantiserum. The anti-MHC-image antibodies made by the host are directed against MHC-image determinants present on some T cells in the inoculum. The anti-MHC-image antibodies may have a V region shape that is similar to the anti-MHC antibodies made in the same host, in that they interact with the same MHC-image antibodies. The V region shapes of anti-MHC-image antibodies are nevertheless somewhat different than those of anti-MHC antibodies; they are not readily absorbed out with the immunogen.

A. The direct cytotoxicity assay detects interactions between the MHC antigens on the target cell and the anti-MHC antibodies in the alloimmune serum. ^{51}Cr-labelled cells were incubated with the alloantiserum, washed and incubated with complement. The amount of ^{51}Cr released into the supernatant is proportional to the amount of antibody plus complement mediated killing.

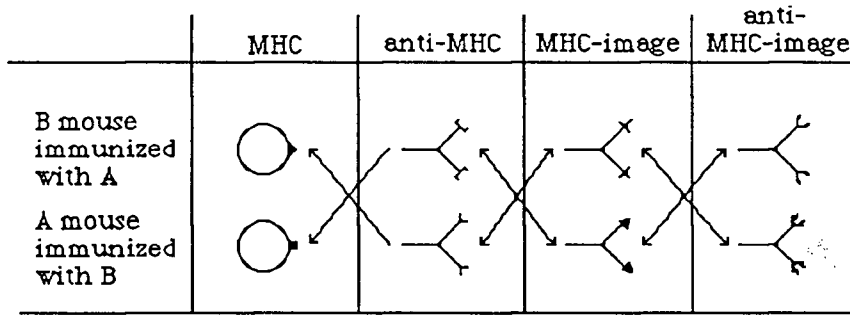
B. The inhibition of cytotoxicity assay detects MHC-image antibodies in one alloimmune serum by the inhibition of cytotoxicity of the reciprocal alloimmune serum against its target cell. For example, MHC-image antibodies made in a A anti-B alloimmune serum (anti-anti-A MHC) will inhibit the cytotoxicity of antibodies in a B anti-A alloimmune serum against target A cells.

C. The MHC-image ELISA detects MHC-image antibodies by the direct interaction with complementary anti-MHC antibodies in the reciprocal alloimmune serum or monoclonal antibodies that are anti-MHC. In this assay, sera containing putative MHC-image antibodies are coated directly to an ELISA plate. The converse alloantiserum is biotinylated, and incubated with the ELISA plates. The biotinylated anti-MHC antibodies that have bound to the plate are detected with avidin-alkaline phosphatase and substrate. There are no target cells in this assay. The signal that results is interpreted as the binding of anti-MHC or anti-MHC-image antibodies in the biotinylated antiserum binding to MHC-image antibodies on the plate. However, interactions between MHC-image antibodies in the biotinylated antiserum and anti-MHC or anti-MHC-image antibodies on the plate may also be detected. (Interactions between MHC-image antibodies and anti-MHC-image antibodies may also be present).

D. The interactions between reciprocal alloimmune, immunogen-absorbed (AIA) sera suggests the presence of a third antibody population with anti-MHC-image activity. Biotinylated AIA serum interacts with the reciprocal alloimmune serum or the reciprocal AIA serum and not with itself. The interactions between these two sera are likely to be MHC-image antibodies in the biotinylated AIA serum binding to the anti-MHC-image antibodies on the plate. Interactions between anti-MHC-image antibodies in the biotinylated serum binding to the MHC-image antibodies in the plate may also be present. MHC-image antibodies and anti-MHC antibodies may interact if the anti-MHC antibodies are not absorbed out with the immunogen.

Assays used to detect anti-MHC, MHC-image and anti-MHC-image antibodies.

Shapes of the MHC and the V regions along the MHC axis of specificities.



Assays		Antibodies detected
A.	direct cytotoxicity	anti-MHC
B.	inhibition of cytotoxicity	MHC-image
C.	MHC-image	MHC-image
D.	anti-MHC-image	anti-MHC-image

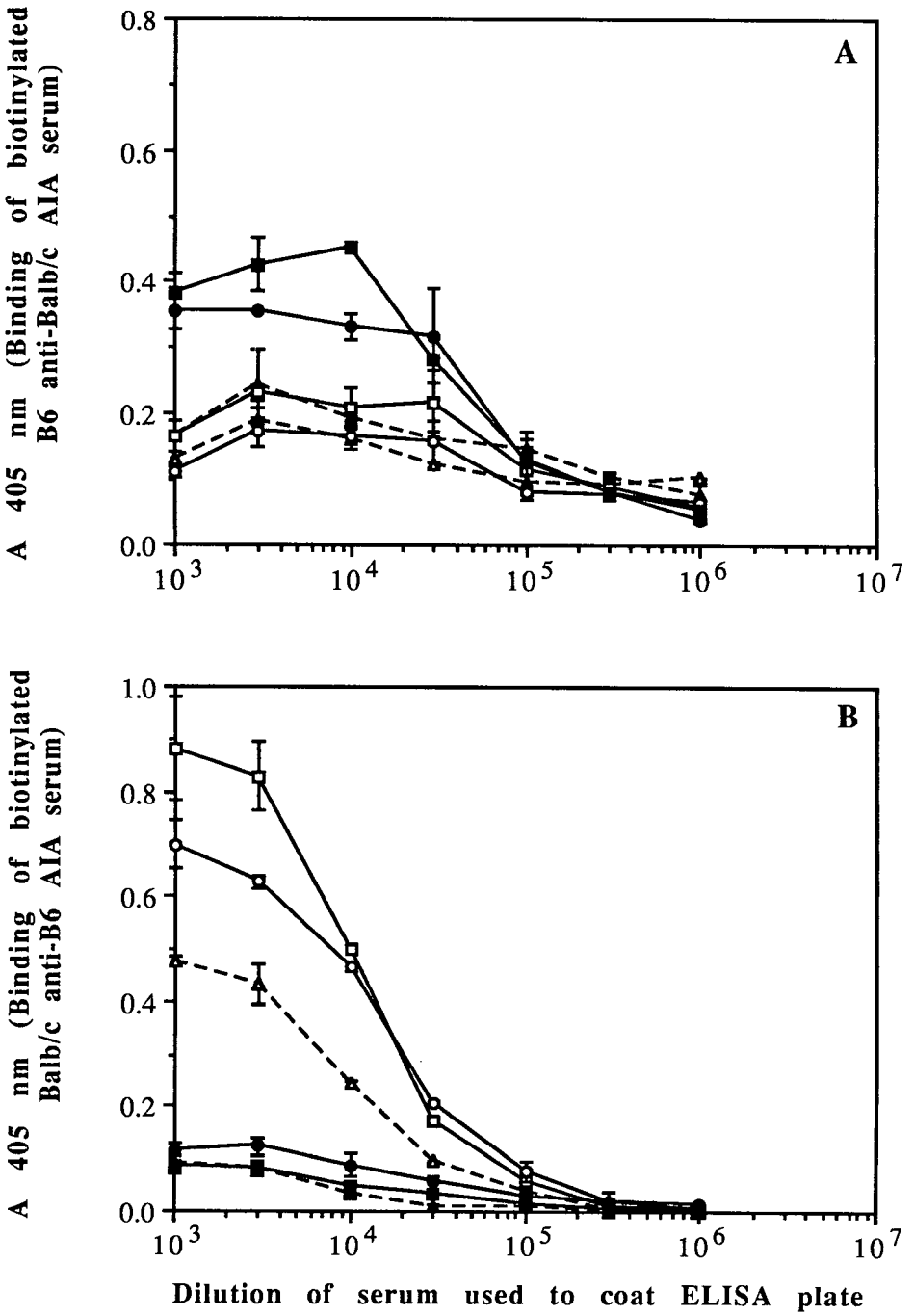
indicates interactions between antibodies and cell surface antigens

indicates interactions between populations of antibodies

indicates an interaction between populations of antibodies if the anti-MHC antibodies were not absorbed out with the immunogen

Figure 13. Biotinylated alloimmune, immunogen-absorbed (AIA) serum interacts with the reciprocal alloantiserum or AIA serum.

Normal, alloimmune or AIA sera were coated directly to ELISA plates at the dilutions indicated. After incubation with biotinylated AIA serum, the bound antibodies were detected with Strep. avidin-alkaline phosphatase conjugate and substrate (t = 40 minutes). The resulting signal is interpreted as resulting from the binding of biotinylated MHC-image antibodies to the anti-MHC-image antibodies on the plate. Each point represents the mean and standard deviation of duplicates. Panel A. Biotinylated Balb/c anti-B6 AIA serum binds to B6 anti-Balb/c antiserum (❑), and B6 anti-Balb/c AIA serum (○), and less strongly to normal B6 serum (△) and Balb/c serum (▲). Additional controls are Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA serum (●). Biotinylated Balb/c anti-B6 AIA serum was diluted to a final concentration of 1/500. Panel B. Biotinylated B6 anti-Balb/c AIA serum binds to Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA serum (●), and less strongly to normal B6 serum (△) or Balb/c serum (▲). Additional controls are B6 anti-Balb/c antiserum (❑) and B6 anti-Balb/c AIA serum (○). Biotinylated B6 anti-Balb/c AIA serum was diluted to a final concentration of 1/500.


A
A 405 nm (Binding of biotinylated B6 anti-Balb/c AIA serum)
0.8
0.6
0.4
0.2
0.0
10^3
10^4
10^5
10^6
10^7
B
A 405 nm (Binding of biotinylated Balb/c anti-B6 AIA serum)
1.0
0.8
0.6
0.4
0.2
0.0
10^3
10^4
10^5
10^6
10^7
Dilution of serum used to coat ELISA plate

AIA serum and not or only weakly to itself, it would indicate that the anti-MHC-image response is likely due to the MHC-image epitopes on the allogeneic T cells. This idea will be addressed in the discussion.

2.3. Discussion.

The above results firstly confirm and extend the previous findings of Hoffmann et al.[35] that alloimmunization of mice induces the production of antibodies with anti-anti-self MHC activity. Anti-anti-self antibodies were interpreted to have MHC-image activity, in that they are able to specifically inhibit the cytotoxicity of the polyclonal anti-MHC antibodies in the reciprocal alloantiserum[35]. MHC-image antibodies sufficiently resemble alloantigens since they also inhibit the idiotypes of anti-MHC antibodies produced in third party strains[35]. MHC-image antibodies in alloantisera were readily detectable with an ELISA at dilutions of sera 100-fold greater than in the inhibition of cytotoxicity assay. The ELISA could be improved by using purified immunoglobulin as the coating antigen and the biotinylated detecting reagent. Although various non-immunoglobulin components coated on the ELISA plate and in the detecting reagent could be interacting with each other, it is unlikely that they could be causing the specific differences in the signals since they would be present in both normal and immune serum. The interpretation that anti-MHC antibodies were interacting with MHC-image antibodies in the reciprocal antiserum was supported by both experiments.

Some additional controls are needed to further confirm the presence and specificity of antibodies with MHC-image activity that are produced

during alloimmunization. For example, an (A x B)F_1 anti-B antiserum would have antibodies of anti-anti-A (A-MHC-image) specificity without the anti-MHC antibodies. The A-MHC-image antibodies prepared in an F_1 host would be expected to inhibit the cytotoxicity of B anti-A antibodies (or bind to biotinylated anti-MHC antibodies) and enhance the survival of A allografts in a B host. Antibodies with such specificities were described in the experiments of Ramseier and Lindemann[32,33], and Binz and Wigzell[34]. Antiserum prepared by the immunization of mice with cell lines (e.g. an anti-MHC CTL) may also generate antibodies with MHC-image activity. However, it is likely that the MHC-image antibodies produced by such an immunization protocol would recognize only a fraction of the idiotypes present in an alloantiserum. The experiment could also have included an antiserum against an irrelevant antigen (e.g. BSA) as a negative control. Such an antiserum would lack the MHC-image activity and would not inhibit the cytotoxicity of an anti-MHC serum nor enhance the survival of skin allografts.

In ELISAs using biotinylated alloimmune, immunogen absorbed (AIA) serum as the detecting reagent, a third population of antibodies with anti-MHC-image activity could be measured in alloantiserum. In this assay, AIA serum (containing MHC-image antibodies but lacking anti-MHC antibodies) was found to react to ELISA plates coated with the reciprocal AIA serum. The anti-MHC-image response is presumably directed against the MHC-image determinants on the T cells in the inoculum.

T cells with MHC-image determinants are thought to play a key role in the regulation of the immune system[42,43]. T cells that have receptors that are weakly complementary to class II MHC antigens are positively selected to

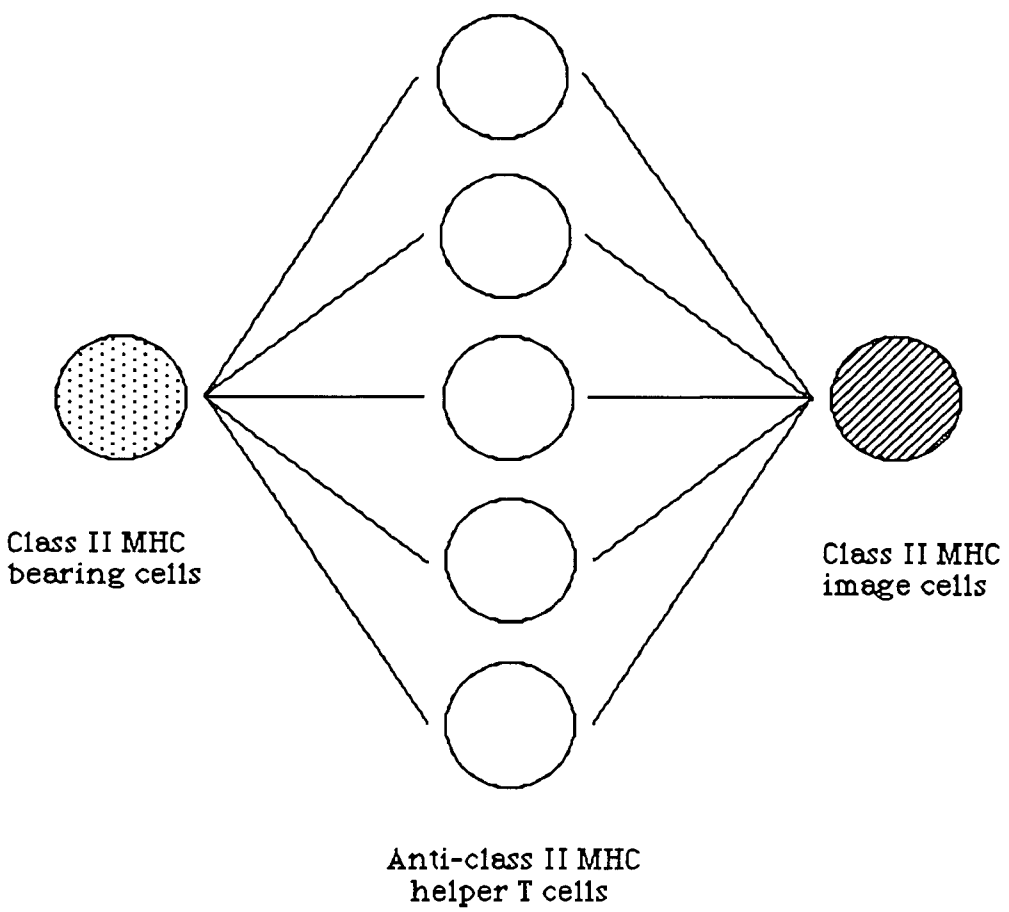
become the helper T cell population. It has been postulated that a population of suppressor T cells are positively selected to have receptors that are complementary to the helper T cell idiotypes[42,43]. The receptors of these suppressor T cells would have epitopes that resemble epitopes on class II MHC molecules from the point of view of the helper T cells; that is, the helper T cell idiotypes interact with class II MHC and the suppressor T cell idiotypes. The interactions between the anti-class II MHC helper T cells and the class II MHC-image suppressor T cells are postulated to be mutually stabilizing. This has led to the idea of a network focussing topology in which class II MHC-image suppressor T cells are idiotypically connected to a large number of helper T cell idiotypes (Figure 14). The helper T cell idiotypes are thus idiotypically connected to a restricted set of suppressor T cell idiotypes, and have lower network connectivity than the suppressor T cells[42,43]. A similar topology was suggested by Grossman based on asymmetrical network interactions but it is not clear that the interactions in his model are idiotype-anti-idiotype[44].

The network focussing topology resulted from an attempt to resolve the I-J paradox. I-J is a controversial serological marker associated with T suppressor cells and their factors. Classical genetic studies had located the I-J gene within the MHC complex[45,46]; however, molecular genetic studies could not identify the gene for I-J[47,48]. Furthermore, the DNA sequences of this region were identical in the two important mouse strains (B10.3R and B10.5R) used in defining I-J[49]. One interpretation is that I-J markers are determinants on the V regions of T cells receptors that have been selected by class II MHC molecules[50-52]. For example, T cells that had differentiated in an irradiated bone marrow chimaera were found to express the I-J

Figure 14. A model for the idiotypic relationship between helper T cells and T cells with class II MHC-image epitopes.

The V regions of helper T cells have low affinity for class II MHC antigens. T cells whose V regions have determinants that are complementary to many helper T cell idiotypes are selected in preference to T cells whose V regions recognize only a few helper T cell idiotypes. At least some suppressor T cells are postulated to be in this group. The interactions between the anti-class II MHC helper T cells and class II MHC-image suppressor T cells are expected to be mutually stimulatory and mutually stabilizing. The helper T cell idiotypes have lower network connectivity than the suppressor T cells bearing class II MHC-image epitopes, which is consistent with what is predicted in symmetrical network theory[14,27]. A simple metaphor has been used to describe this part of the T cell repertoire: a tent consisting of a canvas (helper T cell idiotypes) holding up the centre pole (the class II MHC-image epitopes) and a centre pole holding up the canvas.

From Hoffmann, G. W., Kion, T. A. and Grant, M. D., "An Idiotypic Network Model of AIDS Immunopathogenesis", *PNAS*, 88, 3060 - 3064, 1991 (reference 43).



phenotype of the foreign MHC environment, rather than the I-J phenotype "encoded" by the MHC genes of the stem cells[51]. In Hoffmanns's theory, I-J bearing cells could be class II MHC-image bearing T cells[27] which idiotypcally interact with anti-class II MHC helper T cells.

Although MHC molecules and MHC-image determinants are similar from the point of view of the helper T cells, they may be sufficiently different to elicit different antibody populations. In other words, MHC molecules and MHC-image determinants could be different antigens from the point of view of the B cell population, and yet be similar from the point of view of T cells [see Hoffmann and Tufaro (1989) for a discussion of context dependent similarity][53]. Anti-class II MHC antibodies do not detect class II antigens on the surface of murine T cells. This would imply that I-J determinants on suppressor T cells (which are considered to be an image of class II MHC) and class II MHC molecules are different antigens from the point of view of the B cells. I-J determinants on suppressor T cells could be images of class II MHC antigens from the point of view of helper T cells, and thus idiotypically interact with the anti-MHC receptors on the helper T cells.

Anti-MHC-image antibodies are not readily removed by absorption with the immunogen. It is possible that anti-MHC-image antibodies have a V region shape that is different than the V region shape of anti-MHC antibodies which are removed by such absorptions. An alternative interpretation of these results would be that there are anti-MHC antibodies in the AIA serum that remain even after extensive absorptions with the immunogen, and these anti-MHC antibodies were not detected by the relatively insensitive cytotoxicity assay. These antibodies may not have been absorbed out because the absorbant had been treated with 0.3%

glutaraldehyde, and some epitopes may have been destroyed by the cross-linking of amino groups of cell-surface proteins[54]. Thus, the signal detected would be from anti-MHC/MHC-image interactions. If such antibodies are indeed anti-MHC antibodies, then this assay may provide a more sensitive method for detecting residual anti-MHC antibodies.

2.4. Summary.

The results so far indicate that alloimmunization induces the production of at least three classes of specific antibodies; namely, anti-MHC, MHC-image and anti-MHC-image antibodies. An intruiging possibility is that anti-MHC-image antibodies are related to, or identical with anti-I-J antibodies. Further work is needed to evaluate this possibility.

Chapter 3. Autoimmunity and AIDS.

3.1. Introduction.

Autoimmunity generally refers to the immune responses directed against self molecules. Autoimmune diseases result when these immune responses cause tissue damage or impair normal physiology. Self-reactivity was traditionally thought to be inadmissible and inconsistent with good health. Thus, autoimmunity was considered to be a loss or a breakdown in self-tolerance. Burnet's clonal selection theory proposed that self-tolerance resulted from self-reactive lymphocytes being purged from the developing immune system during ontogeny. Self-reactive lymphocytes that developed after ontogeny were thought to be the result of somatic mutation[1B]. However, immunoregulation and basic immune system functions involve recognition of self molecules. Thus, certain forms of autoimmune responses are not harmful, but are the normal activities of an intact immune system. Several reviews have described two forms of autoimmunity, namely physiological and pathological or pernicious[55-57].

Physiological autoimmunity includes recognition of self molecules that are necessary for immunoregulation and normal immune system functions. The phenomenon of MHC-restriction is based on recognition of self class I or class II MHC molecules by T cells. T cells that have a low affinity for self class I or class II MHC are positively selected for export from the thymus, whereas T cells with a high affinity for self class I or class II MHC are eliminated[58-60]. T cells that recognize self class I MHC tend to develop into $CD4^{-}CD8^{+}$ T cells (CTL and suppressor T cells usually have this

phenotype) and those that recognize self class II MHC tend to develop into $CD4^+CD8^-$ T cells (helper T cells usually have this phenotype)[61]. CTLs that recognize and kill virally infected cells are restricted by self class I MHC molecules[62]. Antigen presentation by macrophages to T cells is restricted by self class II MHC molecules[63,64], as is the regulation of B cell responses by T cells[65]. Idiotypic anti-idiotypic interactions are another form of self-recognition and may be involved in the regulation of the immune response to antigen. The presence of auto-anti-idiotypic antibodies has been associated with down-regulation of antibodies directed to exogenous antigen[66-69] and of auto-antibodies associated with autoimmune diseases such as systemic lupus erythematosus (SLE)[70-73] and rheumatoid arthritis[74,75].

Pathological or pernicious autoimmunity develops when recognition of self components results in injury or disease. Autoimmune responses may be initiated when sequestered antigens are released into the general circulation, or self antigens are modified by an external agent (bacterial, chemical or viral) which make them more immunogenic (reviewed in ref. 76). Molecular mimicry by exogeneous antigens may result in the stimulation of T cells and antibodies that can cross-react with self molecules.

3.1.1. Idiotypic interactions in autoimmunity.

Auto-anti-idiotypic antibodies have been found to occur spontaneously in some autoimmune diseases, and their presence may indicate involvement of the idiotypic network in controlling the development of autoimmunity. For example, anti-anti-DNA antibodies are

often found in patients with SLE when the disease is in remission but not when the disease is active[70-73], and anti-idiotypic antibodies to anti-HLA-DR antibodies in rheumatoid arthritis patients correlate with lower auto-antibody levels and disease activity[74,75].

Anti-idiotypic antibodies have been used to modulate the expression of idiotypes in response to antigens. In some cases, the expression of a particular idiotype may be suppressed, whereas in others, the expression of a particular idiotype may be enhanced. Therefore, the response to anti-idiotypic reagents can "go both ways".

Manipulations with idiotypic or anti-idiotypic reagents can influence the pathology of an autoimmune disease. Injection of a monoclonal anti-DNA antibody (bearing the major idiotype) into young (NZB x NZW)F_1 mice suppressed the development of anti-DNA antibodies and nephritis[77-79]. The injection of an anti-idiotypic antibody (to the major idiotype of anti-DNA antibodies) also prolonged the survival of (NZB x NZW)F_1 mice. The production of auto-antibodies to DNA were only temporarily suppressed, and the auto-antibodies that were produced were of a different idiotype (ie. compensatory production of minor idiotypes)[78]. However, in MRL-*lpr/lpr* and MRL-+/+ mice, the injection of anti-idiotypic antibodies to the H130 idiotype (H130 is a major idiotype of anti-DNA antibodies in MRL mice) was found to enhance production of auto-antibodies and H130-idiotype bearing antibodies[80].

