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LECTIN INDUCED MODULATION OF CELL-MEDIATED CYTOTOXICITY

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LECTIN INDUCED MODULATION OF CELL-MEDIATED CYTOTOXICITY

by

Mark Samuel Schubert

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MOLECULAR BIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1979
I hereby recommend that this dissertation prepared under my direction by Mark Samuel Schubert entitled LECTIN INDUCED MODULATION OF CELL-MEDIATED CYTOTOXICITY be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Dissertation Director 9-27-79

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

Richard Pest 9-27-79
Harris Bernstein 27 Sept 79
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Final approval and acceptance of this dissertation is contingent on the candidate's adequate performance and defense thereof at the final oral examination.

11/78
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ABSTRACT

Spleen cell killing of target cells can manifest through direct cell contact or spleen cell-target cell interaction in the presence of mitogenic lectin, lectin dependent cell-mediated cytotoxicity (LDCC). Spleen cells from C57Bl/6 mice immunized with C3H mouse cells, alloimmunized mouse spleen cells, were found to be capable of cytotoxicity against autologous and other C57Bl/6 spleen cells in the presence of Con-A. Thus, alloimmune spleen cells are capable of an anti-self cytotoxic response in the presence of mitogenic lectin, anti-autologous LDCC.

Further data demonstrating anti-autologous LDCC showed that:
(1) alloimmune cytotoxic spleen cells lost their cytotoxic capability when pre-incubated for six to 24 hours with 10 or 20 µg Con-A per ml; 
(2) the suppression of alloreactivity seen after pre-incubation with Con-A was not due to a toxic effect of Con-A for spleen cells; 
(3) alloimmune spleen cells lysed in the presence of Con-A to a greater extent than did non-immune spleen cells; and 
(4) pre-incubation with colchicine, an inhibitor of cell-mediated cytotoxicity, blocked Con-A induced lysis of alloimmune spleen cells.

Analysis of alloimmune spleen cell subpopulations supported the hypothesis that the LDCC cell is an immature alloimmune cytotoxic cell (pre-killer cell). Potent LDCC was found in alloimmune spleen cell preparations depleted of alloimmune cytotoxic T cells (killer-depleted cells) by three passes on allogeneic cell monolayers genetically
identical with the immunizing cells (homologous allogeneic cell mono-
layers). Such killer-depleted cell preparations have been reported by
others to be rich in pre-killer cells. The LDCC cell was capable of
LDCC against both allogeneic and autologous cells, since killer-
depleted cells lysed both themselves and allogeneic cells in the pres-
ence of Con-A. If the notion that the LDCC cell and the pre-killer
cell are the same cell is correct, then some pre-killers are capable
of binding to homologous allogeneic cell monolayers since alloimmune
spleen cells which bind to homologous allogeneic cell monolayers
(killer-enriched cells) were both suppressed and lysed by incubation
with Con-A.

The cytolytic capability of alloimmune spleen cells was aug-
mented by pre-incubation for 12 or 24 hours with a mitogenic dose of
Con-A. Evidence that this represented maturation of pre-killers to
killers was provided by the observation that killer-depleted cells
showed a greatly enhanced cytotoxic capability after pre-incubation
for 24 hours with a mitogenic dose of Con-A.

Non-immune spleen cells pre-incubated for six and 24 hours with
both mitogenic and super-mitogenic doses of Con-A were stimulated in
their ability to lyse allogeneic target cells. However, the 24 hour
pre-incubation group exhibited a suppressed LDCC capability against
allogeneic targets. Taken together, these data show that both cyto-
lytic and LDCC capabilities of non-immune spleen cells are shifted
after pre-incubation with Con-A.
Thus, Con-A is capable of modulating the cytotoxic response through both anti-autologous and anti-allogeneic LDCC reactions, and maturation of cytotoxic cells. In both cases, the pre-killer cell is presumed to be the target of Con-A induced modulation.
INTRODUCTION

Thymus-derived Lymphocyte Subpopulations

Thymus-derived lymphocytes (T cells) play a key role in the development of immunity to disease, or the rejection of a foreign graft (histoincompatible cells). Subclasses of T cells perform either a regulatory role (acts on other lymphocytes) or effector role (acts on foreign antigen) in the generation of humoral (B cell) or cell-mediated (T cell) immunity. Subclasses of T cells have been described in terms of functional responses and cell surface phenotype. Commonly measured T cell surface antigens in the mouse include receptors for the Fc fragment of immunoglobulin (FcR) (Stout, Waksal and Herzenberg 1976), Ly antigens (Cantor and Boyse 1975a), Ia antigens (Beverly et al. 1976; Stout, Murphy and McDevitt 1977), and the density of Thy-1 antigen (Cantor et al. 1975). In addition, some T cell subclasses can be distinguished by relative sensitivity to anti-lymphocyte serum (ALS) (Cantor et al. 1975). In the human, T cell subclasses are commonly distinguished phenotypically by the presence or absence of FcR (Platsoucas, Good and Gupta 1979), and receptors for sheep red blood cells (Bach 1973). Functional differences are also seen, as in the mouse.

Presently, there are known to exist four basic subclasses of T cells. These are the initiator, helper, cytotoxic, and suppressor T cells.
**Initiator T Cells**

The initiator (Cohen and Livnat 1976), or amplifier (Feldmann et al. 1977) T cell acts first in the chain of responses touched off by exposure to antigen. Initiator lymphocytes are operationally defined as T cells that, when sensitized to allogeneic cells in vitro and injected back into a syngeneic footpad, will recruit other lymphocytes to the footpad with graft-versus-host potential for the sensitizing alloantigen. The initiator T cell requires four to six hours of priming. Cohen and Livnat showed that this cell can be distinguished from other T cells by its radioresistance, resistance to ALS, short life span, and high density of Thy-1 antigen. Feldmann et al. showed that this cell has the Ly-1\(^+\), Ly-2,3\(^-\) phenotype. The function of the initiator T cell is to patrol tissues for foreign antigens, and to amplify antibody and cell-mediated responses through cell recruitment and cell activation.

**Helper T Cells**

Helper T cells are a subclass required during the induction phase of the primary and secondary immune response. They help B cells to produce antibodies (except for T cell-independent antigens), help effector T cells to become activated to respond in an antigen specific fashion, and help suppressor T cells which suppress both B and T effector cell responses (Cantor and Boyse 1975a; Watanabe et al. 1977).

Helper T cells have the cell surface phenotype Ly-1\(^+\), Ly-2,3\(^-\) (Cantor and Boyse 1975a; Cantor, Shen and Boyse 1976; Feldmann et al. 1977). These cells are long lived, are ALS sensitive, and have a
relatively low concentration of Thy-1 antigen (Cantor et al. 1975). Once primed, helper T cells acquire a new surface marker Ala-1.2 (Feeny and Hammerling 1977). Helper T cells are responsible for the DNA synthetic response and mitotic activity when stimulated by admixture with I-region incompatible cells in the mixed lymphocyte reaction (Cantor and Boyse 1975a; Nagy, Elliot and Nabholz 1976). Thus, helper T cells respond to foreign Ia antigens.

Once triggered, the proliferating helper T cells help precytotoxic T cells to mature to cytotoxic T cells directed against H-2K and H-2D antigens on the foreign stimulator cell. Evidence suggests that this help is given via soluble mediators in both the primary (Plate 1976; Sopori et al. 1977; Finke, Orosz and Battisto 1977) and secondary (Tartof and Fitch 1977; Bonavida 1977; Wagner and Rollinghoff 1978) B cell and cytotoxic T cell responses.

Helper T cells can be distinguished by the types of lymphocytes they help, and cell surface phenotype. Helper T cells specific for B effector cells are FcR−, and those for effector T cells are FcR+ (Stout, Waksal and Herzenberg 1976; Stout, Murphy and McDevitt 1977). These authors also showed that at least some helper T cells have Ia antigens on their surfaces. In addition, helper T cells can be subdivided according to the category of B cells which they regulate (Herzenberg et al. 1976; Kimoto et al. 1977). For instance, helper T cells may help either IgG or IgE antibody responses. Antigen specific helper T cells are radiosensitive, whereas non-specific helper T cells triggered by polyclonal T cell stimulation with concanavalin-A (Con-A) are radioresistant (Lawrence, Eastman and Weigle 1978).
Cytotoxic T Cells

A subgroup referred to as the cytotoxic T cell has been ascribed the role of mediating \textit{in vitro} cell-mediated cytolysis of histoincompatible cells (allogeneic or xenogeneic cells), virus infected syngeneic cells, or trinitrophenyl (TNP)-modified syngeneic cells (Cerottini and Brunner 1974; Cantor and Boyse 1976).

Immunization of a mouse with allogeneic or xenogeneic cells either \textit{in vivo} or \textit{in vitro} leads to a recipient T cell-mediated cytolytic response directed against the immunizing cells. This response is specific; only cells identical with the immunizing cells will be lysed (Cerottini and Brunner 1974). Histoincompatible cells whose cell surface phenotypes are unrelated to the immunizing cells will not be lysed. Thus, every cytotoxic T cell is programmed to lyse a particular target cell.

Generation of cytotoxic T cells requires the presence of macrophages (Treves et al. 1976). In addition to macrophages, induction of specific cytotoxic T cells is modulated by the activity of both helper T cells and suppressor T cells. Antibody producing cells (B cells) are not involved in the generation of cytotoxic T cells (Cerottini, Nordin and Brunner 1971; Wagner, Harris and Feldmann 1972).