Idiotypic manipulation may also play a role in the initiation of an autoimmune disease. A SLE-like disease can be induced in mice that do not spontaneously develop an autoimmune disorder by injection of an anti-DNA monoclonal antibody bearing the 16/6 idiotype[81]. 16/6 is a major idiotype of

anti-DNA antibodies in human SLE[82]. Immunization of normal C3H.SW female mice with 16/6-bearing anti-DNA antibody in CFA elicited the production of anti-idiotypic and anti-anti-idiotypic (16/6 bearing) antibodies. Elevated levels of anti-DNA, anti-Sm, anti-RNP and other auto-antibodies were found in such mice, in addition to other non-immunological symptoms associated with SLE. The 16/6 idiotype bearing antibodies occured concomitantly with the production of anti-DNA antibodies[81]. Mice that were similarly immunised with an anti-idiotypic antibody to the 16/6 idiotype also developed symptoms that were similar to the 16/6 idiotype injected mice[83].

3.1.2. The Role of Co-factors in Acquired Immunodeficiency Syndrome (AIDS).

The Center of Disease Control's (CDC) surveillance definition of AIDS is the presence of one or more "indicator" diseases (eg. Kaposi's sarcoma, Pneumocystis carinii pneumonia) with laboratory evidence of infection with the human immunodeficiency virus (HIV) (eg. anti-HIV antibody). However, the diagnosis of AIDS can also be made without laboratory evidence of HIV infection when certain indicator diseases are present in the absence of defined causes of immunosuppression (eg. corticosteroid therapy, congenital immune deficiency)[84]. HIV had been identified as the probable causative agent of AIDS based on the repeated isolation of the virus from the T lymphocytes of AIDS patients[85-87]. The profound immunosuppression and impaired cell-mediated responses of AIDS is attributed to the depletion of $CD4^+$ T lymphocytes, even though only 1/100 $CD4^+$ T lymphocytes are infected

with HIV[88] and active viral expression can be detected in only 0.1 - 1.0% of the infected T lymphocytes[89,90].

Some researchers have questioned the role that HIV has in inducing the profound immunosuppression and impaired cellular immunity of AIDS, and have suggested that there may be several agents that act as co-factors[42,43,91-96]. These co-factors may act synergistically with HIV to cause AIDS. The addition of tetracycline analogs inhibited the lysis of HIV infected cells *in vitro*, leading Montagnier and colleagues to suggest that a tetracycline-sensitive micro-organism such as mycoplasma may be a co-factor[97]. Shearer and collegues noted the similarities of pathologies of AIDS and graft-versus-host disease (which is a form of alloimmunity) and suggested that the exposure to allogeneic lymphocytes cells may be involved in AIDS[91,92]. The involvement of co-factors in triggering the development of AIDS is further supported by the existance of individuals who have been HIV-seropositive for long periods of time and have not developed AIDS (these individuals presumably have not been exposed to agents that can act as co-factors)[98]. Furthermore, there are individuals who have severe $CD4^+$ T lymphocyte depletion in the absence of HIV infection (although other infections may be present in these individuals)[99-101]. One researcher has even claimed that AIDS is the result of immunosuppression caused by the combination of the consumption of drugs (both psychoactive and medical), malnutrition, parasitic infections and other risk factors and that HIV has nothing to do with AIDS[102-104].

Almost all individuals at risk of HIV infection are likely to be exposed to other agents that can be immunosuppressive. Root-Bernstein has listed several agents, some of which are not immunosuppressive in and of

themselves, but which may act synergistically with HIV to cause AIDS[94,95]. Some of these agents are associated with behavioral risks of the individual, and others have been involved in medical treatments. For example, blood transfusions can be immunosuppressive and have been used to enhance the survival of organ transplants[105,106]. Repeated exposure to sperm, lymphocytes and other soluble agents in ejaculates may be immunosuppressive. The injection of sperm cells into experimental animals resulted in a decrease in CTL responses to TNP-modified self antigens and a decrease in natural killer cell activity[107]. A decrease in the effector/suppressor T lymphocyte ratio and functional T cell deficiency (as measured by local graft-versus-host reactions) were observed in individuals that were repeatedly exposed to ejaculates during anal intercourse[108]. Root-Bernstein suggests that HIV is not the sole causative agent of AIDS, but acts synergistically with other agents that are present prior to, concomitant with, or after infection with HIV[94,95]. Duesberg has suggested a risk-AIDS hypothesis in which an excessive combination of agents (eg. frequent sexual contacts, drug abuse, malnutrition, parasitic infections) alone are sufficient to result in profound immunosuppression, and that HIV is not the infectious agent that causes AIDS. Duesberg repeatedly points out that HIV does not pass Koch's postulates for infectious agents[102-104], and that a simple virus-AIDS hypothesis is not "compatible with orthodox viral pathology"[103].

Both Root-Bernstein and Duesberg argue that AIDS is not a new disease; it is a syndrome of immune deficiency in which the patient dies of an opportuntistic infection or a malignancy [an "old" (indicator) disease]. All of the "indicator" diseases had been previously recognized by the CDC. Root-Bernstein noted that the behavioral risk factors of homosexual men (eg.

frequency of sexual contacts, use of inhalant nitrites, etc.) increased during the period of "sexual revolution and gay liberation" preceeding the recognition of AIDS, and that this may explain the apparent sudden emergence of a new disease[94,95]. Other individuals in the high risk groups for developing AIDS (eg. intravenous drug abusers) may be exposed to many of the same risk factors (eg. foreign lymphocytes, other infectious agents, etc) of homosexual men. Duesberg claims the risk-AIDS hypothesis resolves many of the paradoxes of the virus-AIDS hypotheses such as why AIDS is limited to risk groups and not the general population, and why certain "indicator" diseases are associated with certain behavioral-risk factors[102-104].

3.1.3. Autoimmunity models of AIDS.

Autoimmune phenonmena observed in some HIV-seropositive patients indicate that autoimmunity may be involved in AIDS pathogenesis. Clinical similiarities of HIV infection to classical autoimmune diseases such as SLE and reactive arthritis have been recently reviewed[108]. For example, anti-lymphocyte antibodies have been found in the sera of homosexual AIDS patients[108-113], and in patients with SLE and rheumatoid arthritis[75,114-117]. Autoimmunity models of AIDS pathogenesis are based on a possible cross-reactivity between the HIV envelope protein gp120 and class II MHC, which follows from their complementarities to the CD4 antigen. The structures of class II MHC, CD4 and gp120, and the interactions of these molecules is briefly reviewed.

Structure of Class II MHC Molecules

Class II MHC molecules are cell-surface glycoproteins expressed on the surface of B lymphocytes and antigen-presenting cells such as macrophages. Each class II MHC molecule is comprised of two polypeptide chains that are noncovalently associated. The α chain has a molecular weight of 25,000 to 33,000 daltons and the β chain has a molecular weight of 24,000 to 29,000 daltons. Each chain consists of two external domains (α1, α2, and β1, β2 for α and β chains respectively), a transmembrane and a cytoplasmic domain. The β1 domain is about 90 amino acids and contains a disulfide loop of about 60 amino acids, whereas the α1 domain is shorter and does not have a disulfide loop. The α2 and β2 domains are highly conserved and contain a disulfide loop of about 55 amino acids (reviewed in ref. 170). Both α and β chains are members of the immunoglobulin superfamily. Class II MHC molecules in the mouse are I-A and I-E molecules, and are encoded by genes in the H-2I region. In humans, 3 types of class II MHC molecules have been described; namely, DQ, DP and DR. The molecules are encoded by genes in the HLA-D region. DR appears to be the equivalent of I-E and DQ is the equivalent of I-A based on nucleotide and amino acid sequence homologies (reviewed in ref. 170). The β1 and β2 domains of DR shares 63% and 83% amino acid homology with the β1 and β2 domains of I-E, and the β1 and β2 domains of DQ shares 68% and 86% amino acid homology with the β1 and β2 domains of I-A (reviewed in ref. 170). No crystallographic data for the binding of peptide to class II antigens are available. Based on the homologies and similarities in the structure of class II antigens with class I antigen and β2-microglobulin, it is

predicted that the processed peptide on the antigen fits into the groove between the two distal domains of the two chains (reviewed in ref. 170).

Structure of CD4 molecules.

CD4 is a cell-surface glycoprotein with a molecular weight of 55,000 daltons and is expressed on the surface on some subsets of T lymphocytes, and human monocytes, but not mouse macrophages. The CD4 molecule is comprised of a single polypeptide chain of 433 amino acids and is a member of the immunoglobulin superfamily The CD4 molecule has four extracellular domains, a transmembrane domain and a cytoplasmic domain. 3 of the 4 extracellular domains contain loops formed by disulfide bonds (reviewed in ref. 171). The extracellular segment of murine CD4 has approximately 50% amino acid homology and shares a similar structure with its human counterpart[172,173].

Structure of gp120.

HIV gp120 is an envelope glycoprotein of 533 amino acids. Gp120 remains non-covalently associated with gp41, but is not achored in the viral envelope. Althougth gp120 has not been crystallized, the high number of conserved cysteine residues would indicate that gp120 may have several loop structures formed by intrachain disulfide bonds. Gp120 may also exist in multimeric forms on the viral surface by interchain disulfide bonds (reviewed in ref. 174). Gp120 has three areas of homology with the immunoglobulin superfamily; amino acids 261 - 270 and 415 - 451 have

homology with HLA class II antigens (β chains), and the CD4 binding region resembles the complementarity determining region of immunoglobulin V regions (reviewed in ref. 174). The sites of antigenic determinants on gp120 that elicit antibody production and CTL activity in an infected host are distinct from the sites on gp120 that bind to CD4 and share HLA homology (reviewed in ref. 174).

The interaction of CD4 with class II MHC molecules and the interaction of CD4 with gp120.

CD4 is an accessory molecules which stabilizes the interaction of the T cell receptor with the class II MHC molecule/peptide complex on the antigen presenting cell. The site on the class II MHC molecule that binds to CD4 is unknown. The gp120 envelope glycoprotein of HIV also uses the CD4 molecule as a receptor. The site of gp120 that binds to CD4 has been localized to the carboxy-terminus of the molecule. Monoclonal antibodies that bound to gp120 within amino acids 413 - 460 were effective in inhibiting soluble gp120 from binding to CD4[175,176].

The site on CD4 that is involved in binding class II MHC molecules and gp120 has been determined using monoclonal antibodies and amino acid substitution. The binding sites for gp120 and class II MHC were found to overlap in the first domain of CD4[120-122,124,125]. The interactions between CD4 and class II MHC can be blocked by recombinant gp120[123]. Monoclonal antibodies to the first and second domains of CD4 inhibited CD4-class II MHC interactions[177]. The binding of monoclonal antibodies to the first domain of CD4 could be blocked by gp120[177]. Several monoclonal antibodies were used

to map the epitopes involved in CD4-class II MHC and CD4-gp120 binding, and the results suggested that some epitopes were unique to CD4-class II MHC and CD4-gp120 binding and at least two epitopes were shared. It was concluded that CD4-class II MHC interaction involves multiple contact sites within the first and second domains, whereas the binding site for gp120 is located within the most amino-terminal portion of CD4 in the first domain[177]. Substitution of human to murine homologous residues identified some of the critical residues involved in CD4-gp120 binding. Human CD4, but not murine CD4 binds gp120. Changes to amino acids 48, 50 and 51 (within the first domain of CD4) from human to murine homologous residues affected both gp120[121,125] and class II MHC binding[125], whereas other mutations within the first two domains resulted in the loss of binding with class II MHC, but not the loss of binding with gp120[121,125]. Substitution of murine CD4 residues for human CD4 residues 39 - 43 (human- NQGSF- , mouse- QHGKGV) changed the CD4-gp120 binding affinity, and gp120 did not bind to cells with this mutated CD4 molecule. The substitution of residues 48 - 52 (human- PSKLN- , mouse- GSPSQF) changed the binding affinity but not the number of gp120 binding sites per cell[124]. Other substitutions in the middle of the third domain of CD4 had no effect in number of gp120 binding sites. These experiments would suggest that the region that CD4 and gp120 bind is within the first domain involving residues 39 - 52, and overlaps with the region of CD4 that binds to class II MHC. Serological similarities between gp41 of HIV and class II MHC[126] have been described, and sequence homologies have been reported between a conserved region of gp120 of HIV and class II MHC[127] and between Nef of HIV and class II MHC[128].

The area of gp120 that has class II MHC homology and CD4 binding activity is accessible to monoclonal antibodies that block the binding of gp120 to CD4. This may indicate that this portion of gp120 is accessible to the immune system and may stimulate the production of immune responses that cross-react with host class II MHC molecules. Although antibody and CTL responses to this region of gp120 are not produced during natural infection (reviewed in ref. 174), the immune responses are cross-reacting with host tissues and can not be measured by *in vitro* assays.

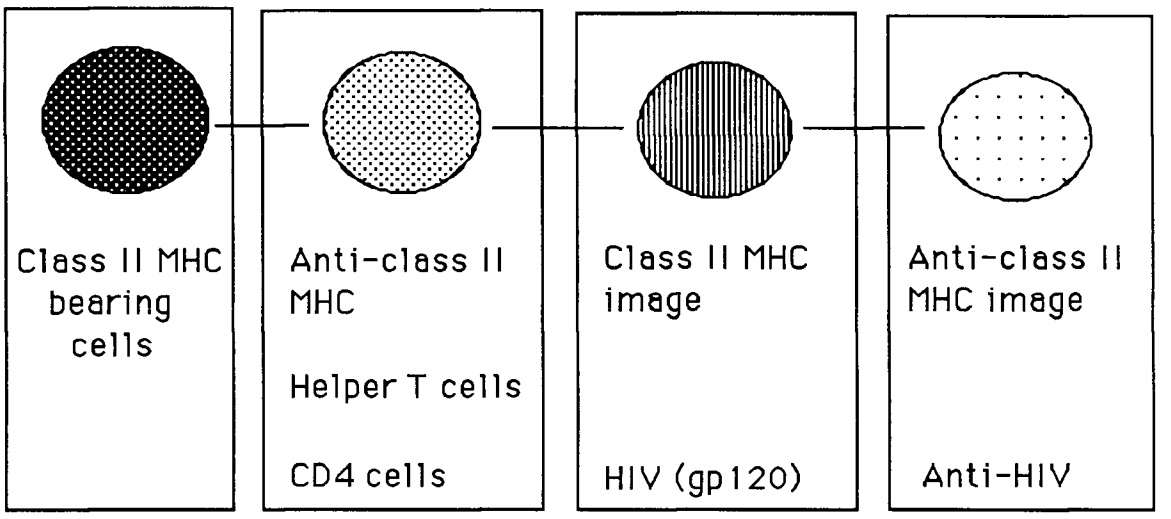
The molecular mimicry of MHC antigens by viruses had been previously suggested by Snell in 1968 to explain how some viruses were able to evade detection by the immune system and become oncogenic in some strains of mice, but did not become oncogenic in mice strains with different MHC haplotypes[131]. Fig. 15 is a schematic drawing showing the relationship between some components of the immune system and components of HIV[43].

Ziegler and Stites[129], and Andrieu et al.[130] suggested that AIDS is an autoimmune disease triggered by immune recognition of the epitopes on envelope proteins of HIV that have homology with class II MHC. Thus, the immune response directed against gp120 may include lymphocytes and antibodies that cross-react with class II MHC, and the concommitant auto-anti-idiotypic response could be directed against CD4[129,130]. These responses were postulated to be involved in blocking communications between CD4 lymphocytes, B lymphocytes and antigen processing cells[129], and could possibly be involved in the destruction of such non-infected class II MHC-bearing cells[130], thus breaking tolerance to self class II MHC.

Figure 15. The relationship between some key components of the immune system and components of HIV.

The receptors or antigens in this sequence have three-dimensional complementarity with their neighbours. 1. Class II MHC is the first level of this sequence. 2. CD4 and helper T cells with receptors that are anti-class II MHC are on the second level. The receptors on allogeneic lymphocytes that are stimulated by host class II are on this level as well. 3. The third level includes T cells that have V regions with epitopes that are images of class II MHC. The viral envelope glycoprotein gp120 of HIV is on this level. Other proteins of HIV that mimic class II MHC would also be on this level. This would include gp41, Nef and possibly p24. 4. The fourth level includes the immune response to HIV as well as the anti-(class II MHC-image) response. It is plausible that anti-HIV responses react with cells bearing MHC-image determinants.

From Hoffmann, G. W., Kion, T. A. and Grant, M. D., "An Idiotypic Network Model of AIDS Immunopathogenesis", *PNAS*, 88, 3060 - 3064, 1991 (reference 43).



3.1.3. MHC-image-anti-MHC-image model of AIDS pathogenesis.

A different autoimmunity model of AIDS pathogenesis has emerged from the symmetrical network model[42,43]. Our model of AIDS pathogenesis involves the immune response to two stimuli, namely HIV and allogeneic lymphocytes (or other MHC mimicking stimuli). The two responses include components that are directed against each other, and are postulated to synergize in a way which causes the collapse of the immune system.

An important component in the symmetrical network theory is the concept of network focussing. It is postulated that the network has the ability to select for clones that are images of particular antigens, some of which include self antigens[42,43]. The V regions of helper T cells have complementarity to self class II MHC molecules. Some suppressor T cells are postulated to have V regions that are complementary to the helper T cell idiotypes. Bidirectional stimulatory interactions of anti-class II MHC helper T cell idiotypes and class II MHC-image suppressor T cell idiotypes could be mutually stabilizing[42,43]. The two sets of cells shown in Figure 14 can be thought of as being like a tent, consisting of a centre pole and a canvas. The helper T cells correspond to the "canvas", and suppressor T cells bearing the internal image of class II correspond to the "centre pole".

Another important concept of the theory is that the immune system has a major axis of specificities that is determined by the antigens of the MHC. Excessive stimulation along this major axis triggered by the combination of HIV and allogeneic stimuli may result in AIDS[42,43]. In people exposed to HIV and allogeneic lymphocytes, there is the possibility of developing two immune responses that are directed against each other,

although they are provoked by two different stimuli. Since gp120 is an "image" of class II MHC (with respect to the epitopes that bind the same regions of CD4 as class II MHC), the response to HIV could include an anti-(MHC-image) response. The response to allogeneic lymphocytes includes an MHC-image response; this is a response to the receptors on the allogeneic lymphocytes that recognize host MHC antigens. (This has been described in detail in Chapter 2). The two responses may be expected to synergize and not only attack HIV and the foreign anti-self idiotypes (on the allogeneic lymphocytes), but also the endogenous "centre pole" (MHC-image cells) and the anti-class II MHC T helper cell idiotypes ("canvas"), thus destabilizing the central T cell idiotypic structure. Since all helper T lymphocytes are stimulated, there is no control preventing help to B cells specific for other self antigens.

People in some high risk groups may be exposed to allogeneic lymphocytes that are present in blood and ejaculates. Shearer noted the similarities of the pathologies of AIDS and GVH disease, thus suggesting a possible connection between alloimmunity and AIDS[91,92,132]. Shearer and his colleagues reported that CTL responses to "self + X" antigens were decreased[133] but CTL responses to HLA alloantigens were increased in some HIV-seropositive homosexual men (with and without clinical evidence of AIDS) when compared to control heterosexual men[133,134]. The increase in CTL responses to HLA alloantigens may have resulted from exposure to alloantigens[115] or by viruses that have epitopes that cross-react with HLA antigens. The repeated exposure to ejaculates (which contains lymphocytes) during anal intercourse can result in alloimmunization[108]. Intravenous drug users may be exposed to only small amounts of allogeneic cells. It is

postulated that this small amount of allogeneic stimuli may be sufficient to trigger the MHC-image clones[42,43]. Clones responding to HIV could then become the stimulus for the MHC-image clones, and the MHC-image/anti-MHC-image responses could become self-perpetuating[42,43].

The idea of complementarity of lymphocytes in the induction of autoimmune disease has been discussed before. Westall and Root-Bernstein proposed a model for experimental allergic encephalitis (EAE) in which one set of lymphocytes is induced by myelin basic protein (MBP) and the other set of lymphocytes is induced by the adjuvant. MBP and the adjuvant are stereochemically complementary, and therefore, the lymphocytes directed against both antigens are complementary[135]. They postulated that the equal and simultaneous stimulation of both sets of lymphocytes is required for the induction of EAE[135].

However, not all AIDS cases have involved exposure to allogeneic lymphocytes. In some cases other factors may play the role of allogeneic stimuli. The association of mycobacterial infections that may include cross-reactions with HLA and autoimmunity have been recently reviewed[136,137]. For example, there is serological cross-reactivity with M. leprae and HLA DR[138] and M. leprae and HIV[139] which may be of significance. The MHC-image response usually triggered by allogeneic lymphocytes may be triggered by epitopes on foreign antigens (eg. M. leprae) that mimic class II MHC, and be further perpetuated by immune responses to HIV.

We have been able to do experiments relevant to this model in mice, even though HIV does not bind to the CD4 molecule of mice. The rationale was that the suppressor T cell centre pole (class II MHC bearing T cells in Figure 14) may be at least partly conserved across species since some MHC

determinants are conserved[140]. During alloimmunizations, anti-centre pole antibodies may be produced from stimulation of anti-MHC-image clones in the host by the MHC-image clones in the inoculum (ie. "image-of-class-II-MHC" suppressor T cells). A reagent that could permit the detection of such anti-centre pole antibodies is gp120 since it also resembles class II MHC. We find that alloimmune mice do indeed make both MHC-image and anti-MHC-image antibodies. The MHC-image response is anti-anti-(self MHC), and the anti-MHC-image response is anti-(foreign MHC-image). The anti-MHC-image response cross-reacts with gp120.

3.2. Results.

3.2.1. Anti-MHC-image (anti-gp120) antibodies in alloimmune sera.

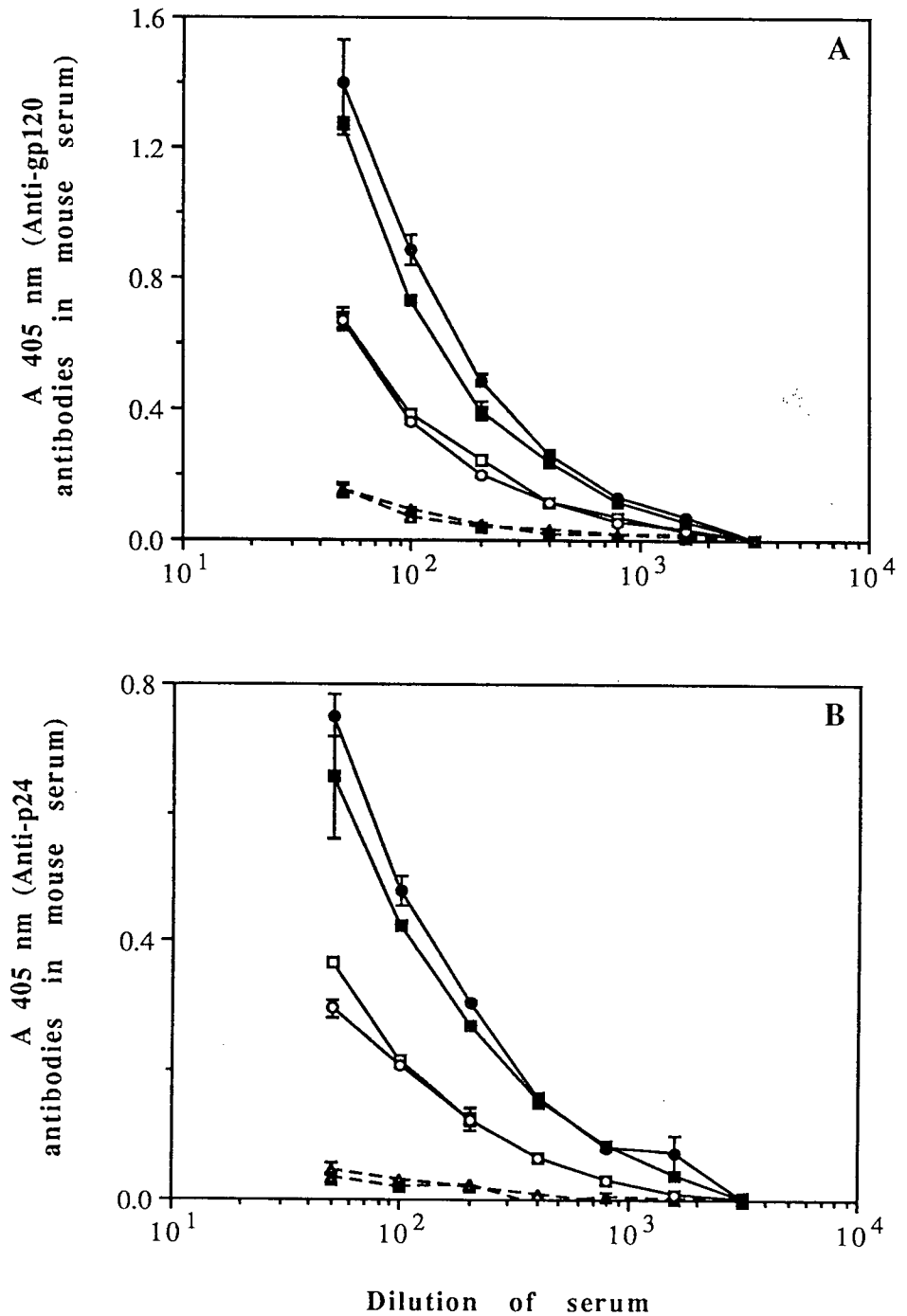
The MHC-image clones in the inoculum of immunocompetent lymphocytes may stimulate anti-MHC-image (anti-centre pole) responses during alloimmunization. Gp120 of HIV may be a suitable reagent for detecting anti-centre pole antibodies as discussed above.

We therefore tested alloimmune murine sera in an ELISA assay for reactivity against recombinant HIV proteins gp120 and p24. Gp120 and p24 were coated directly to ELISA plates at concentrations of 0.4 μg/ml in carbonate buffer. Doubling dilutions of normal and alloimmune sera were made starting at 1/50 and incubated on the plates for 3 hours at 37°C. The bound antibodies were detected with biotinylated goat anti-mouse IgG, Strept. avidin-alkaline phosphatase and the appropriate substrate. Anti-gp120 antibodies and anti-p24 antibodies (Figures 16A and 16B respectively) were

Figure 16. Alloimmune sera and alloimmune, immunogen-absorbed (AIA) sera contain antibodies that bind to gp120 and p24 of HIV.

Recombinant gp120 or p24 of HIV were coated directly to ELISA plates (20 ng/well). After incubation with the sera at the dilutions indicated, the bound antibodies were detected by biotinylated goat anti-mouse IgG, Strep. avidin-alkaline phosphatase conjugate and substrate (t = 3 hours). Each point represents the mean and standard deviation of duplicates. Panel A. Antibodies that bind to gp120 can readily be detected in B6 anti-Balb/c antiserum (❑), B6 anti-Balb/c AIA serum (○), Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA serum (●), but in neither normal B6 serum (△) nor Balb/c serum (▲). Panel B. Antibodies that bind to p24 can readily be detected in B6 anti-Balb/c antiserum (❑), B6 anti-Balb/c AIA serum (○), Balb/c anti-B6 antiserum (■) and Balb/c anti-B6 AIA serum (●), but in neither normal B6 serum (△) nor Balb/c serum (▲).

Statistical analysis of the data (at the 1/50 dilution) by ANOVA.
Panel A. B6 anti-Balb/c and B6 anti-Balb/c AIA sera are significantly different than normal B6 serum with p values of $\leq$ 0.025 and $\leq$ 0.01 respectively. B6 anti-Balb/c and B6 anti-Balb/c AIA sera are different with a p value of $\leq$ 0.25. Balb/c anti-B6 and Balb/c anti-B6 AIA sera are different than normal Balb/c serum with p values of $\leq$ 0.025 and $\leq$ 0.01 respectively. Balb/c anti-B6 and Balb/c anti-B6 AIA sera are different with a p value of $\leq$ 0.25.
Panel B. B6 anti-Balb/c and B6 anti-Balb/c AIA sera are significantly different than normal B6 serum with p values of $\leq$ 0.01. B6 anti-Balb/c and B6 anti-Balb/c AIA sera are different with a p value of $\leq$ 0.25. Balb/c anti-B6 and Balb/c anti-B6 AIA sera are different than normal Balb/c serum with p values of $\leq$ 0.025 and $\leq$ 0.01 respectively. Balb/c anti-B6 and Balb/c anti-B6 AIA sera are different with a p value of $\leq$ 0.25.


A
A 405 nm (Anti-gp120 antibodies in mouse serum)
1.6
1.2
0.8
0.4
0.0
10^1
10^2
10^3
10^4
B
A 405 nm (Anti-p24 antibodies in mouse serum)
0.8
0.4
0.0
10^1
10^2
10^3
10^4
Dilution of serum

present in both B6 anti-Balb/c and Balb/c anti-B6 alloantisera, but not in normal B6 serum nor Balb/c serum. Absorbing the alloimmune sera against the immunogen did not result in the loss of the anti-gp120 or the anti-p24 activity. Anti-gp120 and anti-p24 antibodies could be found in all other alloimmune sera tested, including alloimmune sera made between MHC congeneic mouse strains (Table 1). The sera were tested in an ELISA as described above, but at a dilution of 1/100 and ELISA plates coated with gp120 or p24 at a concentration of 0.2 μg/ml. Since the mice have never been immunised with gp120, a reasonable interpretation is that the anti-gp120 antibodies are anti-MHC-image antibodies. The finding of anti-p24 antibodies in alloimmune mice was unexpected. We also tested the serum of individual alloimmune and normal mice for both anti-gp120 and anti-p24 activities, and found such activity in all alloimmune mice, but not in any of the normal mice (Table 2). Anti-p24 antibodies may also be anti-MHC-image if there are serological cross-reactivities between p24 and class II MHC. Gp120, gp41 and Nef are all similar to class II MHC, so p24 could also be similar to class II MHC if there is evolutionary selection for HIV components to resemble class II MHC. The molecular mimicry of viruses to host cell antigens (as measured by the cross-reactivity of anti-viral antisera and monoclonal antibodies with antigens in host cell extracts or on uninfected host cells) has been described for several viruses[154-157]. Molecular mimicry by a virus may result in persistent infections in which the virus has evaded detection by the host's immune system. The differences in the anti-gp120 reactivities in Tables 1 and 2 may reflect glycosylation differences in the two batches of gp120. The gp120 reagents were found to have different migration patterns in SDS-PAGE[178]. Additional controls that

Table 1. Anti-gp120 and anti-p24 antibodies in several alloimmune sera detected by ELISA.

Sera	A 405 nm on gp120[a,b]	A 405 nm on p24[a,b]	time of incubation (h)
B6 NMS	0.068 ± 0.007[c]	0.030 ± 0.002	3
Balb/c NMS	0.095 ± 0.008	0.018 ± 0.002	3
B6 anti-Balb/c	0.387 ± 0.008	0.215 ± 0.001	3
Balb/c anti-B6	0.731 ± 0.013	0.427 ± 0.000	3
B6 NMS[b]	0.217 ± 0.033	0.092 ± 0.074	2
CBA NMS[b]	0.138 ± 0.016	0.020 ± 0.003	2
B6 anti-CBA[d]	0.867 ± 0.017	0.517 ± 0.006	2
CBA anti-B6[d]	0.692 ± 0.093	0.683 ± 0.063	2
C3H NMS	0.227 ± 0.002	0.036 ± 0.005	2
C3H.SW NMS	0.097 ± 0.004	0.044 ± 0.002	2
C3H anti-C3H.SW	0.389 ± 0.052	0.111 ± 0.008	2
C3H.SW anti-C3H	0.716 ± 0.039	0.203 ± 0.002	2

a) All serum samples were diluted 1/100 and incubated on ELISA plates coated with gp120 or p24 (10 ng/well). Bound antibodies were detected with biotinylated goat anti-mouse IgG and Strep. avidin-alkaline phosphatase and the appropriate substrate. Sera was raised between several mouse strains by repeated immunization with allogeneic lymphocytes as described in Chapter 7.

b) All serum samples tested were tested on recombinant gp120 and p24 from HIV (MicroGeneSys) except where noted.

c) The values calculated are the mean ± standard deviation.

d) This group of sera were tested on recombinant p24 from the National Institute of Allergy and Infectious Disease (NIAID).

Table 2. Anti-gp120 and anti-p24 antibodies in individual normal and alloimmune mice.

Serum	Anti-gp120 (A405 nm)[a]	Anti-p24 (A405 nm)[a]
B6 NMS 1	0.033	0.030
2	0.008	0.019
3	0.025	0.072
4	0.021	0.020
5	0.068	0.051
mean ± standard error	0.031 ± 0.010	0.038 ± 0.010
B6 anti-CBA 1	0.313	0.292
2	0.576	0.498
3	0.277	0.282
4	0.542	0.557
5	0.223	0.227
mean ± standard error	0.376 ± 0.077[b]	0.372 ± 0.064[c]
CBA NMS 1	0.014	0.019
2	0.000	0.024
3	0.000	0.020
4	0.018	0.049
5	0.005	0.048
6	0.021	0.044
mean ± standard error	0.010 ± 0.004	0.034 ± 0.006
CBA anti-B6 1	0.411	0.438
2	0.437	0.505
3	0.787	0.776
4	0.481	0.464
5	0.335	0.299
6	0.821	0.752
mean ± standard error	0.545 ± 0.084[d]	0.539 ± 0.077[e]

a) All serum samples were diluted 1/100 and incubated on ELISA plates coated with gp120 or p24 (NIAID, 20 ng/well). Bound antibodies were detected with goat anti-mouse Ig-alkaline phosphatase conjugate and the appropriate substrate.
b) Significantly different from the normal B6 group with $p \leq 0.005$ by ANOVA.
c) Significantly different from the normal B6 group with $p \leq 0.005$ by ANOVA.
d) Significantly different from the normal CBA group with $p \leq 0.005$ by ANOVA.
e) Significantly different from the normal CBA group with $p \leq 0.0001$ by ANOVA.

could have been done include other viral antigens such as gp70, and conventional protein antigens such as KLH or ovalbulim. This would confirm that anti-gp120 and anti-p24 antibodies are a result of alloimmunization and not simply a result of polyclonal stimulation.

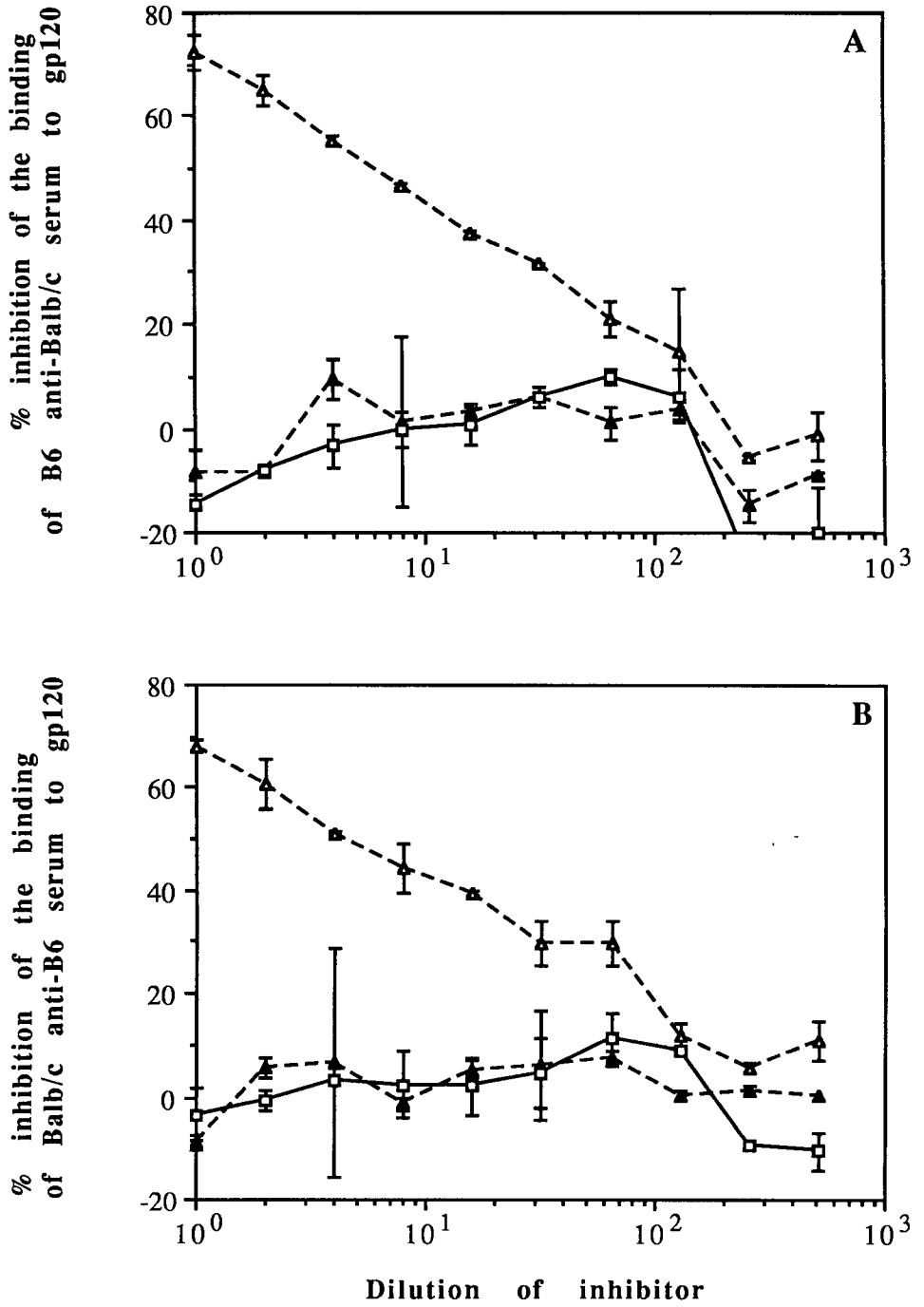
We attempted to inhibit the binding of anti-gp120 antibodies in alloantisera with free gp120, p24 or bovine serum albumin (BSA). Gp120 was coated to the ELISA plate at a concentration of 0.4 µg/ml in carbonate buffer. The alloimmune serum was diluted to a concentration of 1/200 in $PBST_{20}$ (phosphate buffered saline with 0.05% Tween 20) + 1% casein, and 25 µl of serum was added to each well. Gp120, p24 and BSA were diluted in $PBST_{20}$ + 1% casein to a concentration of 2.5 µg/ml (followed by doubling dilutions), and 25 µl of inhibitor was added to each well. The final dilution of antiserum (1/400) gave approximately 50% of the maximum signal observed in the absence of any inhibitors (Figure 16). Antibodies that bound to the gp120 on the ELISA plate were detected with biotinylated goat anti-mouse IgG, Strept. avidin-alkaline phosphatase conjugate and the appropriate substrate. As shown in Figure 17, only free gp120 was able to inhibit the binding of antibodies in alloantisera to bound gp120. The binding of anti-p24 antibodies in alloantisera was not inhibited by free p24, gp120 or BSA at the concentrations tested. ELISAs were done at both 4°C and 37°C, and the concentration of p24 was increased to 5 µg/ml with the alloantisera at a final dilution of 1 in 1000, but no inhibition of binding of anti-p24 was observed (data not shown). These results suggest that the antibody that binds to gp120 is not the same antibody that binds to p24. Alternatively, the p24 binding site could be separate from the gp120 binding site, or the affinity to p24 is much lower than the affinity to gp120. Another possibility is that binding of

antibodies to p24 on the plate is divalent binding, while any inhibition of free antibody with free p24 involves a much weaker monovalent interaction, and hence is not seen in our assay. A second alternative explanation it that the binding of p24 to the ELISA plate may have exposed an epitope on p24 that was not available for antibody binding when p24 was in the soluble form.

Since anti-gp120 antibodies are interpreted to be anti-MHC-image antibodies, free gp120 should inhibit the binding of MHC-image antibodies in biotinylated alloimmune, immunogen-absorbed (AIA) sera from binding to anti-MHC-image antibodies coated on an ELISA plate. For this experiment, we coated ELISA plates with MHC-image antibodies at a dilution of 1/10,000 in carbonate buffer. The AIA serum was diluted to a concentration of 1/100 in $PBST_{20}$ + 1% casein, and 25 µl of serum was added to each well. Gp120, p24 and BSA were diluted in $PBST_{20}$ + 1% casein to a concentration of 2.5 µg/ml (followed by doubling dilutions), and 25 µl of inhibitor was added to each well. Bound antibodies were detected with Strept. avidin-alkaline phosphatase conjugate and the appropriate substrate. Both normal B6 serum and B6 anti-Balb/c AIA serum, but not normal Balb/c serum nor Balb/c anti-B6 AIA serum inhibited the binding of biotinylated Balb/c anti-B6 AIA serum to B6 MHC-image antibodies on the ELISA plate. This result was expected since both normal B6 serum and B6 anti-Balb/c AIA serum had been previously shown to contain MHC-image antibodies (Figures 8 and 10). However, gp120, p24 and BSA (at initial concentrations of 2.5 µg/ml) did not inhibit the binding of biotinylated Balb/c anti-B6 AIA serum to B6 MHC-image antibodies on the ELISA plate (data not shown). Furthermore, neither gp120 nor p24 inhibited the binding of anti-MHC antibodies to MHC-image

Figure 17. Anti-gp120 antibodies in alloimmune sera are inhibited by gp120, but are not inhibited by p24 or BSA.

ELISA plates were coated with gp120 (20 ng/well) as described in Figure 16. The alloimmune serum was diluted in buffer and 25 µl was added to each well. 25 µl of diluted inhibitor [gp120 (△), p24 (▲) or BSA(❑)] was added to each well, and the plates were incubated for 2 hours. The bound antibodies were detected by biotinylated goat anti-mouse IgG, Strep. avidin-alkaline phosphatase conjugate and substrate (t = 2 hours). Each point represents the mean and standard deviation of duplicates. The final dilution of the B6 anti-Balb/c antiserum was 1/150 and the Balb/c anti-B6 antiserum was 1/200, and the final concentration of the inhibitor in the first well was 2.5 µg/ml. The inhibitor was titrated by doubling dilutions. Only gp120 (△) inhibited the binding of anti-gp120 antibodies in alloimmune serum to the gp120 on the ELISA plate. This may suggest that anti-gp120 and anti-p24 antibodies are two different populations, or that gp120 and p24 do not bind to the same site on the antibody. An alternative explanation is that the affinity of anti-MHC-image antibodies to gp120 is higher than the affinity to p24.


% inhibition of the binding of B6 anti-Balb/c serum to gp120
A
80
60
40
20
0
-20
10^0
10^1
10^2
10^3
% inhibition of the binding of Balb/c anti-B6 serum to gp120
B
Dilution of inhibitor

antibodies bound to an ELISA plate (data not shown).

Since these results were unexpected, it may indicate that there is a subpopulation of anti-MHC-image antibodies that cross-react with gp120 or p24, or the affinity of anti-MHC-image antibodies to gp120 is lower than the affinity of anti-MHC-image antibodies to MHC-image antibodies.

3.3. Summary

Alloimmunisation of mice induces the production of at least three classes of specific antibodies; namely anti-MHC, MHC-image and anti-gp120/anti-p24. Antibodies that bind to gp120 are interpreted as being anti-MHC-image, because gp120 is considered to be an image of class II MHC based on its complementarity to CD4. Soluble gp120 inhibited the binding of anti-gp120 antibodies to bound gp120, but did not inhibit the interactions between anti-MHC and MHC-image antibodies, or MHC-image and anti-MHC-image antibodies. These results suggest that anti-MHC-image antibodies contain a population of antibodies that cross-react with gp120 or that the affinity anti-MHC-image antibodies to gp120 is lower than the affinity of MHC-image antibodies. Another possibility is that the alloimmunization of mice stimulates the production of antibodies to an endogenous virus which cross-reacts with gp120 and p24. This possibility could be ruled out by screening pre-immune and immune serum for antibodies to viruses that are typically found in a colony. A second possibility is that alloimmunization results in polyclonal stimulation. This possibility can tested by screening the pre-immune and immune serum against a large panel of antigens, including conventional protein antigens (e.g. KLH) and viral proteins.

Chapter 4. *Systemic Lupus Erythematosus.*

4.1. Introduction.

Systemic lupus erythematosus (SLE or lupus) is a generalized autoimmune disease of unknown etiology. SLE is characterized by B cell hyperactivity (and possibly defects in suppressor T cell functions), in which autoantibody production results in the development of lesions and the clinical manifestations of the disease. The word lupus (Latin for wolf) was used initially to describe the morbid cutaneous lesions rather than to designate a specific disease[141,142].

In 1971, the Arthritis Foundation published a paper in the Bulletin on the Rheumatic Diseases titled "Preliminary Criteria for the Classification of Systemic Lupus Erythematosus". The purpose of this paper was to establish a uniform set of criteria for disease diagnosis. It included fourteen criteria which described the various abnormalities often noted in the diagnosis of SLE. A person was said to have SLE if he had any four or more of the criteria present serially or simultaneously during any interval of observation[143]. In 1982 these criteria were revised to improve disease classification (Table 3). Some manifestations involving the same organ were aggregated into a single criterion, and others were not included because of low sensitivity and specificity[144]. The basis of SLE diagnosis has remained unchanged since then.

Clinical symptoms of SLE include malar rash, discoid rash, photosensitivity, arthritis, serositis, glomerulonephritis, neurological disorders, and hematological disorders. Immunological disorders include

Table 3. Revised Criteria for the Classification of *Systemic Lupus Erythematosus.*

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight
4. Oral ulcers	Oral or nasopharyngeal ulceration
5. Arthritis	Nonerosive arthritis involving 2 or more peripheral joints
6. Serositis	a) Pleuritis - OR b) Pericarditis -
7. Renal disorder	a) Persistent proteinuria - OR b) Cellular casts - may be red cell, hemoglobulin, granular, tubular, or mixed
8. Neurologic disorder	Seizures or psychosis- in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	a) Hemolytic anemia - with reticulocytosis - OR b) Leukopenia - OR c) Lymphopenia -OR d) Thrombocytopenia - in the absence of offending drugs
10. Immunologic disorder	a) Positive LE cell preparation - OR b) Anti-DNA: antibody to native DNA in abnormal titer - OR c) Anti-Sm: presence of antibody to Sm nuclear antigen -OR d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

Adapted from E. M. Tan et al., The 1982 Revised Criteria for the Classification of Systemic Lupus Erthematosus, *Arthritis Rheum.*, 25, pp. 1271 - 1277, 1982 (reference 126).

hypergammaglobulemia, anti-nuclear antibody, and antibody to native DNA or nuclear Sm antigen[144]. SLE is a difficult disease to diagnose. The clinical symptoms and severity of the symptoms can be diverse, and no two patients present with the same collection of symptoms[145]. In some patients SLE is mild and does not affect their longevity, whereas in other patients SLE is violent and fatal. Relapse and remission of SLE is unpredictable.

4.2. MRL Mouse Models of SLE.

Several mouse strains spontaneously develop an autoimmune disease with many of the immunological abnormalities of human SLE. Most of the work on autoimmunity presented in this thesis has been done with the MRL mouse strains.

The MRL mouse strains were developed from a series of crosses involving 4 inbred strains. The MRL genome was estimated to be derived from the following strains in the following proportions: 75% LG/J, 12.6% AKR/J, 12.1% C3H/Di and 0.6% C57Bl/6J[146]. After 12 generations of inbreeding, the line was divided into 2 substrains (MRL/n and MRL/l). There was a 100% occurence of lymphoadenopathy in the MRL/l substrains by the 13th generation. It was estimated that by the 13th generation, the two mouse strains shared 89% of their genome[146]. While the mice retained skin allografts (from the other substrain), it was suggested that the 11% difference in their genomes could influence *in vivo* cell transfers and *in vitro* cell interactions[147]. Breeding experiments confirmed that the massive lymphoproliferation was the result of a single autosomal recessive gene which was called lymphoproliferation, *lpr*[146]. The *lpr* gene was backcrossed

to the MRL/n substrain and to other inbred mouse strains. MRL/n was redesignated MRL/Mp-+/+ (or MRL-+/+) and the congeneic strain was named MRL/Mp-*lpr/lpr* (or MRL-*lpr/lpr*)[146,147]. The residual difference between the genomes of MRL-+/+ and MRL-*lpr/lpr* mice was estimated to be less than 0.1%[146]. By mid-1978, the MRL-*lpr/lpr* strain replaced the MRL/1 strain for distribution by the Jackson Laboratory[147].

MRL-*lpr/lpr* mice develop an early-onset form of disease with 50% mortality in males and females at about 5 to 6 months. The usual cause of death in these mice is subacute proliferative glomerulonephritis[146]. Erythematosus skin lesions and necrosis of the tips of the ears may also be present[147]. The massive lymph node enlargement is the result of proliferation of lymphocytes with admixtures of histiocytes, plasma cells and immunoblasts. Approximately 90% of the proliferating cells are T cells with the $Thy1^+$, Ly 1^-, $Ly23^-$ phenotype, and are weakly responsive to phytohemagglutinin stimulation[146]. Splenomegaly and enlargement of the thymus medulla (following early progressive atrophy of the cortex) also develops in these mice[147]. MRL-+/+ mice develop a late-onset form of the disease with 50% mortality in females and males of 17 and 23 months respectively[146,147].