The surface phenotype of the cytotoxic T cell has been described as Ly-1\textsuperscript{−}, Ly-2,3\textsuperscript{+} (Cantor and Boyse 1975a, 1975b; Shiku et al. 1976). However, a subpopulation of cytotoxic T cells have been found with phenotype Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} (Shiku et al. 1976; Cantor and Boyse 1976). These Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} weakly cytotoxic T cells are relatively immature pre-cytotoxic cells (pre-killer cells) that mature to Ly-1\textsuperscript{−},
Ly-2,3\textsuperscript{+} anti-TMP-modified self killers. Recently it has been suggested that all anti-allogeneic cytotoxic T cells are Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} (Nakayama et al. 1979). The authors suggested that the surface density of the Ly-1 antigen was low, in an attempt to explain why it had not been detected on cytotoxic T cells before.

Suppressor T Cells

The suppressor T cell is capable of suppressing both humoral and cell-mediated immunity (Zembala and Asherson 1973; Baker et al. 1974; Peavy and Pierce 1974). It may be an agent in both high zone and low zone tolerance (Holan, Chutna and Hasek 1978). A lack of suppressor T cells may be involved in the development of autoimmunity and autoimmune disease (Barnes, Willis and Tuffrey 1975).

The suppressor T cell is long-lived, susceptible to ALS (Feldmann et al. 1977), has Ia antigens on its surface (Okumura et al. 1976), and is Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} (Cantor et al. 1976; Feldmann et al. 1977). However, a subpopulation has been described as Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} (Cantor and Boyse 1976; Pickel and Hoffmann 1977). There may be a difference between the Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} and Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} subpopulations in terms of the mechanism of suppression. For example, the authors suggest that Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} suppressor cells are antigen specific and act in some cases by a cytolytic mechanism (also Kedar and Schwartzbach 1979) whereby autologous effector cells are eliminated. However, anti-self cytotoxicity as a mechanism of suppressor cell effect has been disputed (Al-Adra and Pilarski 1978). The Ly-1\textsuperscript{+}, Ly-2,3\textsuperscript{+} cells are non-specific and suppress only the primary B cell response; the mechanism is unclear. Recently, a new subpopulation of suppressors have been found to be
Ly-1+, Ly-2,3- (McDougal, Shen and Elster 1979). Antigen specific (Tada and Taniguchi 1976; Kapp, Pierce and Benacerraf 1977) and non-specific (Rich and Rich 1976) suppressive factors are produced by activated suppressor T cells. Thus, suppressor T cells seem to be heterogeneous with respect to both function and cell surface phenotype.

Mixed lymphocyte reactivity with subsequent development of anti-allogeneic cytolytic ability can be suppressed by co-culture with suppressor cells generated in three basic ways: (1) incubation of non-immune spleen cells for five days in vitro (Hodes and Hitchcock 1976; Kedar and Schwartzbach 1979); (2) alloantigen activated (Truitt, Rich and Rich 1978); and (3) Con-A activated (Peavy and Pierce 1974; Ozato, Ebert and Adler 1976).

**Cell-mediated Cytotoxicity**

Cytotoxic T cells specifically recognize and lyse the foreign histoincompatible cell they have been sensitized to. The major structures that serve as target antigens for cytolysis on the foreign cell are the H-2K and H-2D antigens (Bevan 1975a; Nabholz et al. 1975; Bevan and Hyman 1977). The helper T cell is required during the sensitizing phase (priming phase) of the cytotoxic response, and the reaction of helper T cells can be quantitated by measuring DNA synthesis after spleen cells have been exposed to the foreign cell in vitro for four days. Cytolytic activity, which peaks on day five, will not be induced if there has not been an appropriate stimulation of the helper T cell (Peck, Alter and Lindall 1976). The determinant which stimulates helper T cells is the Ia antigen on the foreign cell's surface. At least 15 minutes in contact with the foreign cell is required for sensitization

Once generated, cytotoxic T cells are specific for the priming alloantigen and require recognition of target before killing occurs. Cytotoxic T cell "A" primed against target cell "B" will not lyse target cell "C", even if "A" and "C" are in very close proximity during and ongoing "A" anti-"B" cytolytic reaction (Cerottini and Brunner 1974). Since it had already been shown that cytotoxic T cells could serve as targets for cytotoxicity as well as effectors (Golstein 1974), Kuppers and Henney (1976) mixed "A" anti-"B" with "B" anti-"C" and observed that cytotoxic T cell "A" killed cytotoxic T cell "B", but not vice versa. Thus, mere proximity to, or contact with, a cytotoxic T cell is not sufficient for a cytolytic response; specificity for the target cell is a requirement.

The specificity for a given target cell resides with the cytotoxic T cell in its receptor for alloantigen (Ramsier 1976; Ramsier, Aguet and Lindenmann 1977). The antigen combining site on the receptor is referred to as the idiotype, as it is for immunoglobulin. The idiotypes found on cytotoxic T cells are different from the idiotypes found on helper T cells after a mouse is alloimmunized (Binz et al. 1979). This is to be expected, since cytotoxic T cells recognize H-2K and H-2D antigens, whereas helper T cells recognize Ia.

Cytotoxic T cell precursors (pre-killers) are clonally pre-committed to respond to a given alloantigen, as with B cells (Miller et al. 1977). The pre-killer frequency is approximately 0.2% and the helper T cell precursor frequency is 0.4% (Ryser and MacDonald 1979).
Once primed, the frequency of specific cytotoxic T cells is between 1 and 2% for a primary response, and 6 to 12% for a secondary response (Engers and Fitch 1979). Under certain conditions, up to 20% of the entire pool of pre-killers can respond and generate mature cytotoxic T cells specific for the same allogeneic cell (Miller et al. 1977). Thus, both proliferation and "plasticity" probably play a role in the high frequency of allospecific cytotoxic T cell maturation. Miller et al. (1977) suggest that the "plasticity" component may be due to either low affinity, cross-reacting cytotoxic T cells, or pre-killers which are multipotential in their responsiveness but become restricted following interaction with alloantigen. Matzinger and Bevan (1977) view the high frequency of allospecific cytotoxic T cells as a result of the cell recognition system for cytotoxic T cells, as discussed later.

Pre-killers were first detected during the decline of the cytotoxic response seen 12 to 18 days post immunization (Kamat and Henney 1975). Spleen cells taken during this phase were reduced in their cytotoxic capability. If, however, these cells were cultured in vitro for 24 hours, specific cytotoxicity was regenerated. This represented maturation of pre-killers to killers. The presence of antigen was not necessary, and neither was protein or DNA synthesis. The pre-killer was distinct from the memory T cell; memory T cells could only be induced to differentiate to cytotoxic cells in the presence of antigen and were adherent to cell monolayers genetically identical with the immunizing cells (homologous allogeneic cell monolayers). Pre-killers were homologous allogeneic cell monolayer non-adherent, a fact disputed
by others (Bach, Bach and Sondell 1976). Kamat and Henney (1976) showed that pre-killers could be detected by day three post immunization, whereas matured cytotoxic T cells were not found until day six or seven. Memory T cells were not found until day nine or ten. After 24 hours of culture, the spleen cells became homologous allogeneic cell monolayer adherent, which was expected if maturation of specific cytotoxic T cells had occurred. The specific cytotoxicity of sensitized spleen cells can also be augmented by 24 hours in vitro with a mitogenic dose of Con-A (Falkoff and Dutton 1977). This is suggestive evidence for Con-A induced maturation of pre-killers. Taken together, these data show that in the alloimmunized mouse there exists a group of cytotoxic T cell precursors. These pre-killer cells are committed to a specific allogeneic target cell, but are unable to bind to, and lyse, that cell. However, they can be induced to become a mature cytotoxic T cell.

Alloimmune cytotoxic T cells (or cytotoxic T cells induced polyclonally with Con-A in vitro) are capable of lysing only one target cell at a time (Henney 1971, 1975b). The cytotoxic mechanism does not require protein synthesis (Thorn and Henney 1976). After the target cell is destroyed, the cytotoxic T cell survives and can continue to lyse more target cells (Zagury et al. 1975).

Three steps which are required for lysis have been described. These are: (1) adhesion formation; (2) programming for lysis; and (3) killer cell-independent lysis (Martz and Benacerraf 1973; Matter 1979). Firm adhesion of killer cell and target cell occurs within one minute after contact. Within six more minutes, the target cell is
programmed to lyse; removal of the killer cell does not prevent subsequent lysis (Martz 1975, 1977). Killer cell-independent lysis reaches completion in several hours (Martz and Benacerraf 1975; Zagury et al. 1975), and is characterized by vigorous zeiosis (blebbing) (Sanderson and Glauert 1979; Matter 1979).

The foreign antigen on the surface of the target cell which is recognized by the cytotoxic T cell may be a combination of the major histocompatibility antigen (H-2K or H-2D) and a minor histocompatibility antigen (non-H-2 antigen, virus, or TNP-modified self) (Bevan 1975b; Zinkernagel et al. 1976; Bevan and Hyman 1977). Thus, the cytotoxic T cell "sees" the H-2 antigen in conjunction with a non-H-2 antigen, and recognition of antigens is "H-2 restricted." There are only two H-2 antigens (H-2K and H-2D) but many non-H-2 antigens. Further, two different cytotoxic T cells each may recognize and lyse the same cell, but what they see may be very different. Cytotoxic T cell "1" may see H-2 antigen K or D plus non-H-2 antigen "X", and cytotoxic T cell "2" may see the H-2 antigen plus non-H-2 antigen "Y". This multiplicity of recognizable antigens on the surface of a foreign cell may explain the high frequency of cytotoxic T cell precursors found to be specific for the same target cell (Matzinger and Bevan 1977).