Serologically, the mice have elevated levels of polyclonal IgG and IgM, circulating immune complexes and autoantibodies that bind to nuclear antigens, double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and retroviral envelope protein gp70. Antibodies to the glycoprotein Sm are present in about 40% of MRL-+/+ mice and 20% of MRL-*lpr/lpr* mice[147].

Swollen joints of the hindlimbs are obvious in some of the older MRL-*lpr/lpr* mice. Deteriorating articular cartilage, synovial cell proliferation,

and pannus formation resembles features found in rheumatoid arthritis[147]. IgG and IgM rheumatoid factors are present in about two-thirds of older MRL-*lpr/lpr* mice. Thus, the MRL-*lpr/lpr* mouse may be suitable for studies in rheumatoid arthritis as well as SLE[147].

4.3. Results and Discussion.

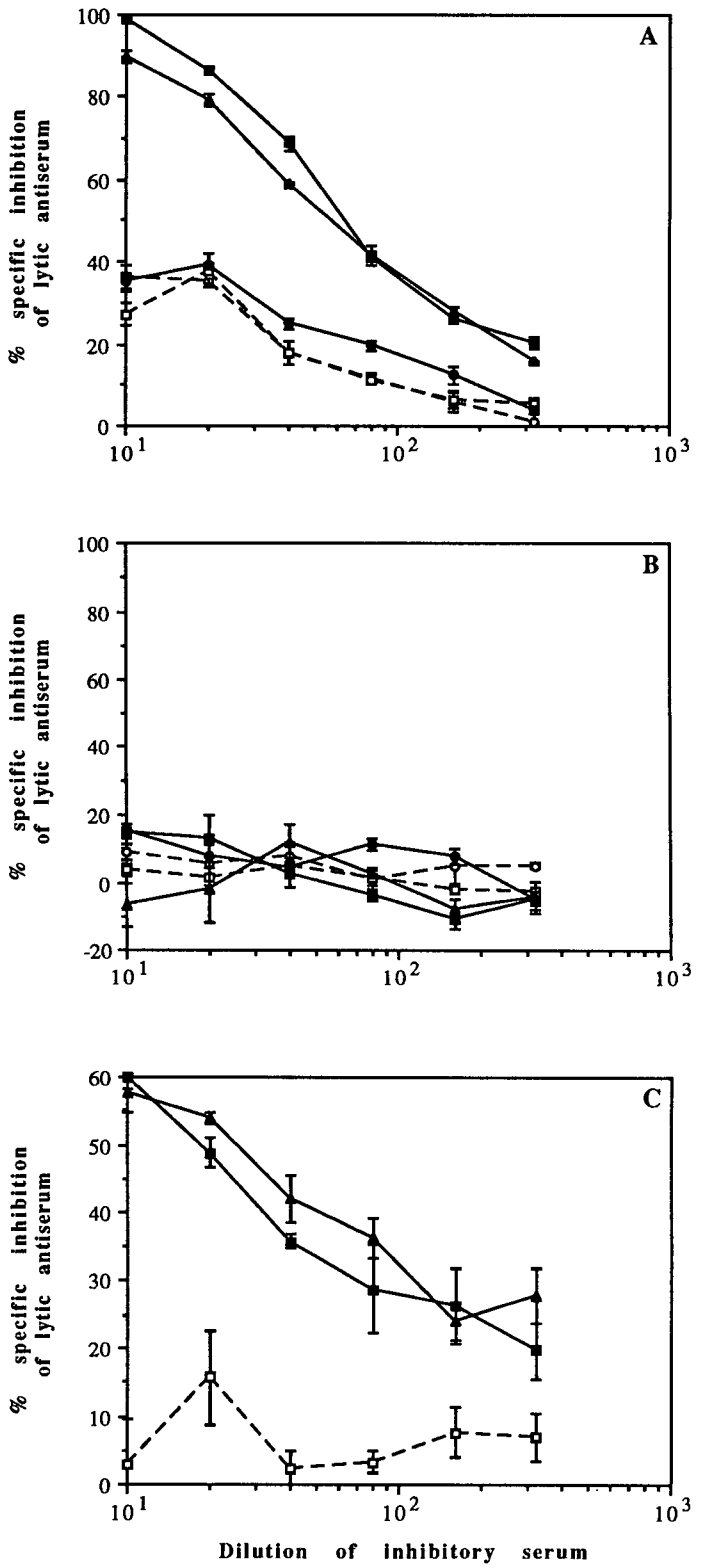
4.3.1. MHC-image antibodies in autoimmune sera.

It seemed conceivable that antibodies with V regions that mimic self MHC molecules could interfere with normal immunoregulatory processes. We therefore assayed the sera of autoimmune MRL mice for anti-anti-self MHC (MHC-image) antibodies based firstly on their ability to inhibit the cytotoxicity of Balb/c anti-CBA and CBA anti-Balb/c antisera (Figure 18). The MHC of MRL mice is of the *k* haplotype, and antibodies with *k* MHC-image activity should be detected by the specific inhibition of anti-*k* cytotoxicity. Sera from age-matched CBA mice (also H-2^k) served as negative controls. For these experiments, the MRL sera were pooled from groups containing 10 to 20 mice and the CBA sera were pooled from groups containing 8 to 10 mice. Serum from 1 month old MRL-*lpr/lpr* mice did not inhibit the cytotoxicity of any of the antisera. Serum from 2 month old MRL-*lpr/lpr* mice inhibited the killing of CBA (H-2^k) lymph node cells by Balb/c anti-CBA antiserum (Figure 18A), but not the killing of Balb/c (H-2^d) lymph node cells by CBA anti-Balb/c antiserum (Figure 18B). The inhibition of an anti-H-2^k alloantiserum prepared from MHC-congeneic mouse strains (B10.D2 anti-B10.BR) further

Figure 18. Antibodies with MHC-image activity can be detected in 2 month old MRL-*lpr/lpr* by the inhibition of anti-H-2^k antibody-mediated cytotoxicity.

^{51}Cr-labelled lymph node cells were incubated with the inhibitory and lytic antisera for 20 minutes, washed, and incubated with complement for 60 minutes. Cell lysis was determined by measuring the amount of ^{51}Cr released into the supernatant. Each point represents the mean and standard deviation of triplicates. Panel. A. The serum from 2 month old MRL-*lpr/lpr* mice (■) specifically inhibits the killing of CBA lymph node cells by Balb/c anti-CBA antiserum. Sera from one month old CBA mice (○), 2 month old CBA mice (❑) and one month old MRL-*lpr/lpr* mice (●) mice does not have this inhibitory activity. The absorption of serum from 2 month old MRL-*lpr/lpr* mice against normal mouse immunoglobulin does not remove this inhibitory activity, indicating that the inhibitory activity is not due to rheumatoid factors (▲). Panel B. The killing of Balb/c lymph node cells by CBA anti-Balb/c antiserum is not inhibited by the serum from 2 month old MRL-*lpr/lpr* mice (■). Additional controls: Sera from one month old CBA mice (○), 2 month old CBA mice (●) and one month old MRL-*lpr/lpr* mice (●), and 2 month old MRL-*lpr/lpr* mice absorbed with normal mouse immunoglobulin (▲). Panel C. The serum from 2 month old MRL-*lpr/lpr* mice specifically inhibits the killing of B10.BR lymph node cells by B10.D2 anti-B10.BR antiserum (■). The absorption of serum from 2 month old MRL-*lpr/lpr* mice against normal mouse immunoglobulin does not remove this inhibitory activity (▲). Additional control: Sera from 2 month old CBA mice (❑). The inhibition of this antiserum indicates that the inhibitory activity is related to the MHC. In the absence of inhibitors, Balb/c anti-CBA antiserum killed 67.3% of target cells, CBA anti-Balb/c antiserum killed 67.0% of target cells and B10.D2 anti-B10.BR antiserum killed 79.1% of target cells.

Statistical analysis of the data (at the1/10 dilution) by ANOVA.
Panel A. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are significantly different than normal CBA serum with p values of ≤ 0.01 and ≤ 0.025 respectively. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are significantly different with a p value of ≤ 0.005.
Panel B. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are different than normal CBA serum with p values of ≤ 0.025 and ≤ 0.25 respectively. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are significantly different with a p value of ≤ 0.05.
Panel C. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are significantly different than normal CBA serum with p values of ≤ 0.005. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are different with a p value of ≤ 0.25.


A
B
C
% specific inhibition of lytic antiserum
Dilution of inhibitory serum
100
80
60
40
20
0
-20
50
30
10
10^1
10^2
10^3

confirmed the inhibitory antibodies had MHC-image activity (Figure 18C). It was concluded that 2 month MRL-*lpr/lpr* mice make specific MHC-image antibodies but one month old mice do not yet do so. The possibility that the inhibition of cytotoxicity is due solely to RF in this assay is ruled out by the specificity of the sera from 2 month old MRL-*lpr/lpr* mice in inhibiting only anti-H-2^k antiserum. Furthermore, absorption of sera from 2 month old MRL-*lpr/lpr* mice against normal mouse immunoglobulin (Ig) coupled to agarose did not remove the specific inhibitory activity (MHC-image antibodies) (Figure 18A).

The sera of 5 month old MRL-*lpr/lpr* mice inhibited the cytotoxicity of both antisera tested (Figure 19). This is due to the presence of an additional activity in the 5 month sera, namely rheumatoid factor (RF), which is specific for the constant part of immunoglobulins. The non-specific component of the inhibitory activity in the sera of 5 month old MRL-*lpr/lpr* mice was shown to be due to RF. Absorption against normal mouse Ig removes the non-specific component, and leaves the specific component intact; the sera then inhibits only anti-H-2^k antiserum as can be seen by comparing the curves of the absorbed sera in Figures 19A and 19B.

Immunoglobulin (and not, say, soluble MHC antigen) is responsible for the inhibitory activity of MRL-*lpr/lpr* sera because the activity is removed by absorption against goat anti-mouse IgG. Table 4 shows that MRL-*lpr/lpr* serum absorbed against goat anti-mouse IgG fails to inhibit the killing of CBA lymph node cells by Balb/c anti-CBA antiserum. MRL-*lpr/lpr* serum absorbed against normal mouse Ig served as the positive control and retained the inhibitory MHC-image antibodies.

Sera from 12 month old MRL-+/+ also contain MHC-image antibodies

Figure 19. Antibodies with MHC-image activity can be detected in 5 month old MRL-*lpr/lpr* by the inhibition of anti-H-2^k antibody-mediated cytotoxicity.

^{51}Cr-labelled lymph node cells were incubated with the inhibitory and lytic antisera for 20 minutes, washed, and incubated with complement for 60 minutes. Cell lysis was determined by measuring the amount of ^{51}Cr released into the supernatant. Each point represents the mean and standard deviation of triplicates. Panel. A. The serum from 5 month old MRL-*lpr/lpr* mice (■) specifically inhibits the killing of CBA lymph node cells by Balb/c anti-CBA antiserum. Sera from one month old CBA mice (○), 5 month old CBA mice (○) and one month old MRL-*lpr/lpr* mice (●) mice does not have this inhibitory activity. The absorption of serum from 5 month old MRL-*lpr/lpr* mice against normal mouse immunoglobulin does not remove this inhibitory activity, indicating that the inhibitory activity is not due to rheumatoid factors (▲). Panel B. The killing of Balb/c lymph node cells by CBA anti-Balb/c antiserum is inhibited by the serum from 5 month old MRL-*lpr/lpr* mice (■), however, this non-specific inhibitory activity is reduced to background levels after absorption with normal mouse immunoglobulin (▲). This non-specific inhibitory activity is presumably due to the presence of RF. Additional controls: Sera from one month old CBA mice (○), 5 month old CBA mice (❑) and one month old MRL-*lpr/lpr* mice (●). In the absence of inhibitors, Balb/c anti-CBA antiserum killed 72.4% of target cells and CBA anti-Balb/c antiserum killed 67.0% of target cells.

Statistical analysis of the data (at the 1/10 dilution) by ANOVA.
Panel A. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are significantly different than normal CBA serum with p values of ≤ 0.0001 and ≤ 0.005 respectively. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are significantly different with a p value of ≤ 0.01.
Panel B. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are different than normal CBA serum with p values of ≤ 0.01 and ≤ 0.25 respectively. MRL-*lpr/lpr* and absorbed MRL-*lpr/lpr* sera are significantly different with a p value of ≤ 0.025.

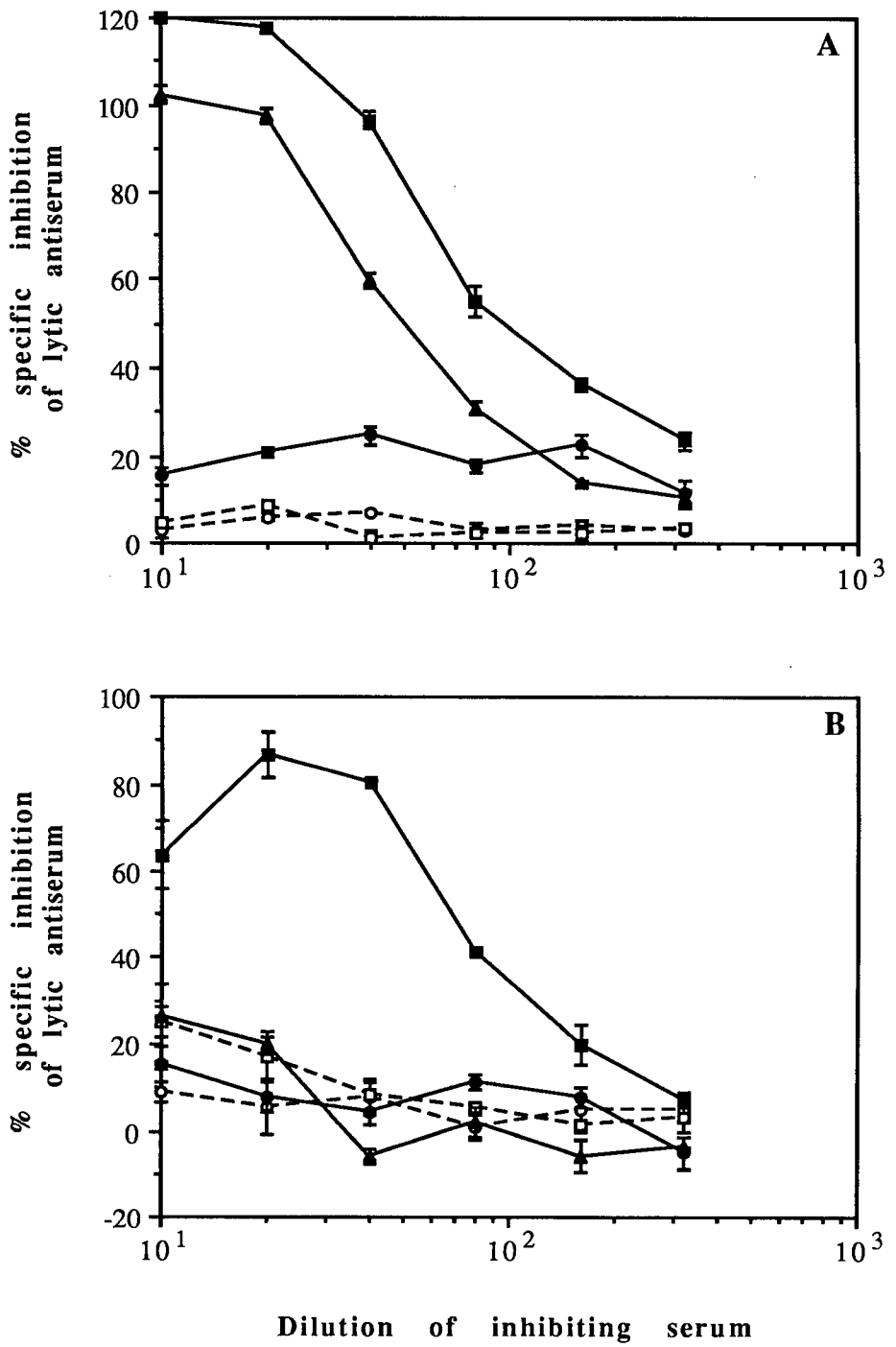

A
B
% specific inhibition of lytic antiserum
% specific inhibition of lytic antiserum
120
100
80
60
40
20
0
-20
10^1
10^2
10^3
Dilution of inhibiting serum

Table 4. MHC-image activity in MRL-*lpr/lpr* sera is immunoglobulin.

Sera	Treatment	% Inhibition[a]
normal CBA[b]	none	1.2 +/- 2.0 [c]
MRL-*lpr/lpr*[d]	none	103.0 +/- 1.7
MRL-*lpr/lpr*	abs. MIg	95.6 +/- 1.8
MRL-*lpr/lpr*	abs. GαMIg	-1.2 +/- 1.6

a) The maximum amount of killing in the absence of any inhibiting serum was 66.0 ± 2.2%.
b) Sera from 5 month old CBA mice was used.
c) The values calculated are the mean ± standard deviation.
d) Sera from 5 month old MRL-*lpr/lpr* mice were used.

that inhibit the cytotoxicity of Balb/c anti-CBA sera (Figure 20). These antibodies could not be absorbed out with normal mouse Ig, but could be absorbed out with an anti-mouse Ig (Figure 20).

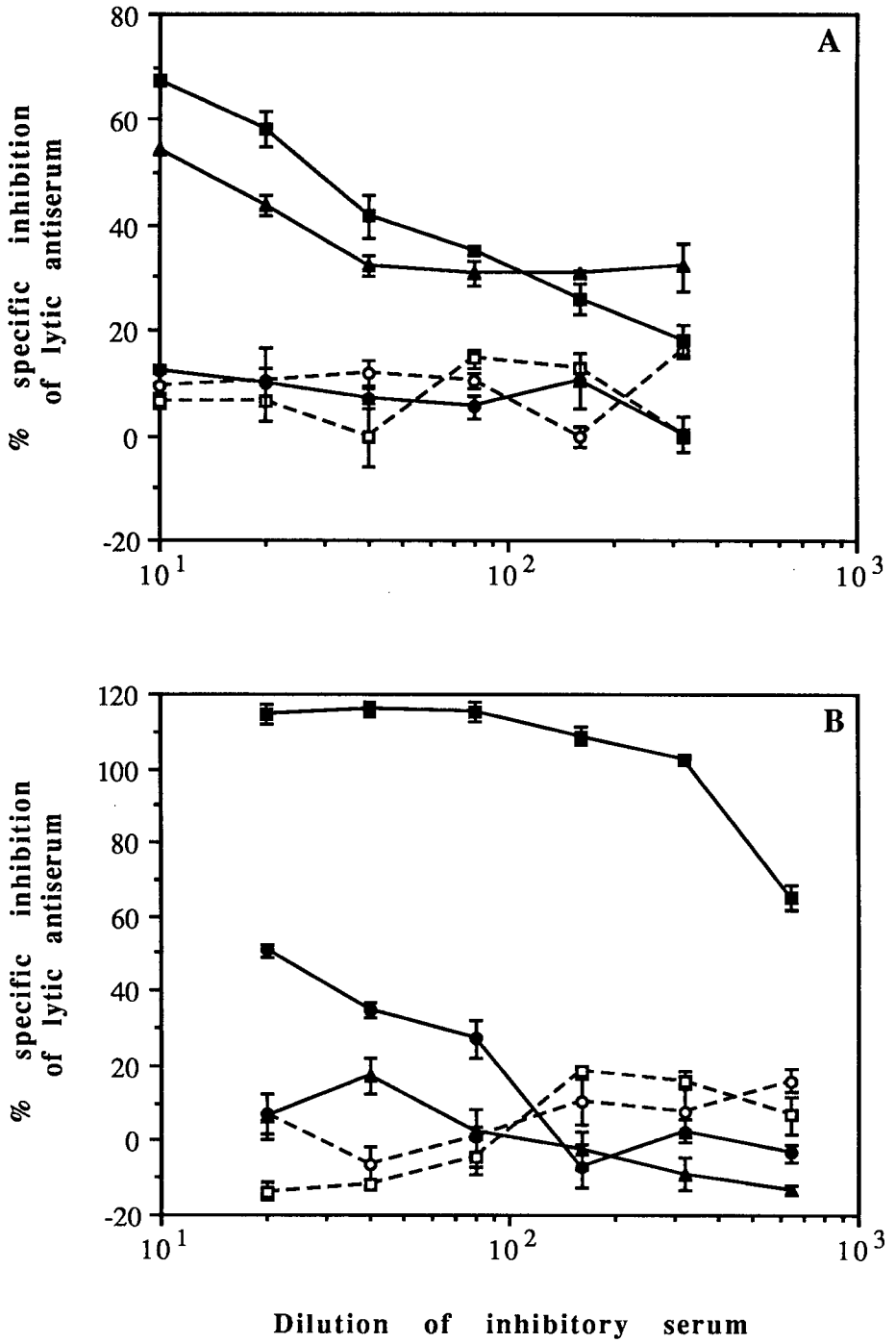
MHC-image antibodies in MRL sera were also detected in a second assay, namely an ELISA using biotinylated IgG purified from polyclonal alloantisera with normal IgG as the control. Sera from normal CBA, MRL-*lpr/lpr* and MRL-+/+ mice were coated directly to ELISA plates at a dilution of 1/10,000. The MRL sera were pooled from groups containing 10 to 20 mice and the CBA sera were pooled from groups containing 8 to 10 mice. After washing and blocking the uncoated binding sites with casein, the ELISA plates were reacted with 100 ng/well of biotinylated IgG purified from normal sera or alloantisera for 3 hours at 37°C. The binding of the biotinylated IgG to the plates was detected with Strep. avidin-alkaline phosphatase conjugate and substrate.

Figure 21 shows the result using IgG purified from normal sera or alloantisera prepared by reciprocal immunization of the congeneic mouse strains C3H (H-2^k) and C3H.SW (H-2^b). In this assay, serum samples were tested from several ages of CBA, MRL-*lpr/lpr* and MRL-+/+ mice. Sera from CBA (panel A), MRL-*lpr/lpr* (panel B) and MRL-+/+ (panel C) showed reactivity with biotinylated IgG purified from alloimmune sera. The strongest signal observed is with the C3H.SW anti-C3H IgG (anti-H-2^k) indicating the presence of H-2^k MHC-image antibodies, and a lower level of binding of C3H anti-C3H.SW IgG which is anti-H-2^b. This indicates that there is lots of cross-reactivity in the MHC-image antibodies as detected in this assay. The lowest signal observed is with IgG from C3H.SW or C3H normal serum, which would indicate that the differential binding is not due to just

Figure 20. Antibodies with MHC-image activity can be detected in 12 month old MRL-+/+ by the inhibition of anti-H-2^k antibody-mediated cytotoxicity.

^{51}Cr-labelled lymph node cells were incubated with the inhibitory and lytic antisera for 20 minutes, washed, and incubated with complement for 60 minutes. Cell lysis was determined by measuring the amount of ^{51}Cr released into the supernatant. Each point represents the mean and standard deviation of triplicates. Panel A. The serum from 12 month old MRL-+/+mice (■) specifically inhibits the killing of CBA lymph node cells by Balb/c anti-CBA antiserum. Sera from one month old CBA mice (○), 12 month old CBA mice (❑) and one month old MRL-+/+ mice (●) mice does not have this inhibitory activity. The absorption of serum from 12 month old MRL-+/+ mice against normal mouse immunoglobulin does not remove this inhibitory activity, indicating that the inhibitory activity is not due to rheumatoid factors (▲). Panel B. The killing of Balb/c lymph node cells by CBA anti-Balb/c antiserum is inhibited by the serum from 12 month old MRL-+/+ mice (■), but this inhibitory activity is removed after absorption with normal mouse immunoglobulin (▲). The partial inhibition of CBA anti-Balb/c antiserum by one month old MRL-+/+ mice (●) is presumably due to the presence of RF. Additional controls: Sera from one month old CBA mice (○) and 12 month old CBA mice (❑). In the absence of inhibitors, Balb/c anti-CBA antiserum killed 73.2% of target cells and CBA anti-Balb/c antiserum killed 72.7% of target cells.

Statistical analysis of the data (at the 1/10 dilution) by ANOVA.
Panel A. MRL-+/+and absorbed MRL-+/+ sera are significantly different than normal CBA serum with p values of $\leq$ 0.005. MRL-+/+ and absorbed MRL-+/+ sera are significantly different with a p value of $\leq$ 0.005.
Panel B. MRL-+/+ and absorbed MRL-+/+ sera are different than normal CBA serum with p values of $\leq$ 0.005 and $\leq$ 0.1 respectively. MRL-+/+ and absorbed MRL-+/+ sera are significantly different with a p value of $\leq$ 0.005.