**Anti-self Immune Responses**

Burnet's clonal selection theory (Burnet 1959) states that autoreactive lymphocytes are eliminated during ontogeny, thus removing the possibility of unwanted anti-self immune responses as an adult. Clonal selection has been challenged in recent years by the mounting
data on autoreactivity not only in immunological disease, but normal
immune functioning. Jerne's network theory (Jerne 1974) more accurately
accounts for autoimmunity. It states that all antibodies (or lympho-
cytes) can recognize two entities (an antigen and another antibody) and
are recognized themselves by another antibody. An antibody which is
recognized, and bound, by another antibody is functionally suppressed
because the binding occurs on the recognized antibody's idiootype. Anti-
body "1" recognizes antigen "A", but can also recognize antibody "2".
Antibody "3" recognizes antibody "1" and antigen "B". Antibody "3" is
also recognized by antibody "4", and so on. This network of anti-self
responses keeps the immune system dormant until the presence of antigen
perturbs the system by combining with the appropriate antibodies. The
antibodies that are "pulled out" of the network are replaced with new ones,
alogous to an equilibrium reaction. Antigen specific antibody levels
will rise due to antigenic pressure and cellular activation only to event-
tually be suppressed by the presence of the network. Thus, the very
essence of the immune system is autoreactivity according to this theory.

During a normal immune response, autoreactive B cells (McHugh
and Bonavida 1978) and lymphocytes bearing autoanti-idiotypic receptors
(Tasiaux et al. 1978) can be detected. L'Age-Stehr and Diamanstein
(1978a, 1978b) showed that autoreactive T cells were cyclophosphamide
resistant, and could be suppressed in vivo by the presence of sup-
pressor T cells. Stimulation of human T cells with Con-A will increase
the number of autoresette forming cells (Fournier and Charreire 1978).

Purified B cells and other non-T cells are capable of stimu-
lating autologous spleen cells in the mixed lymphocyte reaction
(Opelz et al. 1975; Kuntz, Innes and Weksler 1976; Milthorp and Richter 1979). Finke, Ponzio and Battisto (1976) showed that only B cells with IgG₁ on their surface were capable stimulators, as opposed to other B cells. Mitogen activated T cells (J. Miller et al. 1978), but not resting T cells (Takasugi, Kuichi and Opelz 1977; Milthorp and Richter 1979) are capable of stimulating in the autologous mixed lymphocyte reaction.

Data on anti-autologous cytotoxicity are somewhat contradictory. It has been shown that lymphocytes will not respond with DNA synthesis or cytotoxicity to syngeneic leukemic cells in vitro (Zarling et al. 1976; Sondel et al. 1976). However, if an allogeneic cell is added as a third party, a cytotoxic response against the syngeneic leukemic cell can be generated. Thus, the allogeneic cell is providing the proliferative stimulus required for the generation of cytotoxicity. Normal lymphocytes cannot be sensitized to syngeneic normal tissue, even in the presence of an allogeneic cell (Sondel and Bach 1976).

Vande Stowe et al. (1977) showed that anti-autologous proliferation can serve as a trigger for an anti-allogeneic cytotoxic response when the allogeneic cell is incapable of generating proliferation. No cytotoxic response was generated in an autologous mixed lymphocyte reaction when T cells were responding to B cells with proliferation. This proliferative response could serve as a stimulus for cytotoxicity against added allogeneic cells that had been heat treated (which abolished their ability to stimulate a cytotoxic response).

Others have shown that B cells, T cells, and fibroblasts can serve as stimulators and targets for anti-autologous cytotoxic
responses. Miller and Kaplan (1978) have shown that T cell-mediated cytotoxicity could be generated in vitro if autologous B cells were used as stimulators and autologous B cell blasts were used as targets. Priming with autologous B cells generated a cytotoxic response against only autologous B cells; allogeneic B cells were not lysed.

Autoanti-idiotypic cytotoxic T cells specific for either B or T cells have been found (Anderson et al. 1977; Binz and Wizell 1978). These cytotoxic T cells suppress the B or T cell antigen specific response they are directed against.

In the human, naturally occurring cytotoxic lymphocytes directed against autologous fibroblasts have been found (Timonen and Saksela 1977) that are neither mature T nor B cells (Parkman and Rosen 1976), but are FcR⁺ (Osband and Parkman 1978). Osband and Parkman showed that autocytotoxicity is usually difficult to demonstrate because of the presence of a suppressor T cell that inhibits the auto-reactive cell. They were able to isolate these two cells, and showed that the suppressor T cell was unable to inhibit allogeneic cells from their own autocytotoxic responses. Thus, suppression of anti-self responses is self specific.

Rheumatoid arthritis and systemic lupus erythematosis are human autoimmune diseases that are correlated with loss of thymic hormone (Bach, Dardenne and Clot 1975). Without the presence of thymic hormone in vivo, autoreactive lymphocytes are readily detected (Carnaud, Charreire and Bach 1977). Thus, the authors speculate that thymic hormone is an important regulator of suppressor T cells that in turn suppress anti-self immune responses.
New Zealand Black (NZB) mice have high levels of autocytotoxic spleen cells and a high incidence of autoimmunity (Liburd, Russell and Dosseter 1973). In addition, they have an absence of circulating thymic hormone (Bach, Dardenne, Pleau and Bach 1975). Normal mice, but not NZB mice, will generate a positive mixed lymphocyte reaction when spleen cells are stimulated in vitro with purified autologous B cells (Smith and Pasternak 1978). Thus, the autologous mixed lymphocyte reaction may represent triggering of the suppressor T cell that is lost in autoimmune disease.

**Lectin Dependent Cell-mediated Cytotoxicity by Non-immune Cell Populations**

Mitogenic lectins specific for T cells, such as Con-A or phytohemagglutinin (PHA), are capable of amplifying the inherent cytotoxic reactivity of a lymphoid cell population. Two different mechanisms by which lectin can amplify cytotoxicity are: (1) polyclonal maturation of pre-killers to killers after activation of non-immune lymphocytes in vitro with a mitogenic dose of lectin (Moller, Sjoberg and Andersson 1972; Stavy, Treves and Feldmann 1972); and (2) bridging of an effector cell non-specifically to a target cell via the agglutinating properties of the lectin, concomitant with triggering of cytotoxicity via the mitogenic properties of the lectin (Forman and Moller 1973; Muchmore, Nelson, Kirchner and Blaase 1975; Green, Ballas and Henney 1978). Since the later mechanism, but not the former, depends upon the presence of lectin during the cytotoxic reaction, it has been termed lectin dependent cell-mediated cytotoxicity (LDCC) (Davignon and Laux 1978; Green et al. 1978).
The first reports of LDCC described the stimulatory effects of PHA or Con-A on non-immune cytotoxicity (non-immune, background, or baseline killing) when included during the cytotoxic assay (Holm, Perlmann, and Werner 1964; Holm 1967). The effector mechanism of LDCC is dependent on energy requiring processes, but is independent of protein and RNA synthesis. Thus, the LDCC reaction is not dependent upon a typical mitogenic response by the effector cell. The LDCC response shows no antigenic specificity since virtually any target cell can be lysed by non-immune spleen cells in the presence of Con-A or PHA.

The LDCC reaction can occur only if both the effector and target are capable of binding the lectin (Kirchner and Blaese 1973; Muchmore, Nelson, Kirchner and Blaese 1975). Direct binding of the effector to the target is necessary for lysis, since target cells incapable of binding the lectin are not lysed when added to an ongoing LDCC reaction as a third party cell. Thus, a soluble cytotoxic lymphokine, such as lymphotoxin, was ruled out as a cytotoxic mechanism. The role of lectin was postulated to be that of a "glue" or "bridge" between lytic T cells and susceptible target cells (Forman and Moller 1973).

The effector cell responsible for the LDCC response varies depending on the nature of the target cell. When chicken red blood cells (CRBC) are used as targets, B cells, T cells, and macrophages are all lytic in the presence of PHA or Con-A (Muchmore, Nelson, Kirchner and Blaese 1975; Nelson et al. 1976; Jordon et al. 1978). For an optimal response, both non-T and T cells should be present (Wisloff, Froland and
Michaelson 1974). Complex nucleated cells, such as L cells, are killed exclusively by T cells (Muchmore, Nelson, Kirchner and Blaese 1975; Nelson et al. 1976) which are FcR⁻ (Hersey, Edwards and Edwards 1976; Jondal and Targan 1978). In addition, CRBC, but not complex nucleated cells, can be lysed in an LDCC reaction by non-lymphoid cell lines (Muchmore, Nelson and Blaese 1975).

Lectin Dependent Cell-mediated Cytotoxicity
by Immune Cell Populations

It was observed that greatly enhanced levels of LDCC could be obtained by using spleen cells that had either been polyclonally activated with a T cell mitogen (Asherson, Ferluga and Janossy 1973; Bevan and Cohn 1975) or alloimmunized (Moller 1965; Forman and Moller 1973). The LDCC reactivity using alloimmune cells was observed to be 50 to 100 times greater than that from non-immune cells (Gately and Martz 1977). As shown for non-immune LDCC, LDCC from immune cultures showed no target cell specificity; any target cell could be lysed by immune cell cultures in the presence of Con-A or PHA. However, the alloimmune cell cultures did not show an enhancement of cytolysis in the presence of PHA toward the allogeneic target cells they were specifically immunized against. The immune, but not non-immune, spleen cell cultures were also capable of LDCC against syngeneic tumor cells (S-LDCC).

Bevan and Cohn (1975) demonstrated potent anti-allogeneic LDCC by spleen cells that had been stimulated for two days with a mitogenic dose of Con-A. Their conclusion was that Con-A produced LDCC cells by polyclonal activation of progenitor cytotoxic T cells.
Bevan and Cohn also found potent S-LDCC from spleen cells that had been alloimmunized in mixed lymphocyte cultures. The effector cell was a T cell, and it was also capable of lysing to a small extent syngeneic small lymphocytes, but not B or T cell blasts. On the contrary, Bradley and Bonavida (1978) were able to demonstrate LDCC against both syngeneic small lymphocytes and T cell blasts. Published data are consistent with the notion that LDCC and S-LDCC are exacted by the same effector cells; immune LDCC cells, as opposed to non-immune LDCC, are also capable of S-LDCC due to the greater magnitude of the LDCC response. Thus, S-LDCC is a manifestation of the relatively greater LDCC response seen from immune cell cultures.

Generally speaking, Bevan and Cohen, as well as others, could not find enhancement of specific alloreactivity in the presence of T cell mitogens (Bonavida and Bradley 1976; Rubens and Henney 1977). In addition, they found that an LDCC reaction reveals only cytotoxic T cells. Spleen cells immunized with congeneric cells under conditions that produced proliferation but not cytotoxicity were not cytotoxic to any target cell, even in the presence of PHA.

The S-LDCC activity is dependent upon the presence of a T cell mitogenic lectin; B cell mitogens, non-mitogenic cell agglutinants (poly-L-lysine, wheat germ agglutinant, specific anti-serum), and calcium ionophore (mimics early stages of T cell mitogenesis, but is not an agglutinant) will not work (Bonavida and Bradley 1976; Rubens and Henney 1977; Green, Dallas and Henney 1978).

The T cell lectin must be capable of both activating and bridging effector cells to target cells. Lectin coated effector cells will
not lyse uncoated targets (Rubens and Henney 1977; Green, Ballas and Henney 1978). Uncoated but otherwise identical targets are not lysed when added to an ongoing LDCC or S-LDCC reaction as a third party cell. In addition, leukohemagglutinin-A (has the same sugar specificity, agglutinating properties, and T cell mitogenic properties as Con-A) coated effectors will not lyse targets coated with Con-A plus anti-Con-A antibody. Thus, the targets are coated with Con-A but bridging between effectors and targets is blocked by anti-Con-A antibody; the effectors are coated and activated, but bridging cannot take place since all of the specific binding sites (sugars) on the targets are already bound by Con-A. Therefore, together with the data on the requirement for activation, the conclusion is that both activation and bridging are necessary at the same time for lysis to occur.

Data on the nature of the S-LDCC cell are somewhat contradictory. Bonavida and Bradley contend that the S-LDCC cell is the anti-allogeneic cytotoxic T cell. They determined this by noting that homologous allogeneic cell monolayers were able to absorb out both alloimmune and S-LDCC activity. They, as well as Laux and Tunicharoen (1978), also noted that after alloimmunization in vivo, the development of anti-allogeneic cytotoxicity and S-LDCC cells paralleled in time post immunization. Their conclusion was that S-LDCC activity represented alloimmune cytotoxic lymphocytes that had lost their specificity in the presence of lectin.

Contradicting the one cell hypothesis are the results of Rubens and Henney (1977), Laux and Tunicharoen (1978), Davignon and Laux
(1978), and Tsoukas and Martz (1978). These groups contend that the alloimmune cytotoxic lymphocyte and the S-LDCC cell are different cells.

Rubens and Henney were able to separate alloimmune and S-LDCC activity on the appropriate homologous allogeneic cell monolayers as described. Alloreactive cells bound to the monolayers, and cells that did not bind were capable of both LDCC and S-LDCC. Their explanation for Bonavida and Bradley's results was that the technique of monolayer absorption was different. Rubens and Henney also found that S-LDCC activity appeared in vivo earlier after alloimmunization than anti-allogeneic cytotoxicity (three to five days post immunization for S-LDCC, and 10 to 12 days for anti-allogeneic cytotoxicity). Tsoukas and Martz found LDCC activity occurring in mixed lymphocyte cultures sooner than specific cytotoxicity.

Dissociation of the two cell types was also demonstrated by Rubens and Henney's observation that immunization in vivo with sonicated alloantigen, as opposed to whole cells, was able to generate S-LDCC but not anti-allogeneic cytotoxicity. Laux and Tunticharoen found that immunization with syngeneic tumor cells produced cytotoxicity that was detectable only in the presence of Con-A. In addition, Davignon and Laux showed that if mice were immunized in vivo with a low dose (or formaldehyde fixed) of allogeneic cells, S-LDCC but not specific cytotoxicity, was generated. In general, Rubens and Henney noted the similarity between LDCC (or S-LDCC) cells and pre-killer cells; both appear three to five days post immunization in vivo, both are relatively absent from non-immune lymphoid cell pools, and both are non-adherent to sensitizer-cell monolayers.
An LDCC response is probably a result of triggering of the LDCC cell with the resultant cytotoxic response against any target it is agglutinated to; specific recognition is bypassed. Specific anti-allogeneic cytotoxicity and the ability to bind specifically to homologous allogeneic cell monolayers can be inhibited by trypsinization of the effector cell population (which removes the required T cell receptor for alloantigen) (Todd 1975). When Con-A is added, the trypsinized effectors are capable of lysing both syngeneic and allogeneic targets. This is further evidence that specificity of the effector cell is not required for LDCC or S-LDCC. In agreement with this are data showing that target cells do not need H-2K or H-2D antigens on their surface for an LDCC response against them (Golstein et al. 1976; Bevan and Hyman 1977).

Mitogenic lectins can also suppress alloimmune cytotoxicity. It has been shown that incubation of alloimmune spleen cells with PHA for two to four hours, prior to the addition of target cells, reduces both the specific cytotoxic response and S-LDCC (Bradley and Bonavida 1978). The authors hypothesized that pre-incubation of immune cells with PHA suppressed alloreactivity by an anti-autologous, or anti-self, LDCC reaction. They reasoned this because they could demonstrate that syngeneic small lymphocytes and T cell blasts could be lysed by alloimmune and Con-A induced cytotoxic lymphocytes in the presence of Con-A. Evidence that suppression of alloreactivity by pre-incubation with PHA was not due to PHA toxicity was provided by the observation that cell viability of non-immune and Con-A blast cells dropped equally when incubated individually with a suppressive dose of PHA under conditions
which disallowed cell-cell contact (a prerequisite for a cytotoxic response). Examination of published data from other groups supports the contention of reduced cytotoxicity when antigen-specific anti-allogeneic cytotoxicity was measured after immune cells were treated with PHA or Con-A (Gately and Martz 1977; Laux and Tunticharoen 1978).

Experiments were, therefore, undertaken to directly demonstrate anti-autologous LDCC. In addition, attempts were made to delineate the cells which serve as targets and effectors in anti-autologous LDCC reactions. Other Con-A induced modulatory events were also analyzed with respect to immune and non-immune cell-mediated cytotoxic responses.
MATERIALS AND METHODS

Animals

Female C57Bl/6 (H-2^b) and C3HeB/FeJ (H-2^k) mice were purchased (Jackson Labs, Bar Harbor, Maine) and maintained in our facilities. All mice were 7 to 12 weeks of age when used.

Tissue Culture Cells

The continuous line of L929 mouse fibroblasts (L cells) originated from a C3H mouse (H-2^k). These cells were purchased (Flow Labs, Los Angeles, California) and maintained by serial passage in Eagle’s Minimum Essential Medium with Earl’s Salts (MEM; Grand Island Biological Company, Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS; Flow), 2mM glutamine (Flow), penicillin and streptomycin (pen-strep; 50 units per ml and 50 μg per ml, respectively; GIBCO) (supplemented-MEM). These cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air (standard conditions). For passage, a confluent cell monolayer was trypsinized with 0.25% trypsin (GIBCO) and passed at a ratio of 1:4.

Preparation of Lymphoid Cells

Sterile spleen cell suspensions were prepared by finely mincing the spleens in RPMI 1640 (GIBCO) supplemented with 5% heat inactivated FCS (56°C for 45 minutes), 2mM glutamine, and pen-strep (50 units per ml and 50 μg per ml, respectively) (supplemented-RPMI). Clumps were
removed by sedimentation at 1 X g for two or three minutes. Erythrocytes were lysed by a 10 minute exposure at room temperature to an equal volume of sterile tris(hydroxymethyl)aminomethane buffered 0.83% ammonium chloride (pH 7.2). The cells were then pelleted (200 X g for 10 minutes), washed once in supplemented-RPMI, and counted in a hemocytometer. Viability was determined by incubation of a small aliquot of cell suspension with an equal volume of 0.2% trypan blue for two or three minutes. In all cases greater than 90% of the cells were viable as judged by dye exclusion.

**Induction of Immunity**

Mice (C57Bl/6) were immunized in vivo by intraperitoneal injection of 2 X 10^7 L cells that had been washed once in MEM. Ten days later spleens were removed aseptically and spleen cells prepared as described above.

One-way mixed lymphocyte cultures were used for in vitro induction of immunity. Cultures were established in sterile flat bottom Linbro plates (FB-16-24TC; Linbro Scientific, Hamden, Conn.) in 2.5 ml of supplemented-RPMI supplemented with 50 uM 2-mercaptoethanol (Sigma, St. Louis, Mo.) (supplemented-RPMI + 2-ME). Responder cells (10^7 viable C57Bl/6 spleen cells) were cultured with stimulator cells (10^7 viable C3HeB/FeJ spleen cells) that had been X-irradiated with 1000 rads from a 4 MEV linear accelerator (Varian, Palo Alto, Calif.). For irradiation, cells were suspended at a concentration of 10^7 per ml in supplemented-RPMI, and 10 ml were dispensed into 15 ml sterile plastic disposable screw cap centrifuge tubes (Falcon Plastics, Oxnard, Calif.). After irradiation, the cells were washed once in
supplemented-RPMI, then counted and adjusted to 10^7 viable cells per ml in supplemented-RPMI + 2-ME. Cell viability was unaffected by the irradiation. Control cultures consisted of either 10^7 C57B1/6 cells cultured alone, or in the presence of 10^7 irradiated C57B1/6 cells. Both controls gave identical results. In later experiments measuring cytotoxic reactivity of these in vitro immunized cultures, fresh non-immune spleen cells were used as the non-cytotoxic control.