A
B
% specific inhibition of lytic antiserum
% specific inhibition of lytic antiserum
80
60
40
20
0
-20
120
100
80
60
40
20
0
-20
10^1
10^2
10^3
Dilution of inhibitory serum

anti-allotype antibodies or rheumatoid factors in the CBA, MRL-*lpr/lpr* or MRL-+/+ sera coated to the plates. Sera from MRL-*lpr/lpr* and MRL-+/+ mice showed higher levels of specific reactivity (with both alloimmune sera) than did sera from CBA mice, suggesting elevated levels of MHC-image antibodies in these mice strains. The amount of MHC-image antibodies increases with the age of the animal. The level of activity against IgG from normal mouse sera is also slightly elevated in MRL-*lpr/lpr* and MRL-+/+ sera compared to the level for CBA sera, indicating that this assay also detects some RF activity.

MHC-image antibodies were also detected in the same CBA and MRL sera by ELISA using biotinylated monoclonal antibodies purified from ascites or tissue culture supernatants. All monoclonal antibodies used in the experiment of Figure 22 were of the IgG_{2a} isotype. The ELISA plates were coated with sera from CBA, MRL-*lpr/lpr* and MRL-+/+ mice at the dilutions indicated. After washing and blocking the uncoated binding sites with casein, the ELISA plates were reacted with 100 ng/well of biotinylated monoclonal antibody for 3 hours at 37°C. The binding of the biotinylated IgG to the plates was detected with Strep. avidin-alkaline phosphatase conjugate and substrate. Figure 22 shows the reactivity of sera from 2 month old MRL-*lpr/lpr* mice (Panel A) and 12 month old MRL-+/+ mice (Panel B) with anti-I-A^k and anti-H-$2K^kD^k$ monoclonal antibodies. In this experiment, the negative control monoclonal antibodies are anti-I-A^b, anti-H-$2K^bD^b$, anti-$V\beta 8$, and anti-β-2-6-linked fructosan. In both the MRL-*lpr/lpr* and MRL-+/+ sera, specific reactivity at a dilution of greater than 1 in 10^4 is seen with the anti-I-A^k monoclonal antibody. Sera from age matched CBA mice were used as additional controls (2 and 12 months old in Panels C and D respectively) and showed lower reactivities with the monoclonal antibodies than the MRL

Figure 21. MHC-image antibodies in MRL mice can be detected by ELISA.

Sera from CBA mice (Panel A), MRL-*lpr/lpr* mice (Panel B) and MRL-+/+ mice (Panel C) were coated to ELISA plates at a dilution of 1/10,000. The plates were reacted with 100 ng/well of purified biotinylated IgG from normal C3H (■), normal C3H.SW (▩), C3H anti-C3H.SW (▨) (anti-H-2^b) or C3H.SW anti-C3H (□) (anti-H-2^k) and bound IgG was detected with avidin-alkaline phosphatase and substrate (t = 16 hours). The lowest signal is with the IgG from the normal C3H and C3H.SW, which indicates the differences in the binding is not due to anti-allotype antibodies or RF. Although there is cross-reactivity with the anti-H-2^b and the anti-H-2^k reagents, the strongest signal generated is with the anti-H-2^k reagent on CBA, MRL-*lpr/lpr* and MRL-+/+ sera. Sera from MRL-*lpr/lpr* mice has more MHC-image activity than sera from MRL-+/+ mice which in turn has more MHC-image activity than sera from CBA mice. Each bar represents the mean and standard deviation of duplicates.

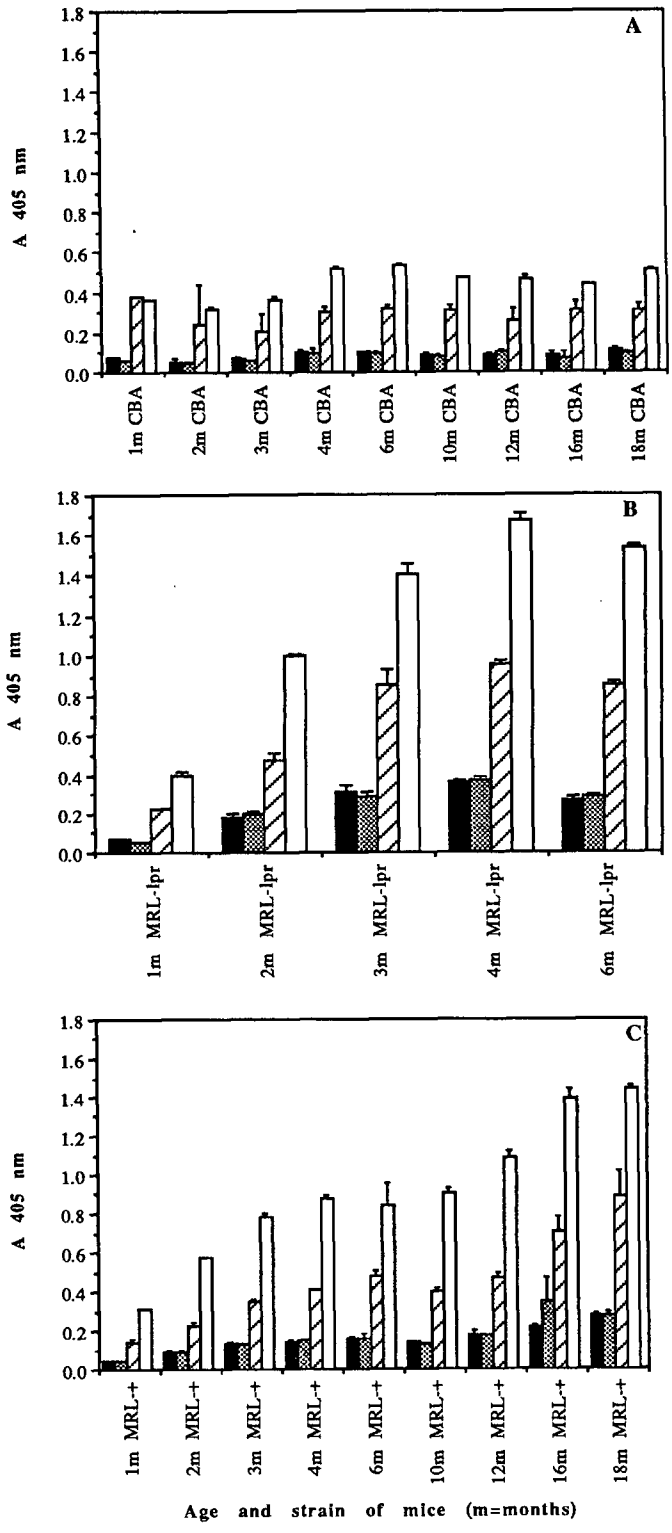
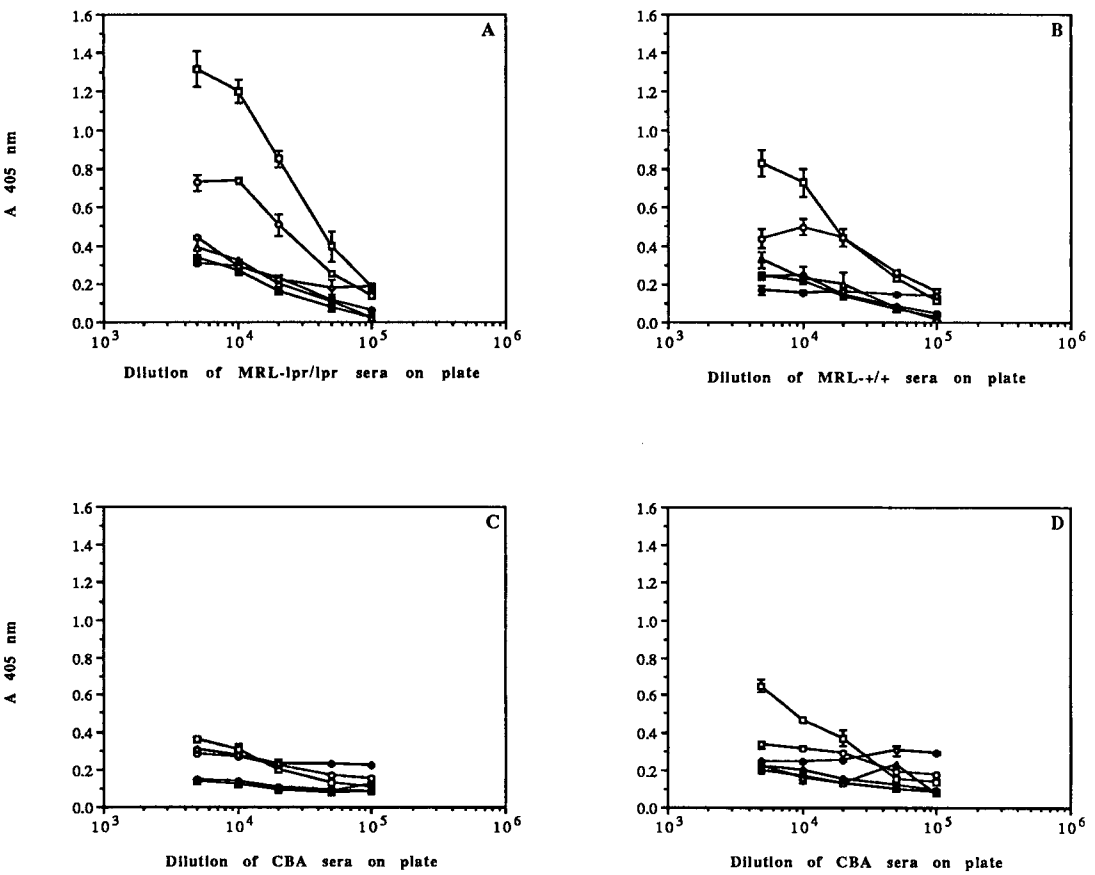

A
A 405 nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1m CBA
2m CBA
3m CBA
4m CBA
6m CBA
10m CBA
12m CBA
16m CBA
18m CBA
B
A 405 nm
1m MRL-lpr
2m MRL-lpr
3m MRL-lpr
4m MRL-lpr
6m MRL-lpr
C
A 405 nm
1m MRL-+
2m MRL-+
3m MRL-+
4m MRL-+
6m MRL-+
10m MRL-+
12m MRL-+
16m MRL-+
18m MRL-+
Age and strain of mice (m=months)

Figure 22. MHC-image antibodies in MRL mice can be detected by ELISA using biotinylated monoclonal antibodies.

Sera from 2 month old MRL-*lpr/lpr* mice (Panel A), 18 month old MRL-+/+ mice (Panel B), 2 month old CBA mice (Panel C) and 18 month old CBA mice (Panel D) were coated to ELISA plates at the dilutions indicated. The plates were reacted with 100 ng/well of purified biotinylated monoclonal antibody. The bound antibody was detected with avidin-alkaline phosphatase and substrate (t = 16 hours). Each point represents the mean and standard deviation of duplicates. The following monoclonal antibodies were used: anti-I-A^k (❑), anti-K^kD^k (○), anti-I-A^b (△), anti-K^bD^b (■), F23.1 (anti-$V_{\beta 8}$) (◇) and anti-β-2-6-linked fructosan (●). All monoclonal antibodies are IgG_{2a}. The sera from CBA, MRL-*lpr/lpr* and MRL-+/+ show reactivity with the anti-I-A^k and anti-K^kD^k monoclonal antibodies, but little reactivity with the other monoclonal antibodies. The sera from MRL-*lpr/lpr* and MRL-+/+ mice have more reactivity with the monoclonal antibodies than the sera from CBA mice.

Statistical analysis of the data (at the 1/3000 dilution) by ANOVA.
Panel A. The reactivities of MRL-*lpr/lpr* serum with anti-I-A^k and anti-K^kD^k monoclonal antibodies compared with anti-I-A^b anti-K^bD^b monoclonal antibodies respectively are different with p values of ≤ 0.05 and ≤ 0.025.
Panel B. The reactivities of MRL-+/+ serum with anti-I-A^k and anti-K^kD^k monoclonal antibodies compared with anti-I-A^b anti-K^bD^b monoclonal antibodies respectively are different with p values of ≤ 0.01.
Panel C. The reactivities of CBA serum with anti-I-A^k and anti-K^kD^k monoclonal antibodies compared with anti-I-A^b anti-K^bD^b monoclonal antibodies respectively are different with p values of ≤ 0.05 and ≤ 0.025.
Panel D. The reactivities of CBA serum with anti-I-A^k and anti-K^kD^k monoclonal antibodies compared with anti-I-A^b anti-K^bD^b monoclonal antibodies respectively are different with p values of ≤ 0.25 and ≤ 0.01.


A
B
C
D
A 405 nm
A 405 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^3
10^4
10^5
10^6
Dilution of MRL-lpr/lpr sera on plate
Dilution of MRL-+/+ sera on plate
Dilution of CBA sera on plate
Dilution of CBA sera on plate

sera. The higher reactivity with anti-I-A^k monclonal antibody (compared with the other monoclonal antibodies) would indicate that sera of normal mice contain some antibodies with MHC-image activity. A lower level of antibodies with MHC-image activity in the sera of normal mice had been previously detected with the inhibition of cytotoxicity assay and ELISA in Chapter 2. Sera from MRL-*lpr/lpr* mice show higher reactivity with the anti-class I MHC and anti-class II MHC monoclonal antibodies than do MRL-+/+ mice. The reactivity with the anti-class II MHC monoclonal antibody was stronger than the anti-class I MHC monoclonal antibody in both MRL mouse strains.

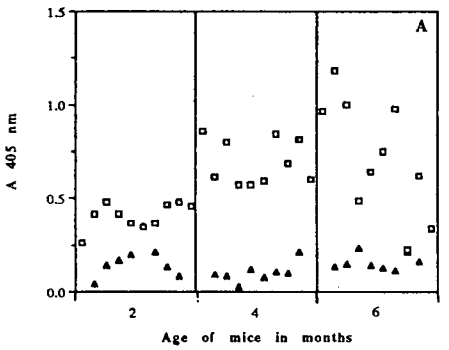
Individual serum samples from CBA, MRL-*lpr/lpr* and MRL-+/+ mice were tested for MHC-image antibodies using biotinylated monoclonal antibodies (Figure 23). This experiment measured the variation in the amount of MHC-image antibodies produced in the three mouse strains at various ages. For each age group, we included 8 samples from CBA mice, and 10 samples each for MRL-*lpr/lpr* and MRL-+/+ mice. The sera of MRL-*lpr/lpr* mice aged 2, 4 and 6 months, and MRL-+/+ mice aged 6, 12, and 18 months (along with sera from aged-matched CBA mice as the control) were monoclonal antibodies. The variation in the amount of reactivity (as indicated by the standard error of the mean) of the sera with the monoclonal antibodies, particularly with the anti-H-2^k monoclonal antibodies, tended to increase with the age of the mice, with MRL-*lpr/lpr* mice having larger increases in the variation than MRL-+/+ and CBA mice. The is also some cross-reactivity of the MHC-image antibodies in MRL-*lpr/lpr* sera with the anti-H-2^b monoclonal antibody.

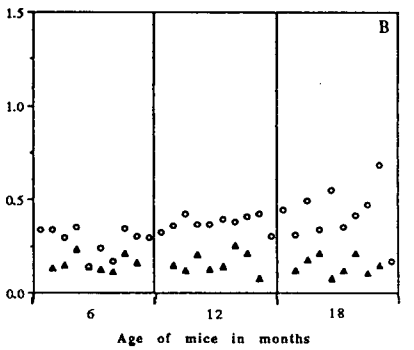
MRL-*lpr/lpr* and MRL-+/+ mice spontaneously produce antibodies

Figure 23. MHC-image antibodies in individual MRL mice can be detected by ELISA using biotinylated monoclonal antibodies.

Panels A, C, E, G. Sera from MRL-*lpr/lpr* mice (❑) aged 2, 4 and 6 months and of age-matched CBA mice (▲) were used to coat the ELISA plate at a dilution of 1/10,000. Panels B, D, F, H. Sera from MRL-+/+ mice (○) aged 6, 12 and 18 months and of age-matched CBA mice (▲) were used to coat the ELISA plate at a dilution of 1/10,000. The plates were incubated with 100 ng/well of purified, biotinylated monoclonal antibodies, and the bound antibodies were detected with avidin-alkaline phosphatase and substrate. The following antibodies were used: anti-K^kD^k (Panel A, B), anti-I-A^k (Panel C, D), anti-β-2-6-linked fructosan (Panel E, F) and anti-K^bD^b (Panel G, H). The sera of MRL-*lpr/lpr* and MRL-+/+ mice showed the highest reactivity with the anti-H-2^k monoclonal antibodies. Each sample was tested in duplicate.

Statistical analysis of the data by ANOVA is described in Table 5.


A
A 405 nm
1.5
1.0
0.5
0.0
2
4
6
Age of mice in months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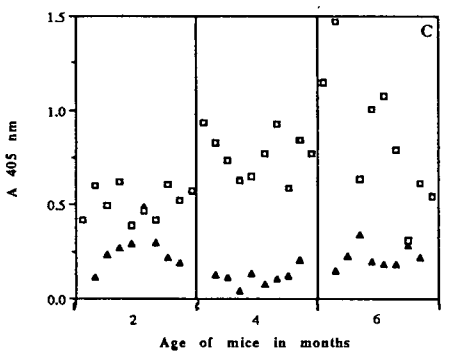
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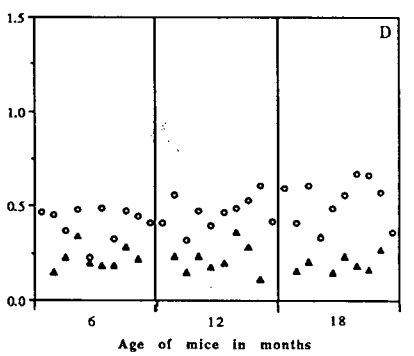
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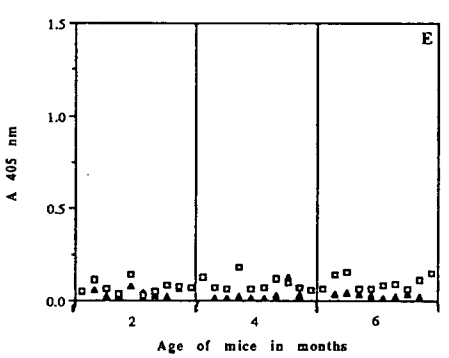
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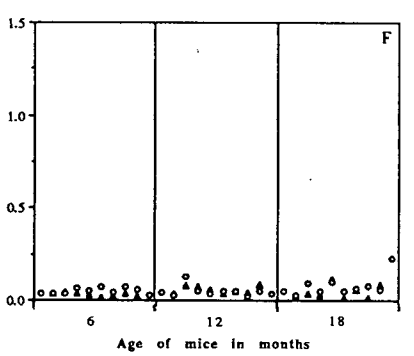
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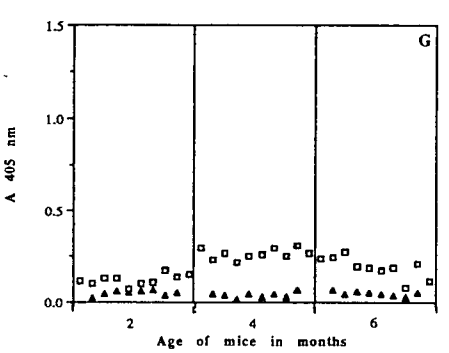
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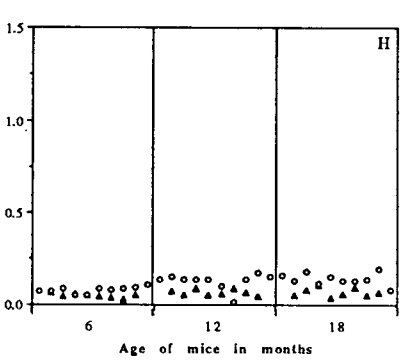
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Age of mice in months

Table 5. MHC-image antibodies in autoimmune mice; summary of data shown in Figure 23.

Mouse[b]	age in months	A 405 nm (mean ± standard error)[a] anti-anti-H-2K^{k}D^k	anti-anti-I-A^k	anti-anti-H-2K^{b}D^b	anti-anti-fructosan (control)
CBA	2	0.167 ± 0.034	0.264 ± 0.038	0.048 ± 0.004	0.042 ± 0.008
MRL-*lpr/lpr*	2	0.407 ± 0.022[c]	0.511 ± 0.028[c]	0.120 ± 0.009[c]	0.071 ± 0.011[e]
CBA	4	0.102 ± 0.018	0.115 ± 0.017	0.036 ± 0.005	0.032 ± 0.014
MRL-*lpr/lpr*	4	0.695 ± 0.038[c]	0.768 ± 0.038[c]	0.260 ± 0.009[c]	0.093 ± 0.013[d]
CBA	6	0.159 ± 0.015	0.224 ± 0.022	0.045 ± 0.004	0.031 ± 0.004
MRL-*lpr/lpr*	6	0.720 ± 0.098[c]	0.894 ± 0.124[c]	0.187 ± 0.018[c]	0.099 ± 0.012[d]
MRL-+/+	6	0.283 ± 0.024[d]	0.412 ± 0.027[c]	0.080 ± 0.005[d]	0.049 ± 0.005[f]
CBA	12	0.162 ± 0.020	0.219 ± 0.028	0.063 ± 0.005	0.052 ± 0.007
MRL-+/+	12	0.375 ± 0.013[c]	0.466 ± 0.027[d]	0.136 ± 0.006[c]	0.051 ± 0.009[g]
CBA	18	0.146 ± 0.018	0.213 ± 0.023	0.066 ± 0.009	0.047 ± 0.013
MRL-+/+	18	0.425 ± 0.045[c]	0.527 ± 0.039[d]	0.136 ± 0.010[d]	0.079 ± 0.018[h]

a) The ELISA plates were coated with CBA, MRL-*lpr/lpr* and MRL-+/+ sera at a dilution of 1/10,000. After blocking the uncoated sites with casein, the plates were incubated with 100 ng/well of biotinylated monoclonal antibody. The bound monoclonal antibodies were detected by avidin-alkaline phosphatase conjugate and substrate. All wells were done in duplicate.
b) The number of mice used in this experiment for each age group: CBA (n=8), MRL-*lpr/lpr* (n=10 for ages 2 and 4 months, and n=6 for age 6 months) and MRL-+/+ (n=10).
c) Significantly different from the CBA group with $p \leq 0.0001$ by ANOVA.
d) Significantly different from the CBA group with $p \leq 0.005$ by ANOVA.
e) Different from the CBA group with $p \leq 0.1$ by ANOVA.
f) Significantly different from the CBA group with $p \leq 0.025$ by ANOVA.
g) Different from the CBA group with $p \leq 0.25$ by ANOVA.
h) Significantly different from the CBA group with $p \leq 0.05$ by ANOVA.

with MHC-image activity as detected first with the inhibition of cytotoxicity assay and later with the ELISA. The specificity of the MHC-image antibodies was confirmed by the inhibition of an alloantiserum produced by immunization of MHC-congeneic mice, and with higher reactivities with anti-H-2 monoclonal antibodies than the monoclonal antibodies directed against non-H-2 antigens. MHC-image antibodies reacted with both anti-class I and anti-class II MHC monoclonal antibodies, indicating MHC-image antibodies contain both class I and class II MHC-image determinants. The experiments of Figures 21 - 23 could have been improved by using purified immunoglobulin to coat the ELISA plates with a uniform amount of immunoglobulin.

The MHC-image antibodies in MRL mice were not RF; they were not removed by absorption with normal mouse Ig and retained the activity that specifically inhibited anti-H-2^k cytotoxicity. The reactivity with the monoclonal antibodies was similar for both the unabsorbed and absorbed (to remove RFs) MRL sera, indicating that the interactions with the monoclonal antibodies were not simply due to RFs.