Cultures were terminated after five days of incubation under standard conditions. Cells were removed from the wells by pipetting up and down a few times, pelleted, and washed twice in supplemented-RPMI. Cells were then counted and diluted to appropriate viable cell concentrations. No glass adherent cells could be found in these cultures after five days of incubation. Viable cell recovery was between 60% to 70% in the mixed cultures, and 10% to 40% in the control cultures.

**Cytotoxicity Assay**

**L Cells as Targets**

Cytotoxicity was measured by the ^{51}Cr release assay (Brunner et al. 1970). The L cells were radioactively labeled for use as targets for cell-mediated cytotoxicity at a dilution of 2.5 X 10^5 per ml. Sodium chromate (^{51}Cr, specific activity 1 mCi per ml; New England Nuclear, Boston, Mass) and mitomycin C (Sigma) were added to a final concentration of 10 uCi and 1 μg per ml, respectively. This cell suspension was then dispensed in 200 μl aliquots into 96 well flat bottom microtiter plates (Falcon Plastics). After incubation for 18
to 24 hours under standard conditions, the wells were individually
washed three times with fresh supplemented-RPMI and lymphoid cells to
be tested for cytotoxicity (effector cells) were added to quadrupli­
cate wells of targets at various effector to target cell ratios in 200
μl of supplemented-RPMI.

After appropriate incubation times of effector cells with
labeled targets, the 200 μl of supernatant from each well was removed
and placed in a 5 ml disposable plastic tube (W. Sourstedt Inc.,
Princeton, N.J.) (one well per tube) for $^{51}$Cr counting in an automatic
gamma counter (Nuclear Chicago Corp., Des Plains, Ill.). The amount
of $^{51}$Cr released into the supernatant from the labeled L cells is pro­
portional to the extent of killing by the effector cells. Spontaneous
release of $^{51}$Cr during the course of the assay was determined by meas­
uring the $^{51}$Cr released from the wells in which no effectors had been
added (0:1 effector to target cell ratio). Spontaneous release (SR)
was approximately 1.3% per hour as calculated from equation (1) below.
Total releasable counts represent the $^{51}$Cr that is available to be re­
leased in the presence of effectors, and excludes spontaneous release.
To measure total releasable counts, supernatants from the 0:1 wells
were removed and replaced with 200 μl of distilled water. After freez­
ing and thawing twice, the microtiter plates were centrifuged at 400 X
g for 10 minutes and the distilled water removed and counted for $^{51}$Cr.

Effector cells were: (1) C57Bl/6 spleen cells immunized
against either L cells in vivo or C3HeB/FeJ spleen cells in mixed
lymphocyte cultures; (2) non-immune C57Bl/6 spleen cells; or (3)
C57Bl/6 effector cells in the presence of Con-A (Calbiochem, San Diego,
Calif.). The cytotoxicity which is dependent upon the presence of Con-A during the cytotoxic reaction (effector cells plus target cells plus Con-A) is referred to as lectin dependent cell-mediated cytotoxicity (LDCC). For LDCC reactions, 0:1 wells also contained Con-A.

The following formulas were used for calculations involving cytotoxicity against L cells:

\[
\text{(1)} \quad \% \ SR = 100 \left( \frac{0:1 \text{ counts released}}{0:1 \text{ counts released} + \text{total releasable counts}} \right)
\]

\[
\text{(2)} \quad \% \text{ immune cytotoxicity} = \frac{\text{counts released in the presence of immune effectors} - \text{counts released in the presence of non-immune effectors}}{\text{total releasable counts}}
\]

\[
\text{(3)} \quad \% \text{ cytotoxicity above SR} = \frac{\text{counts released in the presence of effectors} - \text{0:1 counts released}}{\text{total releasable counts}}
\]

**Assay for Autologous \(^{51}\text{Cr} \text{ Release}**

Lymphoid cells were labeled with \(^{51}\text{Cr} \) for detection of autologous \(^{51}\text{Cr} \) release in the presence of lectin. Chromium labeled lymphoid cells were incubated with or without Con-A and autologous \(^{51}\text{Cr} \) release measured after incubation under standard conditions for four hours. To label the lymphoid cells, \(5 \times 10^7 \) to \(10^8 \) cells were suspended in 900 \(\mu\)l of supplemented-RPMI and 100 \(\mu\)l of \(^{51}\text{Cr} \) was added. This suspension was incubated under standard conditions for one hour, after which the cells were pelleted and washed twice in supplemented-RPMI; in experiments utilizing colchicine-treated (Ranney and Pincus 1976)
target cells, colchicine (GIBCO) was added to a final concentration of 100 \( \mu \text{M} \) for an additional 45 minutes prior to pelleting and washing. The cells were then resuspended in 10 ml and incubated under standard conditions for one hour. Following this, the cells were pelleted, resuspended, counted and adjusted to the appropriate viable cell concentration as described. Cell viability was not affected by \(^{51}\text{Cr} \) or \(^{51}\text{Cr} \) plus colchicine treatment.

Chromium release assays were carried out in triplicate or quadruplicate in sterile disposable plastic culture tubes (Falcon Plastics) at a concentration of \( 5 \times 10^6 \) viable cells per ml. For one ml volumes, 12 X 75 mm tubes were used; for two ml volumes, 17 X 100 mm tubes were used. Results were identical in either case.

To terminate the assays, the tubes were shaken and then pelleted at 200 \( \times \) g for five minutes. An aliquot of the cell-free supernatant was removed and counted for \(^{51}\text{Cr} \) as described. For autologous \(^{51}\text{Cr} \) release assays, the spontaneous release represents the \(^{51}\text{Cr} \) released from the cells not incubated with Con-A. Percent spontaneous release from immune and non-immune cultures was not significantly different (although the immune cells incorporated more \(^{51}\text{Cr} \) than non-immune cells), and ranged from 30% to 40%. Equation (4) was used to calculate total releasable counts. Equations (5a) and (5b) were used to calculate autologous \(^{51}\text{Cr} \) release.

\[
(4) \text{total releasable counts} = \text{total counts incorporated} - \text{SR}
\]
(5a) % autologous $^{51}$Cr release (for immune cultures) =
$$\frac{\text{counts released from immune cells in the presence of Con-A — SR from immune cells}}{\text{total releasable counts in immune cultures}} \times 100$$

(5b) % autologous $^{51}$Cr release (for non-immune cultures) =
$$\frac{\text{counts released from non-immune cells in the presence of Con-A — SR from non-immune cells}}{\text{total releasable counts in non-immune cultures}} \times 100$$

Assay for Anti-autologous LDCC

In experiments where anti-autologous cytotoxic responses were analyzed in the presence of lectin (anti-autologous LDCC), immune and non-immune lymphoid cells were each divided in half. One half was labeled with $^{51}$Cr and colchicine, and served as target cells. The other half was untreated, and served as effector cells. Cytotoxic assays were set up in one ml volumes at a viable cell concentration of $5 \times 10^6$ per ml, at an effector to target cell ratio of 10:1, and at a Con-A concentration of 10 µg per ml. Thus, $4.55 \times 10^6$ effectors were mixed with $4.55 \times 10^5$ targets per culture tube, and 10 µg of Con-A was added to the appropriate tubes. After four hours under standard conditions, assays were terminated in the same manner as autologous $^{51}$Cr release assays. Spontaneous release was calculated from the $^{51}$Cr released from $5 \times 10^6$ target cells cultured without effectors or Con-A; this value was divided by 10.99 to give the spontaneous release value for $4.55 \times 10^5$ targets.

Equations (6a) and (6b) were used to calculate anti-autologous LDCC.
(6a) \( \% \) anti-autologous LDCC (for immune targets) =
\[
\frac{\text{counts released from immune targets in the presence of effectors with Con-A} - \text{counts released from immune targets in the presence of effectors without Con-A}}{\text{total releasable counts in immune targets}} \times 100
\]

(6b) \( \% \) anti-autologous LDCC (for non-immune targets) =
\[
\frac{\text{counts released from non-immune targets in the presence of effectors with Con-A} - \text{counts released from non-immune targets in the present of effectors without Con-A}}{\text{total releasable counts in non-immune targets}} \times 100
\]

**Pre-incubation with Con-A**

Lymphoid cells were pre-incubated in supplemented-RPMI for various lengths of time with several concentrations of Con-A. Cultures were initiated in two ml volumes at 5 \( \times \) 10^6 viable cells per ml in the 17 X 100 mm tubes. At 1.5 hours prior to termination, \( \alpha \)-methylmannoside (Sigma) was added to a final concentration of 50 mM to elute the Con-A from the cells (Bevan 1976). At the termination of the culture the cells were pelleted, washed three times in supplemented-RPMI, and diluted to appropriate viable cell concentrations. Con-A treated cells were then tested for cytotoxic function against L cells either in the absence or presence of fresh Con-A.

**Fractionation of Immune Cell Subpopulations**

Enrichment for anti-L cell cytotoxic effector (killer enriched) cells was accomplished using cellular immunoabsorption followed by elution with ethylenediaminetetraacetic acid (EDTA) (Stulting and Berke 1973). Immune lymphoid cells \( (1.88 \times 10^8) \) in a volume of 25 ml were allowed to adhere for 45 minutes to confluent L cell monolayers in
150 mm tissue culture dishes (Falcon Plastics). The dishes were then swirled, the non-immunoadherent cells pipetted off, and the L cell monolayers washed three times with 20 ml of wash fluid. The first two washes were done with supplemented-RPMI, the third with phosphate buffered saline (pH 7.2) (PBS). The washes were discarded, and 25 ml of PBS supplemented with 5% heat inactivated dialized FCS (Flow) and 10 mM EDTA (PBS-EDTA) were added. The dishes were then incubated under standard conditions for 10 minutes. At this point 50% to 80% of the immunoabsorbed cells could be removed by swirling, pipetting off, and washing two times with 20 ml PBS-EDTA. The L cell monolayer was not removed from the culture dish by this procedure.

The eluted cells were pelleted, washed twice in supplemented-RPMI, counted, and diluted to appropriate viable cell concentrations for measurement of cytotoxicity on L cells or autologous $^{51}$Cr release. Immune lymphoid cells from mixed lymphocyte cultures capable of absorbing to the monolayers constituted approximately 30% of the pre-absorbed population.

To obtain cultures relatively free of cytotoxic effector cells (killer-depleted), immune lymphoid cells were passed three times on L cell monolayers as described above. Each absorption was 45 minutes under standard conditions, followed by swirling, pipetting the non-absorbed cells off, and gently washing the L cell monolayer three times in 20 ml of supplemented-RPMI. All washes from each pass were pooled, the non-absorbed cells pelleted, and then resuspended so that 1.88 x $10^8$ cells could be applied to a fresh L cell monolayer for the next pass. After the final pass, the cells were pelleted and adjusted to
appropriate viable cell concentrations for measurement of L cell cytotoxicity or autologous $^{51}$Cr release.

**Statistical Analysis**

All cytotoxic determinations are reported as percent cytotoxicity ± standard deviation of the mean of triplicate of quadruplicate samples per experiment. Experiments were performed at least three times, and one or more representative experiments are reported. Statistical significance of differences was calculated by Student's t test.
RESULTS

**Lectin Dependent Cell-mediated Cytotoxicity**
**by Spleen Cells on Allogeneic Targets**

The optimal conditions for a lectin dependent cytotoxic response on allogeneic targets was investigated. Non-immune H-2^b spleen cells were measured for their ability to lyse allogeneic L cells in the presence of 2 to 100 μg Con-A per ml (Fig. 1). Three different 51Cr release assay periods are shown. The optimal condition for LDCC was 2 μg Con-A per ml for 24 hours. Between 12 and 24 hours, the 2 μg per ml group more than doubled its killing ability, whereas baseline killing (no Con-A) did not quite double. It is evident by six hours that 2 μg Con-A per ml was the optimum dose, but the magnitude of the responses increased during the 24 hour incubation.

The dose-response kinetics show that by 12 and 24 hours, doses higher than 2 μg per ml caused an inhibition of killing relative to baseline killing. The percent 51Cr release was higher when target cells were exposed to Con-A than when exposed to effectors plus Con-A. Therefore, the presence of effectors protected the target cells from Con-A induced 51Cr release, or toxicity, at higher doses.

Evidence that Con-A was indeed toxic to the L cells is provided by Figure 2. By plotting the 51Cr release values for the groups which consist of Con-A alone acting on the target cells, it could be seen that there was a dose dependent toxicity on L cells which increased with time.
Figure 1. Kinetics of the LDCC reaction. — LDCC reactions by non-immune H-2<sup>b</sup> spleen cells against L cells was calculated by equation (3). Numbers under each bar represent μg Con-A per ml included during the assay. The effector to target cell ratio was 5:1.
Figure 2. Effect of Con-A on L cells.

The cytotoxic effect of 2 (---), 10 (---), 20 (...), and 100 (---) \( \mu g \) Con-A per ml on L cells was assayed in 6, 12, and 24 hour \( ^{51} \text{Cr} \) release assays. For calculations, Con-A replaced effector cells in equation (3).
Figure 2. Effect of Con-A on L cells.
Table 1 shows that α-methylmannoside, a specific Con-A inhibitor (Bevan 1976) inhibited LDCC when included during the reaction. As can be seen, the presence of 50 mM α-methylmannoside greatly reduced the response. This implies that Con-A binding of carbohydrate residues enhances the non-immune killing response.

The $^{51}$Cr released in the presence of non-immune effectors (without Con-A) was contingent to a large extent upon the condition of the target cells on any particular day. Dead effectors were capable of eliciting $^{51}$Cr release from L cells. Therefore, cytotoxicity above spontaneous release (baseline killing) fluctuated from experiment to experiment.

Pre-incubation of Immune Cells (Immunized in vivo) and Non-immune Cells with Con-A

In order to investigate the effect of Con-A on the immune cytotoxic response, H-2$b$ mice were first alloimmunized with L cells in vivo to induce an anti-L cell cytotoxic response. Ten days later, spleen cells were tested for cytotoxicity against L cells in the $^{51}$Cr release assay (Table 2).

The cytotoxic response was a relatively weak one, needing a 24 hour $^{51}$Cr release assay to manifest itself. Little or no response was detected in six or 12 hour assays.

Since it has been reported that the presence of a T cell mitogen during the alloimmune cytotoxic reaction has little or no effect (Bonavida and Bradley 1976; Rubens and Henney 1977 [see Fig. 12, p. 58]), a 24 hour pre-incubation with Con-A was used in an attempt to detect lectin dependent modulation. Alpha-methylmannoside was added
Table 1. Effect of α-methylmannoside on the lectin dependent cell-mediated cytotoxic reaction.$^{a}$

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>α-Methylmannoside (mM)</th>
<th>Con-A$^{b}$</th>
<th>Percent Cytotoxicity Above SR$^{c,d,e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immune</td>
<td>5</td>
<td>-</td>
<td>6 ± 12</td>
</tr>
<tr>
<td>Non-immune</td>
<td>5</td>
<td>+</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>Non-immune</td>
<td>50</td>
<td>-</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Non-immune</td>
<td>50</td>
<td>+</td>
<td>20 ± 3</td>
</tr>
</tbody>
</table>

$^{a}$Non-immune H-2$^{b}$ spleen cells were assayed for an LDCC response against L cells in the presence or absence of Con-A plus α-methylmannoside.

$^{b}$2 μg per ml.

$^{c}$24 hour $^{51}$Cr release assay.

$^{d}$Calculated by equation (3).

$^{e}$Effector to target cell ratio of 25:1.
Table 2. Cytotoxicity of spleen cells from mice immunized *in vivo*.^a^

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Effector/Target</th>
<th>Percent Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Immune^b^</td>
</tr>
<tr>
<td>Immune</td>
<td>10:1</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>Non-immune</td>
<td>10:1</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>Immune</td>
<td>5:1</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>Non-immune</td>
<td>5:1</td>
<td>12 ± 2</td>
</tr>
</tbody>
</table>

^a^H-2^b^ mice were immunized *in vivo* with L cells as described in Materials and Methods. Spleen cell cytotoxicity against L cells was measured in a 24 hour ^51^Cr release assay.

^b^Calculated by equation (2).

^c^Calculated by equation (3).
for 1.5 hours at the end of the pre-incubation period in order to re-
move the Con-A from the cells. The cells were then washed three times.

It can be seen in Table 3 that, first, immune cells pre-treated
with 20 μg Con-A per ml were profoundly suppressed in their ability to
elicit an alloimmune cytotoxic response; second, the addition of
α-methylmannoside to elute the Con-A from the cell surfaces was not
required; third, 50 mM α-methylmannoside had no effect on a normal
cytotoxic response. Concentrations of α-methylmannoside higher than
100 mM made the medium hypertonic, and higher concentrations were not
tested. The combination of the α-methylmannoside plus three washes
was subsequently used to separate the agglutinated cells since it
facilitated resuspension and counting.

Twenty-four hour incubation with 20 μg Con-A per ml reduced
cell viability in both immune and non-immune cell populations. It was
therefore necessary to explore whether the immune suppression induced
by Con-A was due to non-specific toxicity of Con-A for lymphoid cells.

Figure 3 shows that pre-incubation of non-immune cells for 24
hours with from 2 to 20 μg Con-A per ml had a stimulatory effect on
killing above spontaneous release; this effect was seen with as little
as six hours of pre-incubation. Concurrently, in the 24 hour pre-
incubation groups, LDCC was suppressed by prior incubation with Con-A,
indicating that the functional capabilities of the cell population was
shifted after 24 hours of incubation with Con-A.

The viable cell recovery was reduced in the Con-A treated
groups, yet killing above spontaneous release was enhanced. This in-
crease in killing activity argues against a reduced functional capacity
Table 3. Effect of α-methylmannoside on Con-A induced suppression of immune cytotoxicity.a

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell Type</th>
<th>α-Methylmannoside (mM)</th>
<th>Con-A</th>
<th>Percent Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Immune</td>
<td>-</td>
<td>-</td>
<td>37 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>-</td>
<td>37 ± 8</td>
</tr>
<tr>
<td></td>
<td>Immune</td>
<td>-</td>
<td>+</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>+</td>
<td>9 ± 3</td>
</tr>
<tr>
<td></td>
<td>Non-immune</td>
<td>-</td>
<td>-</td>
<td>57 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>-</td>
<td>54 ± 11</td>
</tr>
<tr>
<td></td>
<td>Immune</td>
<td>50</td>
<td>-</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>II</td>
<td>Immune</td>
<td>50</td>
<td>+</td>
<td>7 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>+</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

aH-2b mice were immunized in vivo with L cells as described in Materials and Methods. Spleen cells were then pre-incubated with/without Con-A for 24 hours. At 1.5 hours before the end of the pre-incubation period, α-methylmannoside was/was not added. At the end of 24 hours, the cells were washed three times, and measured for cytotoxic function against L cells in a 24 hour 51Cr release assay.

bPre-incubation with 20 μg per ml.

cIn experiment I, the effector to target cell ratio was 5:1. In experiment II, the effector target cell ratio was 4:1.

dCalculated by equation (2).

eCalculated by equation (3).
Figure 3. Effects of pre-treatment with Con-A on non-immune cytotoxicity and LDGC.