4.3.2. Anti-gp120 and anti-p24 antibodies in MRL mice.

MHC-image antibodies that develop spontaneously in MRL-*lpr/lpr* and MRL-+/+ mice could conceivably stimulate clones with anti-MHC-image activity. Unlike the sera of alloimmune mice, both MHC-image and anti-MHC-image antibodies would be related to self MHC. The sera of MRL-*lpr/lpr* and MRL-+/+ mice were tested for the presence of antibodies that bind to gp120 and p24 using the same method as described for alloimmune sera in

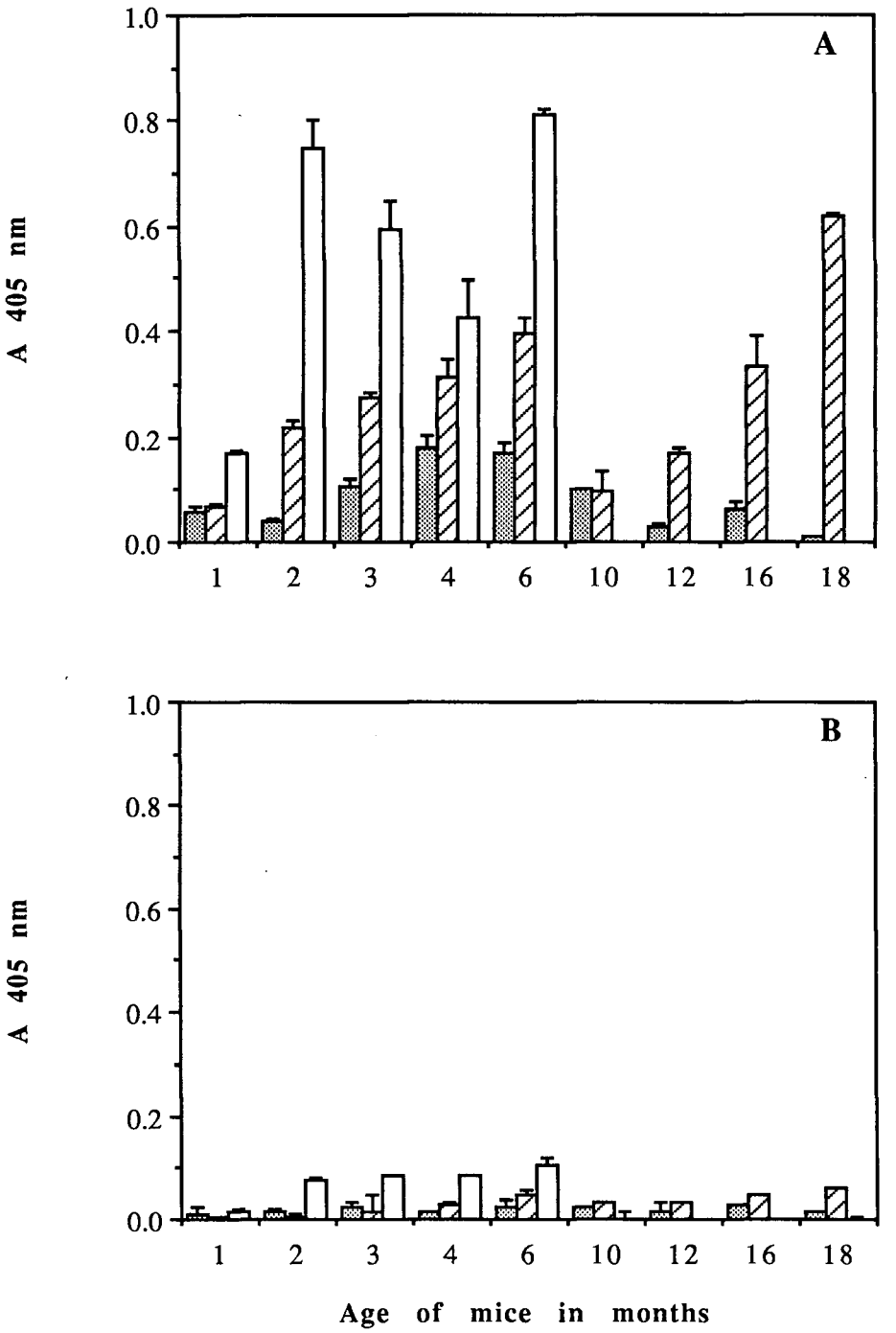
Chapter 3. This was the same pooled sera in which we previously tested for MHC-image antibodies by ELISA (Figure 22). These sera contain anti-gp120 activity which we interpret as anti-MHC-image (regarding gp120 as an image of class II MHC molecules) but little or no anti-p24 activity (Figures 24A and 24B respecitively). The levels of anti-gp120 antibodies initially increased with the age of both MRL-*lpr/lpr* and MRL-+/+ mice. The levels of the anti-gp120 antibodies then decreased in both MRL strains, but then increased again in old MRL-*lpr/lpr* mice (6 months) and MRL-+/+ mice (18 months). The anti-p24 antibodies in MRL-*lpr/lpr* mice did not follow this pattern, and remained at a much lower level (compared with anti-gp120 antibodies). The levels of anti-gp120 antibodies in CBA mice initially increased and then decreased with the age of the mice, and remained at a much lower level than the anti-gp120 antibodies of MRL mice. Anti-gp120 antibodies could be detected in the sera of two other strains of autoimmune mice, namely B6-*lpr/lpr* and B6-*gld/gld* (Table 6).

Individual serum samples from CBA, MRL-*lpr/lpr* and MRL-+/+ mice were tested against gp120 and p24. The MHC-image activity of these samples is shown in Figure 23, and the results are summarized in Table 5. Anti-gp120 antibodies were detected in all MRL-*lpr/lpr* and MRL-+/+ mice (Figure 25); however, unlike the previous experiment in Figure 24 (in which pooled sera was used), high levels of antibodies against p24 could be detected in some, but not all MRL-*lpr/lpr* and MRL-+/+ mice. Table 7 summarizes the results of the experiment in Figure 25. Both MRL-*lpr/lpr* and MRL-+/+ mice had higher anti-gp120 and anti-p24 activities than the aged matched CBA controls, with MRL-*lpr/lpr* mice having more anti-gp120 antibodies than MRL-+/+ mice. The amount of anti-gp120 and anti-p24 antibodies in the mice, and the

Figure 24. Antibodies to gp120 and p24 of HIV in the sera of MRL mice.

Recombinant gp120 or p24 of HIV were coated directly to ELISA plates (20 ng/well). After incubation with the sera diluted 1/300, the bound antibodies were detected by goat anti-mouse Ig-alkaline phosphatase conjugate and the appropriate substrate (t = 4 hours). Pooled sera from CBA (n = 8 - 10), MRL-*lpr/lpr* (n = 10 - 20) and MRL-+/+ (n = 10 - 20) mice at various ages were tested as indicated. For ages greater than 6 months, results are shown only for MRL-+/+ and CBA mice. Panel A. Antibodies that bind to gp120 can be detected in the sera of CBA mice (▩), MRL-*lpr/lpr* mice (□) and MRL-+/+ mice (▨). MRL-*lpr/lpr* mice have higher levels of anti-gp120 antibody than MLR-+/+, which in turn have more anti-gp120 antibodies than CBA mice. There is some fluctuation in the amount of anti-gp120 over time in both MRL-*lpr/lpr* and MRL-+/+ mice. Panel B. Anti-p24 antibodies are barely detectable in CBA mice (▩), MRL-*lpr/lpr* mice (□), and MRL-+/+ mice (▨). Each bar represents the mean and standard deviation of duplicates.

Statistical analysis of data by the ANOVA for anti-gp120 and anti-p24 antibodies in individual mice is described in Table 7.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Age of mice in months

Table 6. Anti-MHC-image (anti-gp120) antibodies occur in other strains of autoimmune mice.[a]

Age of mouse in months	B6	B6-*lpr/lpr*	B6-*gld/gld*	MRL-*lpr/lpr*
1	0.022 ± 0.000[b]	0.020 ± 0.001	0.015 ± 0.001	0.169 ± 0.002
2	0.044 ± 0.001	0.048 ± 0.002	0.076 ± 0.000	0.748 ± 0.022
3	0.017 ± 0.001	0.182 ± 0.041	0.221 ± 0.027	0.542 ± 0.016
4	0.079 ± 0.000	0.278 ± 0.028	NT	0.427 ± 0.041
6	0.030 ± 0.005	0.134 ± 0.008	0.907 ± 0.123	0.810 ± 0.012

NT - not tested

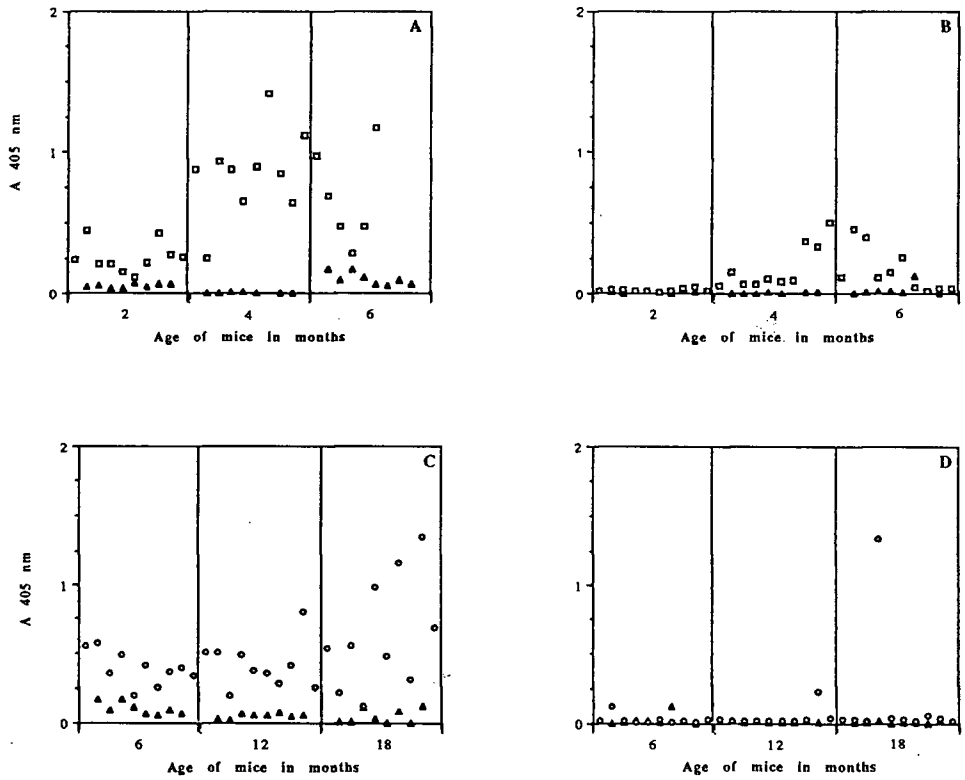
a) Sera diluted 1/300 is incubated on ELISA plates coated with gp120 (20 ng/well), and bound antibodies are detected with biotinylated goat anti-mouse IgG and avidin-alkaline phosphatase. All wells were done in triplicate. Pooled sera from B6, B6-*lpr/lpr*, B6-*gld/gld* and MRL-*lpr/lpr* mice were tested at the ages indicated.

b) The values calculated are the mean ± standard deviation of the A405 nm values.

Figure 25. Anti-gp120 and anti-p24 antibodies in individual CBA, MRL-*lpr/lpr* and MRL-+/+ mice.

Recombinant gp120 or p24 of HIV were coated directly to ELISA plates (20 ng/well). After incubation with the sera diluted as indicated, the bound antibodies were detected by biotinylated goat anti-mouse IgG, Strep. avidin-alkaline phosphatase conjugate and substrate (t = 20 minutes). Sera from the three strains of mice at various ages were tested as indicated. Panel A. Antibodies to gp120 can be detected in the serum samples of all MRL-*lpr/lpr* mice (❑) aged 2, 4 and 6 months. The levels of anti-gp120 antibodies increases with the age of the mice, and there is some variation in the amount of anti-gp120. Controls: Serum from CBA mice (▲) aged 2, 4 and 6 months. Panel B. Anti-p24 antibodies can be detected in the serum samples of some MRL-*lpr/lpr* mice (❑) aged 4 and 6 months, but not at 2 months of age. Controls: Serum from CBA mice (▲) aged 2, 4 and 6 months. Panel C. Antibodies to gp120 can be detected in the serum samples of all MRL-+/+mice (○) aged 6, 12 and 18 months. Again there is some variation in the amount of anti-gp120 produced in individual mice, and there is an increase in the amount of anti-gp120 antibodies produced with the age of the animals. Controls: Serum from CBA mice (▲) aged 6, 12 and 18 months. Panel D. Anti-p24 antibodies are only detected in the occasional 6, 12 and 18 month old MRL-+/+ mouse (○). Controls: Serum from CBA mice (▲) aged 6, 12 and 18 months. Each sample was tested in duplicate.

Statistical analysis of the data by ANOVA is described in Table 7.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Age of mice in months

Table 7. Anti-gp120 and anti-p24 antibodies in autoimmune mice: summary of data shown in Figure 25.

Mouse[b]	age in months	A 405 nm[a] (mean ± standard error) anti-gp120	anti-p24
CBA	2	0.054 ± 0.004	0.012 ± 0.003
MRL-*lpr/lpr*	2	0.253 ± 0.034[c]	0.025 ± 0.004[e]
CBA	4	0.003 ± 0.001	0.003 ± 0.001
MRL-*lpr/lpr*	4	0.851 ± 0.097[d]	0.183 ± 0.050[a]
CBA	6	0.105 ± 0.015	0.025 ± 0.014
MRL-*lpr/lpr*	6	0.513 ± 0.105[c]	0.163 ± 0.049[c]
MRL-+/+	6	0.396 ± 0.039[d]	0.027 ± 0.011[e]
CBA	12	0.049 ± 0.007	0.013 ±0.003
MRL-+/+	12	0.420 ± 0.055[d]	0.023 ±0.002[e]
CBA	18	0.045 ± 0.019	0.011 ± 0.003
MRL-+/+	18	0.641 ± 0.128[c]	0.163 ± 0.131[e]

a) Sera diluted 1/100 is incubated on ELISA plates coated with recombinant gp120 or p24 from HIV (20 ng/well), and bound antibodies are detected with biotinylated goat anti-mouse IgG, avidin-alkaline phosphatase conjugate and substrate. All wells were done in duplicate.
b) The number of mice used in this experiment for each age group: CBA (n=8), MRL-*lpr/lpr* (n=10 for ages 2 and 4 months, and 6 for ages 6 months) and MRL-+/+ (n=10).
c) Significantly different from the normal CBA group with $p \leq 0.005$ by ANOVA.
d) Significantly different from the normal CBA group with $p \leq 0.0001$ by ANOVA.
e) Different from the normal CBA group with $p \leq 0.25$ by ANOVA.

variation in the amount of these antibodies (as indicated by the standard error of the mean) tended to increase with age in both MRL-*lpr/lpr* and MRL-+/+ mice.

Some additional controls that should have been included would involve using other viral proteins (e.g. gp70) and some irrelevant proteins (e.g. KLH) as antigens on the ELISA plate. This would show that anti-gp120 antibodies in MRL mice are not due simply to polyclonal activation.

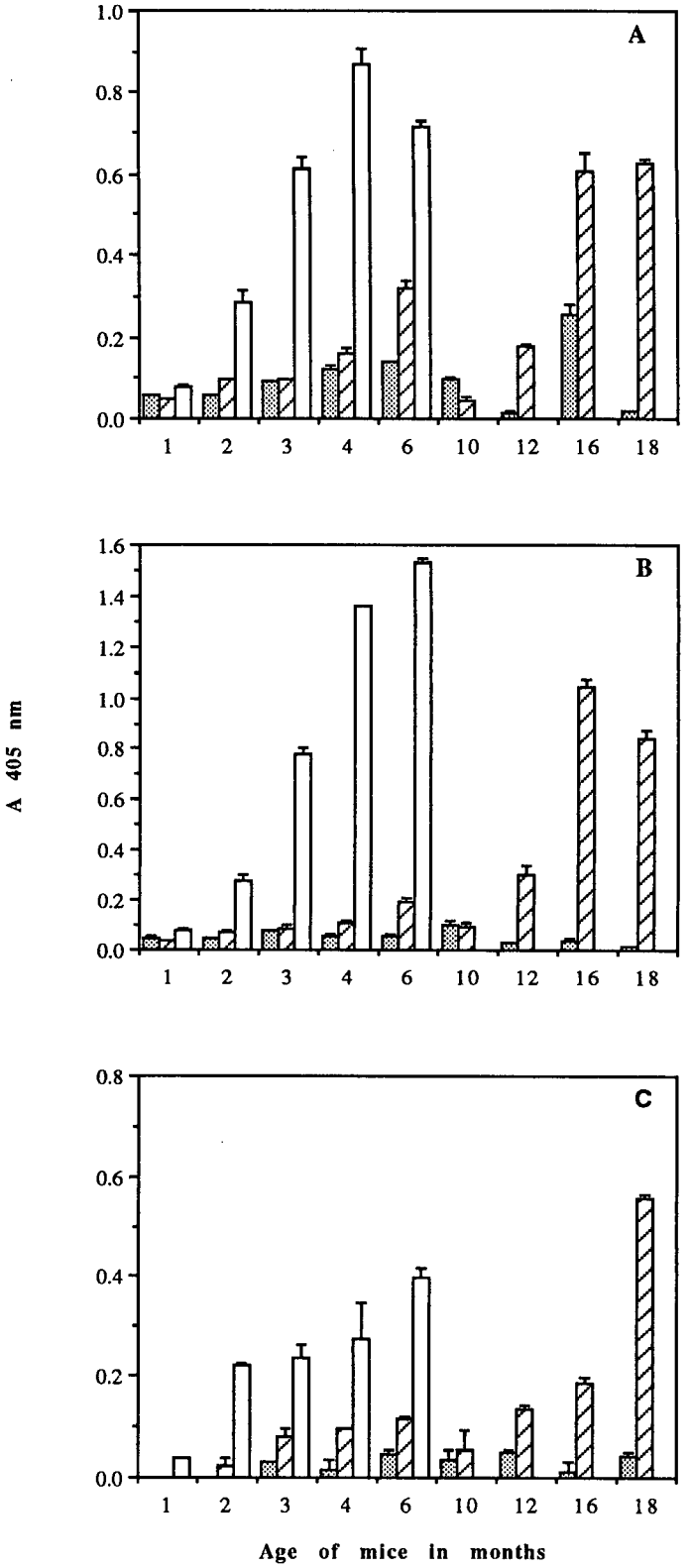
4.3.3. Typical autoantibodies produced in MRL autoimmune mice.

The sera of MRL-*lpr/lpr* and MRL-+/+ mice were tested for the production of various autoantibodies that are typically associated with SLE to assess the development of autoimmune disease. The sera of age-matched CBA mice were used as negative controls. Figure 26 shows the levels of anti-dsDNA, anti-RNP.Sm and anti-collagen antibodies in sera from CBA, MRL-*lpr/lpr* and MRL-+/+ mice at a dilution of 1/300. The ELISA protocols are described in detail in Chapter 7. These are the same pooled sera samples in which MHC-image antibodies were detected by ELISA in Figure 22, and anti-MHC-image antibodies were detected by ELISA in Figure 24. The amount of anti-collagen (Panel A), anti-dsDNA (Panel B) and anti-RNP.Sm (Panel C) antibodies typically increases with the age of the mice, and MRL-*lpr/lpr* mice typically have higher levels of the autoantibodies than MRL-+/+ mice. Serum samples from individual CBA, MRL-*lpr/lpr*, and MRL-+/+ mice were tested at various ages for anti-collagen and anti-DNA activity (Figure 27A and 27B respectively). Table 8 summarizes the results of the experiment in Figure 27. These same samples had been previously tested for MHC-image

Figure 26. Anti-collagen, anti-dsDNA and anti-RNP.Sm autoantibodies in MRL-*lpr/lpr* and MRL-+/+ mice.

ELISA plates were coated with collagen, type III, (50 ng/well), dsDNA (1 μg/well) or RNP.Sm (1 μg/well). After incubation with the sera diluted 1/300, the bound antibodies were detected by goat anti-mouse Ig-alkaline phosphatase conjugate and substrate (t = 1 hour for collagen and dsDNA, 2 hours for RNP.Sm). Pooled sera from CBA (n = 8 - 10), MRL-*lpr/lpr* (n = 10 - 20) and MRL-+/+ (n = 10 - 20) mice at various ages were tested as indicated. Each bar represents the mean and standard deviation of duplicates. Panel A. Anti-collagen antibodies are detected in the sera of MRL-*lpr/lpr* mice (□) and MRL-+/+ mice (▨). CBA mice (▩) typically have lower levels of anti-collagen antibodies. The amount of anti-collagen antibodies tends to increase with the age of the animal. Panel B. Anti-DNA antibodies can be detected in MRL-*lpr/lpr* mice (□) as young as 2 months of age, but are seen at high levels in older MRL-+/+ mice (▨). The levels of anti-DNA antibodies increase with the age of the animal. The controls include sera from CBA mice (▩). Panel C. Anti-RNP.Sm antibodies can be detected in MRL-*lpr/lpr* mice (□) as young as 2 months of age, but are seen at high levels in older MRL-+/+ mice (▨). The levels of anti-RNP.Sm antibodies also increase with the age of the animal. The controls include sera from CBA mice (▩).

Statistical analysis of data by the ANOVA for anti-collagen and anti-DNA antibodies in individual mice is described in Table 8.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Age of mice in months

antibodies (Figure 23) and anti-MHC-image antibodies (Figure 25) with the appropriate ELISAs. As is seen with the pooled serum results, the amount of anti-DNA and anti-collagen antibodies increases with the age of the animal, and MRL-*lpr/lpr* mice have higher autoantibody activity than MRL-+/+ mice. As with MHC-image and anti-MHC-image antibodies, the level of the autoantibodies can vary within both mouse strains.

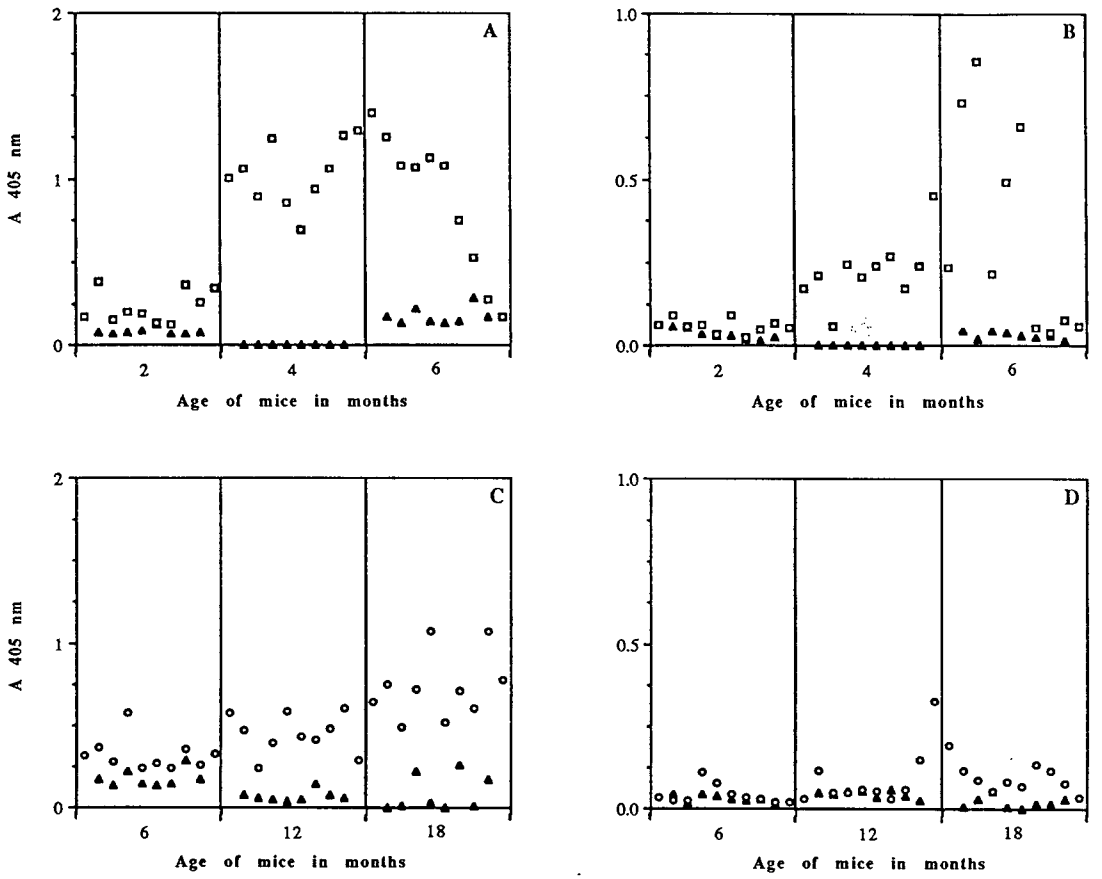
4.4. Summary.