Non-immune H-2^b spleen cells were pre-incubated for six or 24 hours with various doses of Con-A as described in Materials and Methods. Cytotoxicity was then measured against L cells in a 24 hour ^{51}Cr release assay in the absence (□) or presence (□) of 2 µg Con-A per ml. An effector to target cell ratio of 5:1 was used, and cytotoxicity was calculated by equation (3).
Figure 3. Effects of pre-treatment with Con-A on non-immune cytotoxicity and LDCC.
after Con-A pre-treatment. Con-A toxicity could be the explanation for the suppression of immune cytotoxicity only if the toxicity were specific for immune cytotoxic cells as opposed to non-immune cytotoxic cells.

It has previously been shown that immune cytotoxic assays of 24 hours in length detect a heterogeneous group of killer cells, most probably T cells in combination with macrophages (Lohrmann-Matthes and Fisher 1973). Short term assays, such as four to six hours, measure predominantly T cell function (Henney 1973b; Golstein and Blomgren 1973). It can be seen in Table 4 that a seven hour $^{51}$Cr release assay could not detect killing by cells from the in vivo immunized mice. In a 24 hour assay, immune cytotoxicity and its suppression by Con-A were seen. Since the cytotoxic response was low and probably a product of multiple cell types, an alternate form of generating immune cytotoxic cells was used.

**Cytotoxicity Generated from Mixed Lymphocyte Cultures and its Suppression with Con-A**

Responder H-2$^b$ spleen cells were mixed with X-irradiated allogeneic H-2$^k$ stimulator cells at a ratio of 1:1. After five days of incubation under standard conditions, these mixed lymphocyte cultures were tested for cytotoxicity against L cells which were H-2$^k$ in origin. Control cells consisted of H-2$^b$ spleen cells cultured alone or with X-irradiated H-2$^b$ spleen cells.

Figure 4 shows the kinetics of anti-L cell cytotoxicity generated in this manner. The six hour $^{51}$Cr release assay was sufficient. By 24 hours, killing by cells from non-immune animals at 10:1 and 25:1 effector to target cell ratios was sufficiently great to reduce the
Table 4. Effect of Con-A on cytotoxicity of spleen cells from mice immunized in vivo.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Con-A\textsuperscript{b}</th>
<th>Assay Time\textsuperscript{c}</th>
<th>Percent Immune Cytotoxicity\textsuperscript{d,e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune</td>
<td>-</td>
<td>7 hrs.</td>
<td>-5 ± 2</td>
</tr>
<tr>
<td>Immune</td>
<td>+</td>
<td>7 hrs.</td>
<td>-5 ± 4</td>
</tr>
<tr>
<td>Immune</td>
<td>-</td>
<td>24 hrs.</td>
<td>24 ± 9</td>
</tr>
<tr>
<td>Immune</td>
<td>+</td>
<td>24 hrs.</td>
<td>-4 ± 3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}H-2\textsuperscript{b} mice were immunized in vivo with L cells, and the immunized spleen cells, prior to measuring cytotoxic function against L cells, were pre-incubated with/without Con-A for 24 hours as described in Materials and Methods.

\textsuperscript{b}Pre-incubation with 20 \&mu;g per ml.

\textsuperscript{c}Length of the \textsuperscript{51}Cr release assay.

\textsuperscript{d}Calculated by equation (2).

\textsuperscript{e}Effector to target cell ratio of 5:1.
Figure 4. Cytotoxicity generated by mixed lymphocyte cultures.

$H^{-2}_b$ anti-$H^{-2}_k$ cytotoxic lymphocytes were generated in mixed lymphocyte cultures, and cytotoxicity against L cells was calculated by equation (2). Six (□), 12 (○), and 24 (△) hour $^{51}Cr$ release assays are shown.
Figure 4. Cytotoxicity generated by mixed lymphocyte cultures.
percent immune cytotoxicity calculated by equation (2). This was seen less dramatically when fresh non-immune spleen cells were used for the non-cytotoxic control, because spontaneous killers arise after five days of culture (Klimpel, personal communication 1978). Immune cytotoxic cells were generated in this manner for all further experiments, and $^{51}$Cr release assays were subsequently run for six hours.

Figure 5 shows the effects that pre-incubation with Con-A has on in vitro generated cytotoxic cells. Pre-incubation was for 6, 12, and 24 hours with 2, 10, and 20 µg Con-A per ml. As can be seen, the immune cytotoxic response was stimulated with 2, but suppressed with 10 and 20 µg Con-A per ml. Cell viability, as shown in Figure 6, dropped in the Con-A treated groups, but was not necessarily an indicator of suppression since 2 µg per ml caused stimulation of cytotoxicity concomitant with a loss of cell viability.

Detection of Autologous $^{51}$Cr Release

The reduction in lymphoid cell numbers and suppression of cytotoxic function in the presence of Con-A might be explained by an anti-self, or anti-autologous, LDCC reaction. By this theory, loss of cytotoxic capability would be due to a loss of cytotoxic cells through an internal anti-autologous cytotoxic response (Bradley and Bonavida 1978). Immune cells are profoundly suppressed by pre-treatment with Con-A, as opposed to non-immune cells. Therefore, anti-autologous LDCC responses would be more prevalent in immune cell cultures than non-immune cultures. It would be expected that, firstly, chromated immune lymphoid cells would lyse in the presence of Con-A to a greater extent than non-immune lymphoid cells.
Figure 5. Con-A induced suppression of mixed lymphocyte culture cytotoxicity.

H-2b anti-H-2k cytotoxic lymphocytes were generated in mixed lymphocyte cultures, and pre-incubated for 6, 12, and 24 hours with 0 (□), 2 (△), 10 (●), and 20 (○) μg Con-A per ml as described in Materials and Methods. Cytotoxicity was then assayed against L cells in a six hour ⁵¹Cr release assay using equation (2) for calculations. The effector to target cell ratio was 5:1.
Figure 5. Con-A induced suppression of mixed lymphocyte culture cytotoxicity.
Figure 6. Viable cell recovery after Con-A treatment.

The percent viable cell recovery after Con-A pre-treatment was measured by trypan blue exclusion on the cells from Figure 5 prior to assay for cytotoxic function. Non-immune cells (□), immune cells (△), immune cells plus 2 (○), 10 (■), and 20 (▲) μg Con-A per ml.
Figure 6. Viable cell recovery after Con-A treatment.
Figure 7 shows that chromated immune lymphoid cells did indeed release more of their own $^{51}$Cr than did non-immune lymphoid cells in the presence of an immune-suppressive dose of Con-A. The non-immune cells were from five day cultures ($H-2^b$ cells cultured alone). Five day control cultures were used for the non-immune control only for one experiment. Because of the poor viable cell recovery from five day control cultures, this experiment was repeated using fresh non-immune spleen cells as the non-immune control.

Figure 8 shows the effects of various doses of Con-A on autologous $^{51}$Cr release using fresh non-immune spleen cells as the non-immune control. Results were in agreement with Figure 7. As can be seen, the immune cytotoxic cell population was lysed more extensively than were non-immune cells at all doses of Con-A tested. Fresh non-immune spleen cells were used as the non-cytotoxic control for all further experiments.

**Autologous $^{51}$Cr Release in the Presence of Con-A Represents an Anti-autologous Cytotoxic Response**

If an anti-autologous cytotoxic response were responsible for the suppression seen with Con-A treated immune lymphoid cells, then this response should be blocked by agents which interfere with cytotoxic reactions. Colchicine disrupts microtubules at low concentrations (Mizel and Wilson 1972) and profoundly suppresses the effector mechanism of the cytotoxic response (Plaut, Lichenstein and Henney 1973). Colchicine will not affect the ability of target cells to be recognized and lysed in the $^{51}$Cr release assay (Ranney 1977); therefore, the
Figure 7. Autologous $^{51}$Cr release from immune and non-immune (five day control) cells. $H^{-2b}$ anti-$H^{-2k}$ cytotoxic lymphocytes (□) were generated in mixed lymphocyte cultures, and autologous $^{51}$Cr release was assayed in a four hour $^{51}$Cr release assay with 20 μg Con-A per ml. Autologous $^{51}$Cr release was calculated using equations (5a) and (5b). Non-immune $H^{-2b}$ lymphocytes (□) are from five day control cultures ($H^{-2b}$ cultured alone).
Figure 8. Autologous $^{51}$Cr release from immune and non-immune cells.

H-2$^b$ anti-H-2$^k$ cytotoxic lymphocytes (□) were generated in mixed lymphocyte cultures, and autologous $^{51}$Cr release was assayed in a four hour $^{51}$Cr release assay with various doses of Con-A. Autologous $^{51}$Cr release was calculated using equations (5a) and (5b). The doses of Con-A in μg per ml are written above the bars. Non-immune H-2$^b$ lymphocytes are represented by (□).
Figure 8. Autologous $^{51}$Cr release from immune and non-immune cells.
effect of colchicine in inhibiting cytotoxicity is on the effector cell and not the target cell. Colchicine does not interfere with the binding of Con-A to the cell surface (Ranney and Pincus 1976), and so can be used to specifically block the cytotoxic effector mechanism in LDCC reactions.

Figure 9 shows the data from an experiment in which immune and non-immune cells were treated with 100 μM colchicine prior to measuring autologous $^{51}$Cr release in the presence of Con-A. As can be seen, colchicine did block autologous $^{51}$Cr release, suggesting very strongly that $^{51}$Cr release from immune cells in the presence of Con-A represented an anti-autologous cytotoxic response.

Further evidence that anti-autologous cytotoxic responses existed is seen in Figure 10. Immune and non-immune cell cultures were each divided in half. One half served as target cells, the other half as effector cells. If immunization induces a population that attacks autologous cells in the presence of Con-A, then immune target cells should lyse only in the presence of immune effector cells. In order to insure that the immune cells serving as targets did not destroy themselves, they were pre-treated with colchicine to block self-lysis. Immune or non-immune effector cells were then added to $^{51}$Cr labeled colchicine treated immune or non-immune target cells at an effector to target cell ratio of 10:1, and in the presence of 10 μg Con-A per ml. As predicted, immune cells were capable of lysing autologous immune cells, and non-immune cells were not capable of anti-autologous or anti-syngeneic lysis. That the immune target cells were not self-lysing is shown by the low level of $^{51}$Cr release from immune
Figure 9. Effect of colchicine on autologous $^{51}$Cr release.