MHC-image and anti-MHC-image antibodies were found to develop spontaneously in MRL-*lpr/lpr* and MRL-+/+ mice. MHC-image antibodies were demonstrated using inhibition of cytotoxicity assay and ELISAs, and were found to be both class I and class II MHC-image antibodies as defined by the specific reactivity of the sera of MRL-*lpr/lpr* and MRL-+/+ mice with anti-K^kD^k and anti-I-A^k monoclonal antibodies. However, the MRL sera were found to show a lot of cross-reactivity with the other monoclonal antibodies tested. The sera of MRL-*lpr/lpr* and MRL-+/+ mice reacted specifically with gp120 of HIV and unlike alloimmune mice, very few MRL mice produced antibodies that reacted with p24 of HIV. Antibodies that bound to gp120 may be anti-MHC-image since gp120 is considered to be an image of class II MHC molecules. It is possible that the anti-gp120 antibodies could cross-react with gp70, which is known to be produced in MRL mice. This could be tested by inhibiting anti-gp120 antibodies with gp70 and vice versa. It is also possible that anti-gp120 antibodies are the result of polyclonal stimulation, and this could be tested by screening the serum against several conventional protein antigens. The presence and levels of MHC-image and the putative anti-MHC-

Figure 27. Anti-collagen and anti-dsDNA autoantibodies in individual MRL-*lpr/lpr* and MRL-+/+ mice.

ELISA plates were coated with collagen, type III (50 ng/well) or dsDNA (1 µg/well) as in Figure 26. After incubation with the sera diluted 1/100, the bound antibodies were detected by biotinylated goat anti-mouse IgG, Strep. avidin-alkaline phosphatase conjugate and substrate (for collagen, t = 15 minutes, for dsDNA, t = 1 hour). Sera from the three strains of mice at various ages were tested as indicated. Anti-collagen (Panel A) and anti-dsDNA (Panel B) antibodies can be detected in all MRL-*lpr/lpr* mice (❑) aged 4 and 6 months, with very low levels of these antibodies in 2 month old mice. The control was sera from aged-matched CBA mice (▲). Anti-collagen antibodies (Panel C) can be detected in MRL-+/+ mice (○) aged 12 and 18 months, with low levels of anti-collagen antibodies in 6 months old mice. Low levels of anti-DNA antibodies (Panel D) were detected in MRL-+/+ mice (○) aged 6, 12 and 18 months. The control was sera from aged-matched CBA mice (▲). Each sample was tested in duplicate.

Statistical analysis of the data by ANOVA is described in Table 8.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Age of mice in months

Table 8. Anti-collagen and anti-DNA antibodies in autoimmune mice; summary of data in Figure 27.

Mouse[b]	age in months	A 405 nm[a] (mean ± standard error) anti-collagen	anti-DNA
CBA	2	0.080 ± 0.006	0.033 ± 0.005
MRL-*lpr/lpr*	2	0.233 ± 0.031[c]	0.059 ± 0.007[e]
CBA	4	0.001 ± 0.001	0.001 ± 0.000
MRL-*lpr/lpr*	4	1.032 ± 0.062[d]	0.226 ± 0.031[d]
CBA	6	0.174 ± 0.019	0.030 ± 0.004
MRL-*lpr/lpr*	6	0.875 ± 0.133[d]	0.341 ± 0.100[c]
MRL-+/+	6	0.323 ± 0.032[c]	0.042 ± 0.009f
CBA	12	0.069 ± 0.012	0.044 ±0.004
MRL-+/+	12	0.448 ± 0.038[c]	0.091 ±0.029[f]
CBA	18	0.088 ± 0.039	0.019 ± 0.006
MRL-+/+	18	0.733 ± 0.068[d]	0.095 ± 0.014[c]

a) Sera diluted 1/100 is incubated on ELISA plates coated with recombinant gp120 or p24 from HIV (20 ng/well), and bound antibodies are detected with biotinylated goat anti-mouse IgG and avidin-alkaline phosphatase.
b) The number of mice used in this experiment for each age group: CBA (n=8), MRL-*lpr/lpr* (n=10 for 2 and 4 months, and n=6 for 6 months) and MRL-+/+ (n=10).
c) Significantly different from the CBA group with $p \leq 0.005$ by ANOVA.
d) Significantly different from the CBA group with $p \leq 0.0001$ by ANOVA.
e) Significantly different from the CBA group with $p \leq 0.01$ by ANOVA.
f) Different from the CBA group with $p \leq 0.25$ by ANOVA.

image antibodies paralleled the presence and levels of the autoantibodies typically associated with SLE.

Chapter 5. The Injection of Low Doses of HIV Proteins to Prevent Autoimmunity.

5.1. Introduction.

Autoimmunity models of AIDS pathogenesis suggest that the immune response to HIV and the concomittant anti-idiotypic response may cross-react with self components[129,130]. The immune responses to allogeneic cells may include a component which can further stimulate the immune response induced by HIV[42,43]. The immune response to allogeneic cells includes an MHC-image response[35], and the immune response to the MHC-mimicking portions of HIV is an anti-MHC-image response[42,43]. The MHC-image response and anti-MHC-image responses are complementary to each other by definition and may therefore mutually stimulate and synergize with each other to result in the destabilization of the immune system. Therefore, the induction of tolerance to the MHC-mimicking portions of HIV rather than immunity may be important in protecting individuals at risk of developing AIDS[42,43].

MRL-*lpr/lpr* mice were found to spontaneously produce antibodies that bound to gp120 of HIV, even though these mice had never been exposed to HIV. Since many autoimmune phenomenon are associated with HIV infection[108b], we considered whether inducing tolerance to gp120 in these mice may prevent the production of anti-gp120 antibodies and delay the development of autoimmunity. To address this hypothesis, young MRL-*lpr/lpr* mice were immunized with doses of recombinant gp120 and p24 of HIV that were below the immunogenic dose (1 μg) stated by the supplier[149].

The mice received two doses (10 ng/injection) of gp120 or p24 in PBS and control mice were injected with PBS or were not immunized at all. The mice were injected intraperitonally at three and four weeks of age, and bled four weeks after the second injection.

5.2. Results.

5.2.1. The injection of gp120 or p24 into young MRL-*lpr/lpr* mice suppresses the production of autoantibodies.

The sera of MRL-*lpr/lpr* mice immunized as described above was tested for the presence of anti-gp120, anti-collagen and anti-dsDNA antibodies and MHC-image antibodies. Antibodies that bind to gp120 are interpreted as having anti-MHC-image activity since gp120 is considered to be an image of class II MHC molecules. For this experiment, all sera samples were diluted 1 in 100, and incubated on ELISA plates coated with gp120, collagen or dsDNA. The bound antibodies were detected by biotinylated goat anti-mouse IgG, Strept. avidin-alkaline phosphatase conjugate and the appropriate substrate. Table 9 shows the result of such an assay. Mice that were injected with gp120 had approximately a 50% reduction in the amount of anti-gp120 antibodies (Experiment 1). Furthermore, the sera of the same mice also had approximately a 60% reduction in the amount of antibodies that bound to collagen and a slight reduction in the amount of anti-DNA antibodies (Experiment 1). Surprisingly, the injection of p24 also suppressed the production of the same antibodies (by approximately 75% and 50% respectively) in MRL-*lpr/lpr* mice. The experiment was repeated with a

Table 9. The injection of recombinant gp120 or p24 of HIV suppresses the production of autoantibodies in MRL-*lpr/lpr* mice.

Experiment 1.

Antigen injected into MRL-*lpr/lpr* mice[a]	anti-gp120 (A405 nm)[b]	anti-collagen (A405 nm)[b]	anti-DNA (A405 nm)[b]
PBS (n = 4)	0.425 ± 0.033[c]	0.497 ± 0.055	0.533 ± 0.054
gp120 (n = 4)	0.248 ± 0.046[d]	0.216 ± 0.015[f]	0.400 ± 0.088[g]
p24 (n = 4)	0.155 ± 0.066[f]	0.130 ± 0.012[f]	0.274 ± 0.056[d]

Experiment 2.

Antigen injected into MRL-*lpr/lpr* mice[a]	anti-gp120 (A405 nm)[b]	anti-collagen (A405 nm)[b]	anti-DNA (A405 nm)[b]	anti-p24 (405 nm)[b]
PBS (n = 15)	0.231 ± 0.017[c]	0.355 ± 0.031	0.298 ± 0.046	0.023 ± 0.002
gp120 (n = 10)	0.148 ± 0.009[f]	0.185 ± 0.008[f]	0.140 ± 0.027[d]	0.007 ± 0.001[i]
p24 (n = 11)	0.154 ± 0.010[f]	0.186 ± 0.019[f]	0.169 ± 0.036[h]	0.008 ± 0.001[i]

a) MRL-*lpr/lpr* mice were injected with 10 ng recombinant gp120 or p24 from HIV (MicroGeneSys) in PBS. The control mice were injected with PBS. All injections were done i.p. at ages 3 and 4 weeks.
b) All serum samples were diluted 1/100 (Experiment 1) or 1/200 (Experiment 2) and incubated on ELISA plates coated with gp120 (20 ng/well), collagen (50 ng/well) or dsDNA (1 μg/well). Bound antibodies were detected with goat anti-mouse Ig-alkaline phosphatase conjugate and the appropriate substrate. The ELISA methods are described in detail in Chapter 7.
c) The values calculated are the mean and the standard error of the mean.
d) Significantly different from the PBS-injected group with $p \leq 0.025$ by ANOVA.
e) Significantly different from the PBS-injected group with $p \leq 0.0005$ by ANOVA.
f) Significantly different from the PBS-injected group with $p \leq 0.005$ by ANOVA.
g) Different from the PBS-injected group with $p \leq 0.25$ by ANOVA.
h) Different from the PBS-injected group with $p \leq 0.05$ by ANOVA.
i) Significantly different from the PBS-injected group with $p \leq 0.001$ by ANOVA.

second group of mice which were immunized and bled as described for Experiment 1. The resulting sera were tested against a panel of antigens which included gp120, p24, collagen and dsDNA. The results in Table 9 show that the mice that were injected with gp120 or p24 again had lower levels of anti-gp120, anti-collagen and anti-dsDNA antibodies than PBS-injected mice, and that all MRL-*lpr/lpr* had very low levels of anti-p24 antibodies (Experiment 2). According to the idiotypic network model of Hoffmann et al. the immune system network has the ability to select clones with V regions that are complementary to MHC molecules or are images of MHC molecules. The immune system network can be destabilized by excessive mutual stimulation of clones with complementary MHC-image and anti-MHC-image V regions[43]. The injection of subimmunogenic doses of gp120 (which has epitopes that are MHC-mimicking) may have stabilized the interactions between MHC-image and anti-MHC-image clones. The apparent stabilization of these interactions by p24 may be explained if p24 is similar to gp120 in that it has epitopes that mimics portions of class II MHC molecules or epitopes that are anti-MHC-image. These interactions may have been stabilized by stimulating suppressor T cells bearing MHC-image determinants. An alternative explanation is that gp120 and p24 are immunosuppressants which act to non-specifically suppress the immune system.

5.2.2. The injection of gp120 or p24 into young MRL-*lpr/lpr* mice does not suppresses the production of MHC-image antibodies.

The serum of the MRL-*lpr/lpr* mice was coated directly to the ELISA plate at a dilution of 1/10,000. MHC-image antibodies were measured by the binding of biotinylated anti-H-$2K^kD^k$ and anti-$I\text{-}A^k$ monoclonal antibodies as described in Chapter 4. The bound monoclonal antibodies were detected with Strept. avidin-alkaline phosphatase conjugate and substrate. There was no difference in the levels of MHC-image antibodies in gp120-injected or p24-injected mice when compared to the control PBS-injected mice (Table 10).

5.2.3. The injection of gp120 or p24 enhances the survival of MRL-*lpr/lpr* mice.

The MRL-*lpr/lpr* mice from the experiment described in Experiment 1 of Table 9 were allowed to live out their lives and the dates of their deaths were recorded. Figure 28 shows the survival curve of the mice up to one year. The control mice include the four PBS-injected mice together with an additional fifteen uninjected MRL-*lpr/lpr* mice. At one year of age, 2 of 4 mice in both the gp120 and the p24 groups were alive, compared to the average lifespan of 6.3 months for the control mice. Although the groups are small, the differences of the survival times of the gp120-injected and p24-injected mice compared with the control mice are significant.

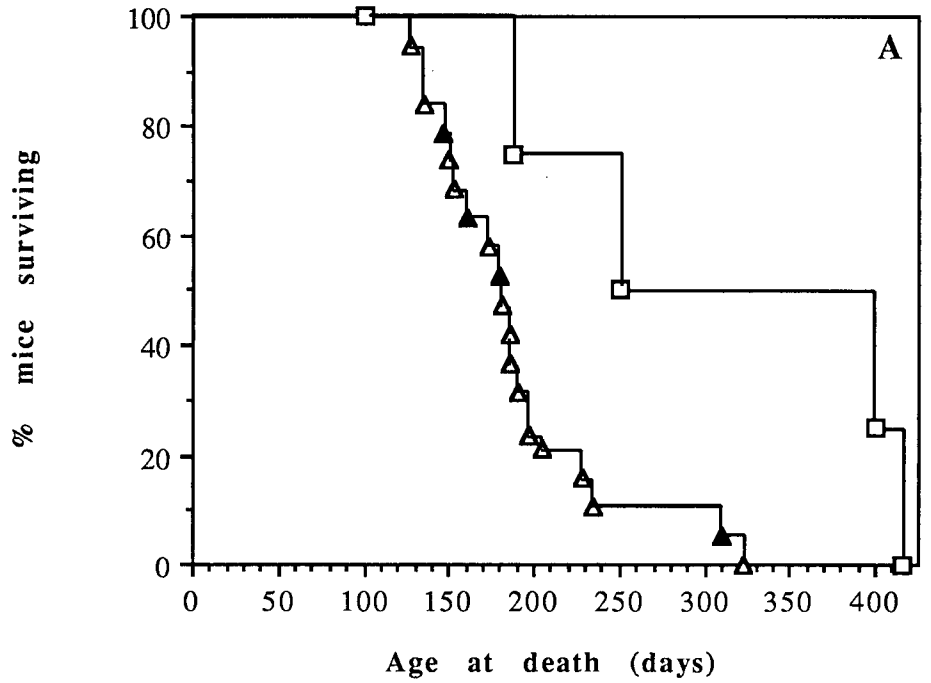
Table 10. The injection of recombinant gp120 or p24 of HIV did not suppress the development of MHC-image antibodies in MRL-*lpr/lpr* mice.

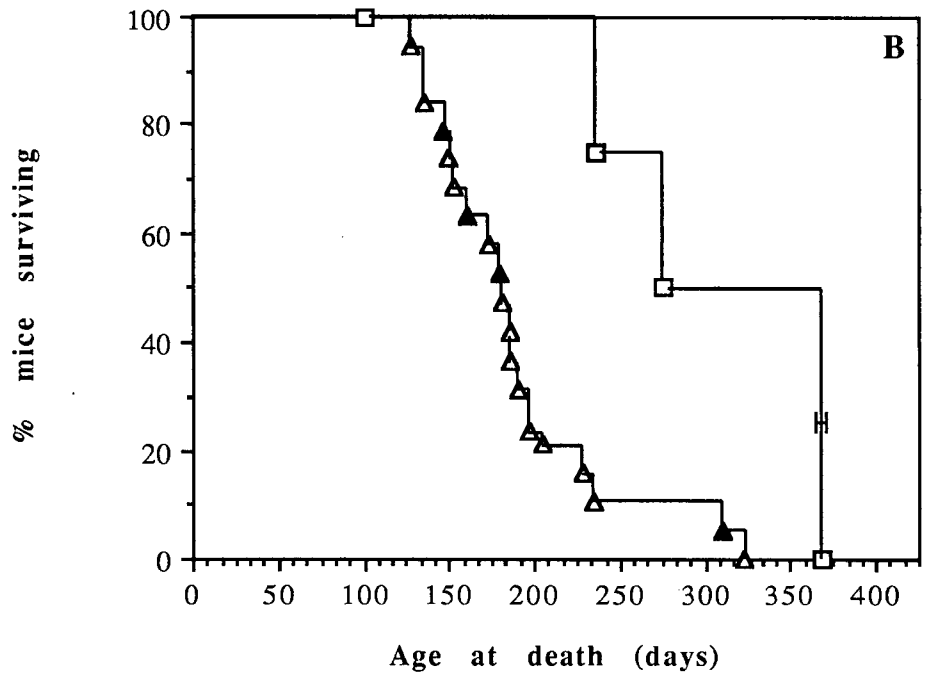
Antigen injected into MRL-*lpr/lpr* mice[a]	reactivity with anti-H-2K^{k}D^k MAb (405 nm)[b]	reactivity with anti-I-A^k MAb (405 nm)[b]
PBS	0.559 ± 0.067[c]	0.543 ± 0.039
gp120	0.528 ± 0.036	0.579 ± 0.027
p24	0.575 ± 0.032	0.635 ± 0.044

a) MRL-*lpr/lpr* mice were injected with 10 ng recombinant gp120 or p24 from HIV (MicroGeneSys) in PBS. The control mice were injected with PBS. All injections were done i.p. at ages 3 and 4 weeks. There were 4 mice in each group. The mice were bled 4 weeks after the injections.
b) All serum samples were diluted 1/10,000 and coated directly onto ELISA plates. The plates were incubated with 100 ng/well of purified biotinylated monoclonal antibodies. Bound antibodies were detected with Strept. avidin-alkaline phosphatase and the appropriate substrate. The ELISA methods are described in detail in Chapter 7.
c) The values calculated are the mean and the standard error of the mean.

Figure 28. The injection of recombinant gp120 or p24 of HIV into young MRL-*lpr/lpr* mice enhances the longevity of these mice.

Young MRL-*lpr/lpr* mice were injected i.p. with 10 ng of gp120 or p24 at ages 3 and 4 weeks. The control mice were injected with PBS (4 mice) or were not injected at all (15 mice). Panel A. Mice injected with gp120 (4 mice, ❑) survived significantly longer than the PBS-injected mice (▲) and uninjected mice (△) with $p \leq 0.005$ by ANOVA. After 1 year, 2 of 4 mice were surviving. Panel B. Mice injected with p24 (4 mice, ❑) survived survived significantly longer than the PBS-injected mice (▲) and the uninjected mice (△) with $p \leq 0.005$ by ANOVA. 2 of 4 mice survived to one year of age, and died between days 365 and 370 as indicated by the error bar (the exact dates of their particular deaths are not known). The survival times for the control mice was 184.5 ± 12.8 days (mean ± S. E. M.), for the gp120-injected mice, 313.8 ± 56.0 days and the for p24-injected mice, 319.0 ± 31.5 days.


A
% mice surviving
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
Age at death (days)


B
% mice surviving
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
Age at death (days)

5.3. Discussion.

SLE patients are usually treated with corticosteroids in conjunction with anti-inflammatory or cytotoxic drugs[148]. This non-specific form of therapy suppresses the anti-self immune responses, but may increase the susceptibility to infections or result in other serious complications[148]. Other forms of non-specific immunotherapy described in animal models of SLE have involved the use of monoclonal antibodies. For example, weekly injections of large doses (2 mg/injection) of an anti-L3T4[161] or an anti-Ia[162] monoclonal antibody suppressed the development of autoimmunity and prolonged the life of (NZB x NZW)F_1 mice. A similar experiment was done with MRL-*lpr/lpr* mice. The mice were injected twice weekly with anti-L3T4 monoclonal antibody (1 mg/injection) or an isotype-matched control monoclonal antibody. In this study, autoantibody production and lymphoadenopathy were greatly alleviated[179]. It is possible that the large doses of monoclonal antibodies depleted the cells bearing the antigens, or that the antigens were blocked by the monoclonal antibodies and that the cells could not be activated. Specific immunotherapies such as the induction of tolerance to the autoantigens by oral antigen therapy has also been described in some experimental models of autoimmune disease (reviewed in reference 163).

It is plausible that the pathogenesis of AIDS and SLE may be similar since they share many clinical and immunological similarities[108b]. MRL-*lpr/lpr* mice spontaneously develop antibodies that react with the envelope glycoprotein gp120 of HIV, and these antibodies are interpreted as having anti-MHC-image activity based on the similarities of gp120 to class II MHC

molecules[125,127]. The induction of tolerance to gp120 in MRL-*lpr/lpr* mice could be an effective method of suppressing the development of the MHC-image and presumptive anti-MHC-image antibodies that occur with autoimmunity. Young MRL-*lpr/lpr* mice were injected with low doses (i.e. subimmunogenic doses) of gp120, and the sera were tested for the presence of anti-gp120, anti-dsDNA and anti-collagen antibodies. Gp120-injected mice had lower levels of these antibodies compared with PBS-injected mice (Table 9). A second group of MRL-*lpr/lpr* mice were injected with the core protein p24 of HIV. This group of mice also produced lower levels of anti-gp120, anti-dsDNA and anti-collagen antibodies compared with the PBS-injected mice (Table 9). This result could be understood if p24 also resembled class II MHC molecules. The MHC-image responses of these mice were not changed by treatment with either gp120 or p24. Although tolerance to gp120 was not achieved in the MRL-*lpr/lpr* mice injected with either gp120 or p24 (i.e. some mice produced antibodies that bound to gp120), these mice survived longer than mice injected with PBS or mice that were not injected at all (Figure 28).

An explanation for these findings is that the subimmunogenic doses of gp120 and p24 stabilized the interactions between MHC-image and presumably anti-MHC-image clones, which in turn, stabilized other clones (producing autoantibodies) that were idiotypically connected to the MHC-image and anti-MHC-image clones and suppressed the development of autoimmunity in MRL-*lpr/lpr* mice.

Additional controls that should have been done would include groups of mice injected with an irrelevant viral protein, an auto-antigen (e.g. thyroglobulin), other antigens of the immunoglobulin superfamily (e.g.

CD4) and conventional protein antigens. This would determine if the effects of gp120 and p24 were specific in stabilizing interactions between MHC-image and anti-MHC-image clones, or whether they were non-specific immunosuppressants.

AIDS is postulated to be the consequence of the destabilization of the immune system following excessive stimulation of MHC-image and presumptive anti-MHC-image clones. The induction of tolerance to the class II MHC-mimicking portions of HIV proteins may stabilize the MHC-image and anti-MHC-image clones that are activated during alloimmunity and autoimmunity. The immunization with subimmunogenic doses of these proteins may be effective in protecting individuals with autoimmune disease or AIDS.

Chapter 6. Discussion.

Autoimmunity models of AIDS have suggested that the immune response to HIV may be involved in the depletion of CD4 T lymphocytes that is typically observed in AIDS patients. Several components of HIV have been shown to have epitopes that are cross-reactive with class II MHC (HLA) molecules[103,105-107,155,156]. The anti-HIV response may include antibodies and T lymphocytes that cross-react with cells bearing class II MHC molecules (both uninfected and infected cells) and the concomitant anti-idiotypic response may cross-react with the CD4 antigen on T lymphocytes[109,110].

The idiotypic network model of AIDS of Hoffmann et al. suggests that the immune responses to different stimuli, namely HIV and allogeneic lymphocytes (or other MHC-mimicking stimuli), includes components that are complementary to each other, and may stimulate and synergize with each other in such a way as to result in the destabilization of the immune system. In this model, the normal immune system network is postulated to be able to select clones that have V regions that are complementary to MHC molecules and clones that have V regions that are images of MHC molecules. A central part of the regulatory T cell repertoire consists of anti-class II MHC helper T cells and class II MHC-image suppressor T cells. The interactions between these two T cell populations are expected to be mutually stabilizing. AIDS is postulated to be an autoimmune disease that can be caused by excessive stimulation of the clones in the regulatory T cell repertoire by HIV and allogeneic stimuli[42,43].

HIV is often introduced into the body with (or within) lymphocytes that are present in both ejaculates and blood or with MHC (HLA) antigens or

immunoglobulin in factor VIII concentrates[158]. The immune response to the class II MHC-mimicking portions of HIV may include an anti-MHC-image response. The immune response to allogeneic lymphocytes includes an MHC-image response as described in Chapter 2. The MHC-image response and the anti-MHC-image part of an anti-HIV response are complementary to each other (by definition) and may synergize with each other to destabilize the central part ot the regulatory T cell network. Although, complementary responses may stabilize the immune system (e.g. the oscillations between idiotypic and anti-idiotypic antibodies during relapse and remission of SLE[70-73]), they may also lead to the disruption of the immune system. The idea of complementarity of lymphocytes in the induction of autoimmune disease has been discussed before by Westall and Root-Bernstein. They postulated that the equal and simultaneous stimulation of both sets of lymphocytes is required for the induction of EAE[135]. HIV infection and alloimmunization may be a similar case for AIDS. The latency period between HIV infection and AIDS may result from the immune system being a normally self-stabilizing network in which the regulatory T cells are stabilized by many different clones[42,43]. The destabilization of the central part of the regulatory T cell network could take a long time[43]. The other immunosuppressive agents discussed by both Root-Bernstein and Duesberg (eg. malnutrition, drug use) could certainly have additional devasting effects on the immune system.

Evidence for the involvement of alloimmunity in AIDS pathogenesis has been obtained in our laboratory and in other laboratories. Anti-collagen antibodies were found in the sera of many homosexual men, both HIV-negative (32%) and HIV-positive (66%) and in the sera of 100% of

homosexual AIDS patients[43,151]. Anti-collagen antibodies react preferentially with denatured collagens, and such antibodies are rarely seen in the arthritides[43,151]. Anti-collagen antibodies can also be detected in graft-versus-host disease, which is a form of alloimmunity. Shearer and colleagues noted the similarities of pathologies of AIDS and graft-versus-host disease and suggested that the exposure to allogeneic lymphocytes may be involved in AIDS pathogenesis[91,92]. It was suggested that anti-collagen antibodies are a consequence of alloimmunity, and are independent of HIV infection[43,151]. Since all homosexual AIDS patients made anti-collagen antibodies, the findings are consistent with the idea that alloimmunity is an important co-factor in the development of AIDS in homosexuals[43,151]. Anti-collagen antibodies are not detected in HIV-negative hemophiliacs, and only rarely in HIV-positive hemophiliacs (13%). However, hemophiliacs were found to make anti-anti-CD4, anti-anti-CD8 and anti-anti-gp120 antibodies, and the expression of these antibodies increased after HIV infection[151]. CD4 and CD8 are complementary to class II MHC and class I MHC antigens respectively, and it follows that the immune systems of hemophiliacs have also been perturbed by MHC molecules or by immunoglobulins with V regions that are images of MHC molecules. Antibodies with MHC-image V regions may be part of the normal antibody repertoire[35,41,42]. Antibodies with MHC-image activity have been demonstrated in normal serum (Chapter 2, also reference 35) and in the culture supernatants of LPS-stimulated spleen cells[41]. The production of the anti-anti-CD4 and anti-anti-CD8 antibodies may be stimulated by the presence of immunoglobulin (up to 10% of total protein) in factor VIII concentrates[150].

MHC-image responses were shown to be involved in alloimmunity by our laboratory and several other laboratories[31-38]. These responses developed in animals that were repeatedly immunized with large doses of allogeneic or semi-allogeneic lymphocytes. MHC-image responses were also shown to be involved in autoimmunity (Chapter 4). These responses developed spontaneously in the MRL mouse strains, a model for the human disease SLE. Anti-gp120 antibodies were found in both alloimmune and autoimmune mice (Chapters 3 and 4 respectively). These results might not have been expected since the animals were not exposed to HIV. Based on the similarities of epitopes of class II MHC and epitopes of HIV (and the similarities between human and murine MHC molecules), these antibodies were interpreted as being anti-MHC-image antibodies. The anti-MHC-image responses in alloimmune mice were presumably directed against the MHC-image determinants on the foreign lymphocytes in the inoculum, whereas the anti-MHC-image responses in autoimmune mice are presumably related to self MHC. Gp120 did not inhibit the binding of MHC-image antibodies to anti-MHC-image antibodies, which may indicate that only a sub-population of anti-MHC-image antibodies bind to gp120. An alternative explanation is that the binding affinity of anti-MHC-image antibodies to gp120 is lower than that to MHC-image antibodies. This could be expected since the mice were not directly immunized with gp120.

Immunization protocols that typically result in MHC-image responses have also been shown to result in the production of autoantibodies[152]. The injection of F_1 hybrid mice with coisogenic spleen cells (the only difference between F_1 and both parents was a class II MHC molecule) resulted in the production of anti-DNA, anti-erythrocyte and anti-nuclear antibodies and

other symptoms of SLE. Although these investigators did not test for the presence of MHC-image antibodies (or "anti-recognition structure" antibodies), their immunization protocol was similar to that of Ramseier and Lindemann[31-33], and Binz and Wigzell[34] in which antibodies with MHC-image activity were made.

The presence of antibodies with MHC-image activity has been reported in rheumatoid arthritis (RA). Searles and colleagues reported that anti-anti-HLA antibodies could be detected in the sera of RA patients in remission, but not when the patients have active disease[74,75]. The presence of MHC-image antibodies coincided with the development of autoimmune disease in MRL mice (Chapter 4). MHC-image antibodies may also be involved in the down-regulation of autoimmune disease; MHC-image antibodies were effective in down-regulating the immune response to skin allografts[35].

Anti-p24 antibodies were detected in all alloimmune mice, but rarely in the autoimmune mice. Anti-p24 antibodies may also be anti-MHC-image antibodies if p24 is also an image of class II MHC molecules. There is some evidence that gp120, gp41 and Nef all have some similarity to class II MHC, so p24 could also be similar to class II MHC if there is evolutionary selection for HIV components to have determinants that resemble class II MHC.

Autoimmunity models of AIDS pathogenesis suggest that establishing tolerance to the class II MHC-mimicking portions of HIV rather than immunity may be important in protecting individuals at risk for contracting AIDS. This hypothesis was tested by injecting young MLR-*lpr/lpr* mice with low doses of gp120 or p24. The production of anti-gp120, anti-DNA and anti-collagen autoantibodies was suppressed in these mice. Furthermore, the mean survival times of gp120-injected or p24-injected mice were found to be

more than 100 days longer than mean survival times of uninjected or PBS-injected mice. 2 of 4 mice in both the gp120-injected and the p24-injected groups survived for one year or longer compared with the average survival of 6.3 months for the control (uninjected or PBS-injected) mice.

In the context of autoimmunity models, MHC-image and anti-MHC-image responses may synergize to destabilize the network, even though they may be triggered by different stimuli. The inherent instability of the immune system in the MRL mice strains that leads to autoimmunity occurs together with MHC-image and presumptive anti-MHC-image antibodies. Injection of young MRL-*lpr/lpr* mice with low doses of gp120 or p24 suppressed autoantibody production and enhanced the survival of these mice. This supports an MHC-image-anti-MHC-image model of pathogenesis of lupus for these autoimmune mice. The presence of both kinds of antibodies in alloimmune mice suggests that synergy between immune responses to allogeneic cells and HIV is important in at least some groups that are at risk for AIDS.

Chapter 7. Material and Methods.

7.1 Mice. Balb/cJ (Balb/c), B10.D2/oSnJ (B10.D2), B10.BR/SgSnJ (B10.BR), C57BL/6J (B6), CBA/J (CBA), C3H/HeJ (C3H), C3H.SW/SnJ (C3H.SW), MRL Mp/J-*lpr/lpr* (MRL-*lpr/lpr*), MRLMp/J-+/+ (MRL-+/+), C57BL/6J-*lpr/lpr* (B6-*lpr/lpr*) and C57Bl/6J-*gld/gld* (B6-*gld/gld*)mice were bred in our animal care facility from breeding pairs obtained from The Jackson Laboratory (Bar Harbour, ME). Mice used for immunizations were 6 - 8 weeks old at the beginning of the immunizations.

7.2 Alloantisera. Mice were immunized with 6 weekly intraperitoneal (i.p.) injections of 5 x 10^7 allogeneic lymphoid cells (lymph node, spleen and thymus) in phosphate buffered saline, pH 7.2 (PBS) and bled 7 days after the last injection. Thereafter, mice were injected with 1 x 10^7 allogeneic lymphoid cells (i.p.) 7 days prior to bleeding. The antisera were pooled from groups of 10 to 20 mice, heat inactivated at 56°C for 30 minutes and stored at -20°C until use. Reciprocal sets of antisera were raised between the following pairs of mice: Balb/c and CBA, B6 and CBA, B10.D2 and B10.BR, and C3H and C3H.SW. Normal sera were taken from age and sex-matched mice.

7.3 Autoimmune sera. Autoimmune mice were bled at the ages indicated. The sera were heat inactivated at 56°C for 30 minutes and stored at -20°C until use. CBA mice were used as controls for MRL-*lpr/lpr* and MRL-+/+ mice, and B6 mice were used as controls for B6-*lpr/lpr* and B6-*gld/gld* mice.

7.4 Skin grafting. Mice were injected i.p. with 20 µl of normal or anti-anti-self sera diluted to 200 µl with PBS on days -7, -4, -1 and 0 relative to grafting. Animals were anaesthetized with Somnotol for the procedure. The animals were shaved and the skin was cleaned with 70% EtOH in water. A 0.5 cm^2 graft bed was prepared by pulling up the skin with forceps and snipping off the skin. The graft bed was fitted with a piece of donor skin (turned 180^o so the hair would grow in the opposite orientation) and covered with Cicratrin antibiotic powder and a vaseline-coated gauze bandage. The bandage was removed after 7 days and the graft assessed daily for signs of rejection. Grafts were considered rejected when there was no viable skin. Technical failures were reported if the graft had slipped during bandaging or was pulled off when the bandage was removed.

Donor grafts were prepared from larger pieces of skin that had been freed of fat and panniculus carnosus by scraping with a scapel. The grafts were stored in cold PBS and cut to the appropriate size.

7.5 RNP.Sm preparation. RNP.Sm was generously provided by Dr. J. D. Waterfield at the beginning of this project. Additional RNP.Sm was prepared by the following protocol.

i) Isolation. RNP.Sm was isolated from rabbit thymus acetone powder (Pel-freez Biologicals, Rogers, AR) by the method described by Billing et al[158,159]. The powder was dissolved in PBS + 10 mM PMSF and stirred overnight at 4^oC. The material was centrifuged and solid ammonium sulfate was added to a final concentration of 33.3%, stirred for 30 minutes at 4^oC and centrifuged at 10,000 x g. Ammonium sulfate was added to the supernatant to a final concentration of 60% and centrifuged again. The pellet was resuspended in

PBS and dialyzed overnight at 4°C against several changes of PBS. The dialysate was added to a column of human anti-Sm immunoglobulin coupled to Sepharose 4B (provided by Dr. Waterfield) and the material was eluted with 3 M guanidine-HCl, pH 2.4. The RNP.Sm was dialyzed against PBS, concentrated and redialyzed against PBS. Protein concentration was determined by a bicinchoninic acid (BCA) assay for 15 minutes at 60°C. The RNP.Sm was stored in aliquots at -20°C until use.

ii) Western blot. RNP.Sm was electrophoresed in a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) under reducing conditions in a BIO-RAD (BIO-RAD, Mississauga, Ontario) minigel apparatus under constant voltage (100V) for 1 hour. The gel was washed in a transfer buffer (25 mM TRIS, 192 mM glycine and 15% MeOH, pH 8.4) for 30 minutes prior to electroblotting to nitrocellulose paper (BIO-RAD, pore size 45 μm) under constant voltage (100V) for 1 hour. The nitrocellulose membrane was blocked overnight at 4°C with 5% casein in $TBST_{20}$ (TRIS-buffered saline with 0.05% $Tween_{20}$). The nitrocellulose membrane was washed twice with $TBST_{20}$ (15 minutes each wash) with gentle agitation and then cut into strips. Normal human serum or serum from a lupus patient was diluted 1/500 in $TBST_{20}$ and the strips are incubated for 1 hour at room temperature, then washed twice with $TBST_{20}$. The strips were then incubated with biotinylated goat anti-human immunoglobulin (BRL/Gibco, Gaitherburg, MD, diluted 1/1000 in $TBST_{20}$) for 1 hour at room temperature, were washed twice with $TBST_{20}$. The strips were incubated with Strep. avidin-alkaline phosphatase conjugate (avidin isolated from *Streptomyces avidinii* and conjugated with alkaline phosphatase, Calbiochem, San Diego, CA, diluted 1/1000 in $TBST_{20}$)

for 1 hour at room temperature. The strips were washed twice with $TBST_{20}$, and once with the substrate buffer. The strips were incubated with nitro blue tetrazolium (NBT) and bromochloroindolyl (BCIP) in the appropriate buffer at room temperature for about 30 minutes. The reaction was stopped by incubating the strips in PBS + 10 mM disodium ethylene diamine tetraacetate·$2H_2O$ (EDTA) for 20 minutes. The strips were washed with dH_2O and allowed to air dry. BRL/Gibco prestained SDS-PAGE markers were used to make a standard curve. A plot of log molecular weight versus the distance each band migrated was used to calculate the approximate molecular weight of the four bands in the RNP.Sm preparation. The bands were calculated to have the approximate molecular weights of 69.9, 40.7, 36.7 and 13.2 kD.

7.6 Absorptions. The following absorptions were carried out on alloimmune and autoimmune sera.

i) Anti-MHC antibodies were removed from alloimmune serum by five serial absorptions against 2 x 10^9 glutaraldehyde-fixed lymphocytes/ml serum. Each absorption was done at room temperature for 1 hour with occasional mixing. Anti-MHC antibodies in the absorbed serum could no longer be detected by a cytotoxicity assay. We refer to such absorbed serum as alloimmune, immunogen-absorbed (AIA) serum. The lymphocytes were fixed with 0.3% glutaraldehyde for 15 minutes at room temperature, washed once with medium containing 15% fetal bovine serum and twice with PBS before being used for absorptions.

ii) For Figures 18-20 and Table 4, autoimmune serum from MRL-*lpr/lpr* and MRL-(+/+) mice was absorbed against normal Balb/c immunoglobulin (3 mg/ml) coupled to Sepharose 4B (Sigma). To remove all antibodies,

autoimmune serum was absorbed against rabbit anti-mouse immunoglobulin (12 mg/ml) bound to Sepharose 4B (Sigma). Approximately 200 µl of serum was absorbed with 200 µl of absorbant for 1 hour at room temperature.

7.7 Measurement of alloantibody by ^{51}Cr release assay. The assay used for these experiments is the same as the one previously described[35] but is reviewed briefly here. The medium used for the assay is RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with HEPES buffer (Sigma, 25 mM), 0.37% sodium bicarbonate and 15% fetal bovine serum (Gibco). A single cell suspension of lymph node cells was prepared in medium and labelled for 90 minutes at 37°C in $Na_2{}^{51}CrO_4$ (Amersham Corporation, Arlington Hts., IL), washed twice in medium and resuspended to 2 x 10^5 cells/ml.

Serum was diluted in medium and further doubling dilutions were prepared in round bottom microtitre trays (Cooke) in a final volume of 50 µl. 50 µl of the cell suspension was added and the plates were incubated at 37°C for 20 minutes. 100 µl of medium was added to each well, the plates were centrifuged at 1000 rpm (IEC CRU-5000 centrifuge-130 x g) for 5 minutes and the medium was flicked off. 25 µl of Low Tox-M rabbit complement [(Cedarlane, Hornby, Ontario), previously absorbed with 1 x 10^8 mouse spleen cells per ml of complement on ice for 1 hour and stored at -20°C until use, diluted 1/20 in medium for the assay] was added to each well and the plates were gently vortexed, then incubated for 60 minutes at 37°C. 175 µl of medium was added to each well, the plates were centrifuged at 1500 rpm (300 x g) for 5 minutes and 100 µl of supernatant was removed for γ counting. Controls included incubating the cells in the presence of complement only. Maximum release was determined by incubating the cells during the second

step in 25 µl of 5% Triton X-100 in HEPES-buffered salt solution (HBSS). All samples were run in triplicate. Percent lysis was calculated as:

$$\% \text{ lysis} = \frac{\text{observed cpm - C' cpm}}{\text{total cpm - C' cpm}} \text{ X } 100\%$$

7.8 Measurement of anti-anti-self (MHC image) antibodies by the inhibition of lytic antibodies detected with ^{51}Cr release. ^{51}Cr-labelled cells were prepared as described for the above assay. Sera (inhibitory or normal) were diluted in medium to a volume of 25 µl and 25 µl of diluted "lytic" antibody was added and incubated for 30 minutes at 37°C. The dilution of lytic antibody required to give between 50 and 70% killing was determined by the above assay. The cells were added to the plates and the assay was developed as previously described[35]. An additional control was the presence of cells and lytic antibody in the absence of any inhibitors. Percent inhibition was calculated as:

$$\% \text{ inhibition} = 1 - \frac{\text{observed cpm - C' cpm}}{\text{lytic Ab cpm - C' cpm}} \text{ X } 100\%$$

7.9 Monoclonal antibodies (MAb). The following cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in tissue culture and ascitic form: 16-1-2N (anti-H-2K^kD^k), 15-5-5S (anti-D^k), 16-3-1N (anti-K^k), 28-8-6S (anti-H-2K^bD^b), AF6-120.1.2 (anti-I-A^b), 10-3.6.2 (anti-I-A^k), 14-4-S (anti-I-E^k) and S-S.1 (anti-SRBC). UPC 10 (anti-β-2-6-linked fructosan) was purchased as ascites from Sigma (St. Louis, MO). The F23.1 (anti-Vβ8) cell line was the generous gift of Dr. H.-S. Teh.

Bg1/314.7.5 (anti-ferrodoxin) ascites was the generous gift of Dr. J. G. Levy. All monoclonal antibodies were of the IgG_{2a} subclass.

7.10 Purification of monoclonal antibodies. Goat anti-mouse IgG and goat anti-mouse IgM immunoabsorbant columns were purchased from Sigma. The ascites or culture supernatant were dialyzed against three changes of 0.5 M NaCl-PBS pH 7.3, loaded onto the columns in the same buffer. The monoclonal antibodies were eluted with 0.15 M NaCl, 0.1 M HCl-glycine, pH 2.3, and immediately neutralized with 1.0 M TRIS base, pH 8.0. All concentrations were done with Centriprep-30 (Amicon Corporation, Lexington, MA) cartridges at 4°C, followed be dialysis in PBS at 4°C.

7.11 Biotinylation of antibodies. Purified antibody in PBS or antisera diluted 1 in 10 in PBS were biotinylated by adding a 0.1 volume of biotinylation reagent [5 mM biotinamidocaproyl N-hydroxysuccinimide ester (Sigma) in dimethyl-formamide] and incubating at room temperature for 4 hours with occasional mixing. The dimethyl-formamide and unused biotinylation reagent were removed by dialysis against several changes in PBS^{160}.

7.12 MHC-image ELISA. Normal or alloimmune sera were coated directly to Immulon 2 ELISA plates in carbonate buffer at 4°C overnight. All washes were done with PBS containing 0.05% $Tween_{20}$ ($PBST_{20}$). The plates were blocked with 5% fat-free casein (BDH Chemicals Ltd., Poole, England) in $PBST_{20}$ for 1 hour at 37°C. For alloimmune sera, the plates were incubated with diluted biotinylated antisera in $PBST_{20}$ with 1% casein (50 µl/well) for 3 hours at 37°C. Bound antibody (anti-MHC) was detected with Strept. avidin-

alkaline phosphatase conjugate (Calbiochem) diluted 1/2000 in $PBST_{20}$ with 1% casein and the appropriate substrate. The plates were incubated with the substrate for 0.5 - 2.0 hours at 37°C. All wells were done in duplicate. For autoimmune sera, the plates were incubated with 100 ng/well of biotinylated monoclonal antibody or purified Ig (normal Ig or anti-MHC) in $PBST_{20}$ with 1% casein (50 µl/well) for 3 hours at 37°C. Bound antibody was detected with Strept. avidin-alkaline phosphatase conjugate (Calbiochem) diluted 1/2000 in $PBST_{20}$ with 1% casein and the appropriate substrate. The plates were incubated with the substrate for 16 - 20 hours 37°C. All wells were done in duplicate.

7.13 Gp120 and p24 ELISA. Recombinant gp120 or p24 (MicroGeneSys, West Haven, CT) were coated directly to Immulon 2 ELISA plates at 20 ng/well in carbonate buffer at 4°C overnight. All washes were done with $PBST_{20}$. The plates were blocked with 5% fat-free casein (BDH) in $PBST_{20}$ for 1 hour at 37°C. Sera were diluted in $PBST_{20}$ with 1% casein and incubated on the plates for 3 hours at 37°C. Bound antibodies were detected with biotinylated goat anti-mouse IgG (BRL/Gibco), Strept. avidin-alkaline phosphatase conjugate (Calbiochem) diluted 1/2000 in $PBST_{20}$ with 1% casein and the appropriate substrate. Plates were incubated with the substrate for 3 hours (alloimmune sera) or 1 hour (autoimmune sera) at 37°C. All wells were done in duplicate.

7.14 Inhibition ELISAs. ELISA plates were prepared as for the corresponding direct assays as described above (7.12 or 7.13). 25 µl of inhibitor and 25 µl of diluted biotinylated antisera (both in $PBST_{20}$ with 1% casein) were added per well and incubated for 2 hours at 37°C. Bound antibodies (i.e. antibodies that

were not blocked by the inhibitor and bound to the antigen on the plate) were detected with Strept. avidin-alkaline phosphatase conjugate (Calbiochem) diluted in $PBST_{20}$ with 1% casein and the appropriate substrate. In the ELISAs where anti-MHC antibodies were inhibited (by antigen) from binding to immobilized MHC-image antibodies, the ELISA plates were incubated with the substrate for 40 minutes at 37°C and all wells were done in triplicate. In the ELISAs where anti-gp120/anti-p24 antibodies were inhibited (by antigen) from binding to immobilized gp120/p24, the ELISA plates were incubated with substrate for 3.5 hours at 37°C and all wells were done in duplicate. In some of the ELISAs where we tried to inhibit the anti-p24 antibodies from binding to immobilized p24, we did all of the incubations at 4°C.

7.15 Collagen ELISA. Immulon 2 ELISA plates were coated overnight with 500 ng/well of calf skin collagen, Type III (Sigma) in carbonate buffer, pH 9.6. (The collagen was solubilized by overnight incubation in 0.1 M acetic acid and denatured by incubation at 60°C for 30 minutes). After washing with $PBST_{20}$, the plates were blocked with 5% casein in $PBST_{20}$. Sera were diluted in $PBST_{20}$ with 1% casein and incubated on the plates for 3 hours at 37°C. Anti-collagen antibodies were detected with goat anti-mouse Ig conjugated with alkaline phosphatase (BRL/Gibco) diluted 1/3000 in $PBST_{20}$ with 1% casein. Plates were incubated with the substrate at 37°C for 1 hour and all wells were done in duplicate. In some experiments the bound antibodies were detected with biotinylated goat anti-mouse IgG (BRL/Gibco, diluted 1/1000) and Strept. avidin-alkaline phosphatase conjugate (Calbiochem,

diluted 1/2000) in $PBST_{20}$ with 1% casein and the appropriate substrate. All incubations were done at 37°C, and all wells were done in duplicate.

7.16 DNA ELISA. Immulon 2 plates were precoated with poly-L-lysine (2.5 µg/well; Sigma) for 2 hours at 37°C, then washed with PBS. 1 µg/well of calf thymus DNA (Sigma, rehydrated in PBS + 10 mM EDTA and passed through a nitrocellulose filter to bind single stranded DNA) in PBS + 10 mM EDTA was coated to the plates for 2 hours at 4°C, then washed with PBS. The plates were then blocked with 1% BSA in PBS overnight at 4°C. After washing with $PBST_{20}$, the plates were incubated with the primary antibody diluted in $PBST_{20}$ with 1% casein for 2 hours at 4°C, washed with $PBST_{20}$ and incubated overnight at 4°C with goat anti-mouse immunoglobulin conjugated with alkaline phosphatase (BRL/Gibco) and diluted 1/3000 in $PBST_{20}$ with 1% casein. The plates were then developed with the substrate for 3 hours at 37°C, and all wells were done in duplicate. In some experiments the anti-DNA antibodies were detected with biotinylated goat anti-mouse IgG (BRL/Gibco, diluted 1/1000), Strept. avidin-alkaline phosphatase conjugate (Calbiochem, diluted 1/2000) in $PBST_{20}$ with 1% casein and the appropriate substrate. All incubations were done at 37°C, and all wells were done in duplicate.

7.17 RNP.Sm ELISA. 1 µg/well of RNP.Sm diluted in PBS + 10 mM EDTA was coated directly to Immulon 2 ELISA plates for 2 hours at 37°C, then washed with PBS. The plates were blocked with 1% BSA in PBS overnight at 4°C. After washing with $PBST_{20}$, the plates were incubated with the primary antibody diluted in $PBST_{20}$ with 1% casein for 2 hours at 37°C, washed with $PBST_{20}$ and incubated overnight at 4°C with goat anti-mouse immunoglobulin

conjugated with alkaline phosphatase (BRL/Gibco) diluted 1/3000 in $PBST_{20}$ with 1% casein. The plates were then developed with the substrate at 37°C, and all wells were done in duplicate.

7.19 Gp120 and p24 injections. MRL-*lpr/lpr* mice were injected i.p. with recombinant gp120 or recombinant p24. Each mouse was injected with 10 ng of protein at 3 and 4 weeks of age. All injections were done in 0.2 ml of PBS. Control mice were injected with 0.2 ml PBS, or did not receive any injections.

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