$H-2^b$ anti-$H-2^k$ cytotoxic lymphocytes were generated in mixed lymphocyte cultures, and autologous $^{51}$Cr release was assayed in a four hour $^{51}$Cr release assay with $10 \mu$g Con-A per ml. Autologous $^{51}$Cr release was calculated by equations (5a) and (5b). Cells were pre-treated with colchicine as described in Materials and Methods.
Figure 9. Effect of colchicine on autologous $^{51}$Cr release.
Figure 10. Anti-autologous and anti-syngeneic cytotoxic responses.

H-2b anti-H-2k cytotoxic lymphocytes were generated in mixed lymphocyte cultures, and anti-autologous LDCC assayed as described in Materials and Methods. Anti-autologous LDCC was calculated using equations (6a) and (6b).
Figure 10. Anti-autologous and anti-syngeneic cytotoxic responses.
target cells in the presence of non-immune effectors. In addition, immune cell cultures were capable of lysing syngeneic non-immune lymphoid cells.

**Anti-autologous Cytotoxic Responses from Fractionated Immune Cell Subpopulations**

An attempt was made to isolate relatively purified populations of L cell killers present in immune cell cultures. Immune cells were allowed to adhere to L cell monolayers for 45 minutes. The monolayers were then washed several times, leaving an enriched population of anti-L cell cytotoxic cells adhering to the monolayers (killer enriched). These cytotoxic cells were then removed with PBS-EDTA as described in Materials and Methods, and tested for the ability of pre-incubation with Con-A to suppress their cytotoxic function.

It was envisioned that possibly a cell other than the anti-allogeneic cytotoxic cell was the effector cell for the anti-autologous cytotoxic response, with the anti-allogeneic cell serving as the target cell. This anti-self cytotoxic cell would not be present in the lymphoid cell population found adhering to L cell monolayers due to its specificity for self as opposed to the allo-target. Therefore, it was expected that killer-enriched cells would not be suppressed when treated with Con-A.

Table 5 shows that killer-enriched cells were suppressed in their ability to mount an anti-L cell cytotoxic response after pre-treatment with 20 μg Con-A per ml. Autologous $^{51}$Cr release was also demonstrable for killer-enriched cells (Fig. 11). Even though 2 μg Con-A per ml stimulated the anti-allogeneic cytotoxic response
Table 5. Effect of pre-incubation with Con-A on killer-enriched cell cytotoxicity.a

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Con-A</th>
<th>Percent Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune</td>
<td></td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Immune</td>
<td>+</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Immune</td>
<td>-</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>Killer-enriched</td>
<td>-</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Killer-depleted</td>
<td>-</td>
<td>7 ± 2</td>
</tr>
</tbody>
</table>

H-2<sup>b</sup> anti-H-2<sup>k</sup> cytotoxic lymphocytes were generated in mixed lymphocyte cultures, fractionated on L cell monolayers, and then pre-incubated with/without Con-A for 24 hours as described in Materials and Methods. The resultant cells were tested for cytotoxicity against L cells in a six hour ⁵¹Cr release assay at an effector to target cell ratio of 1:4:1.

Pre-incubation with 20 µg per ml.

Calculated by equation (2).
Figure 11. Autologous $^{51}$Cr release from fractionated immune cell subpopulations.

$H^{-2}_{-2b}$ anti $H^{-2}_{-2k}$ cytotoxic lymphocytes were generated in mixed lymphocyte cultures, and fractionated on L cell monolayers. Autologous $^{51}$Cr release was assayed in a four hour $^{51}$Cr release assay with various doses of Con-A. Autologous $^{51}$Cr release was calculated using equations (5a) and (5b). Two separate experiments are represented by A and B.
Figure 11. Autologous $^{51}$Cr release from fractionated immune cell subpopulations.
(Fig. 5), this dose of Con-A also induced autologous $^{51}\text{Cr}$ release from killer-enriched cells. In addition, it can be seen that killer-depleted cells, purified by three passes on L cell monolayers, were also capable of autologous $^{51}\text{Cr}$ release in the presence of Con-A. Taken together, these data suggest that anti-autologous LDCC cells are found in both killer-enriched and killer-depleted cell cultures.

**Stimulation of Immune Reactivity by Con-A is Due to Enhancement at the Cytotoxic Cell Precursor Level**

Immune cytotoxic cell cultures pre-treated with 2 $\mu$g Con-A per ml were enhanced in their ability to lyse L cell targets. At this dose, however, the pattern of loss of cell viability concomitant with autologous $^{51}\text{Cr}$ release was similar to that seen with higher doses of Con-A. In addition, the subpopulation enriched for L cell killers was susceptible to autologous $^{51}\text{Cr}$ release in the presence of Con-A. To explain the stimulation in anti-allogeneic cytotoxicity with 2 $\mu$g, it was postulated that a shift from immature, non-functional cytotoxic cells to mature, functional cells was occurring. Thus, a Con-A induced maturational event was adding functional anti-L cell cytotoxic cells to the existing cytotoxic cell pool. This would result in an enhancement of the overall cytotoxic response due to increased numbers of cytotoxic effector cells. The maturation of effector cells would be greater than the loss of effector cells due to the anti-autologous cytotoxic response; this would result in a net enhancement of the anti-allogeneic cytotoxic response. Immature cytotoxic cells would be expected to lack the appropriate kinds or number of receptors specific for firm binding
to the allogeneic target cell, which is a prerequisite for lysis (Kuppers and Henney 1976; Berke 1977). Such cytotoxic cell precursors should be found in the killer-depleted group (Kamat and Henney 1975, 1976).

Support for this hypothesis is shown in Figure 12. Killer-depleted cells were fractionated out of the immune cell culture and pre-incubated for 24 hours with 2 and 10μg Con-A per ml, along with untreated immune and non-immune unfractionated populations. Cytotoxic responses were then measured against L cells in the absence and presence of Con-A (LDCC). Both six and 24 hour 51Cr release assays are shown.

In examining the six hour 51Cr release data (A), it was seen that pre-incubation of killer-depleted cells with 2 μg Con-A per ml did enhance their ability to lyse L cells. Pre-incubation with 10 μg Con-A per ml had a suppressive effect on the residual cytotoxic capability of the killer-depleted group. It was also apparent that there were potent lectin dependent anti-allogeneic cytotoxic cells in the killer-depleted group. The LDCC response for killer-depleted cells occurred sooner and was higher in magnitude than for non-immune cells. As discussed earlier, unfractionated immune cells showed only a very small LDCC ability on the allogeneic targets, suggesting that the proportion of anti-L cell cytotoxic cells was higher than lectin dependent anti-allogeneic cytotoxic cells in unfractionated immune cell cultures.

By 24 hours of assay (B) the untreated killer-depleted cells had developed a potent anti-L cell cytotoxic capability. These same cells pre-treated with 10 μg Con-A per ml, which were suppressed in
Figure 12. Effect of Con-A on killer-depleted cell responses.

H-2<sup>b</sup> anti-H-2<sup>k</sup> cytotoxic lymphocytes were generated in mixed lymphocyte cultures, fractionated on L cell monolayers, and then pre-incubated for 24 hours with various doses of Con-A as described in Materials and Methods. The resultant cells were assayed for cytotoxicity against L cells in the presence (▴) or absence (□) of 2 µg Con-A per ml, and at an effector to target cell ratio of 5:1. Cytotoxicity was calculated using equation (3). A and B represent six and 24 hour <sup>51</sup>Cr release assays, respectively.
Figure 12. Effect of Con-A on killer-depleted cell responses.
Figure 12—Continued.
the six hour $^{51}$Cr release assay, developed a substantial cytotoxic ability in the 24 hour assay. The LDCC reactions generally showed a suppression relative to LDCC independent killing, except for the familiar LDCC response seen with non-immune cells.
DISCUSSION

The data presented here on Con-A mediated suppression of allo-reactivity confirm and extend the observations made by others either explicitly (Bradley and Bonavida 1978) or implicitly (Gately and Martz 1977; Laux and Tunticharoen 1978). Bradley and Bonavida showed that the antigen specific cytotoxic capability of in vivo immunized lymphocytes could be suppressed by pre-incubation with either 10 µl PHA per ml, or 100 µg Con-A per ml, for four hours. In addition, they found that $^{51}$Cr labeled Con-A blasts lysed in the presence of an immunosuppressive dose of PHA. There was no difference in lectin mediated reduction in cell viability of blasts as compared to non-immune cells when each was diluted so that cell-cell contact could not occur. Thus, their hypothesis was that lectin induced suppression of cytotoxicity and lysis of blasts was due to an anti-autologous LDCC response.

Similar observations are reported here. The cytolytic capability of H-2$^b$ spleen cells alloimmunized to L cells in vivo was suppressed by incubation for 24 hours with 20 µg Con-A per ml (Tables 2, 3, and 4). Similarly the cytolytic capability of H-2$^b$ spleen cells alloimmunized to H-2$^k$ spleen cells in vitro was suppressed by incubation for six, 12, or 24 hours with 10 or 20 µg Con-A per ml (Fig. 5). There was a loss of cell viability concomitant with suppression (Fig. 6). Suppression was not directly attributable to the loss of cell viability or a non-specific toxic effect of Con-A for lymphocytes as
shown by an overall stimulation of non-immune cytotoxicity by pre-incubation for six and 24 hours with 10 and 20 μg Con-A per ml despite a loss of cell viability (Fig. 3). If toxicity were responsible for the immunosuppression, then immune cells were selectively affected and non-immune cells were unaffected.

Investigation of the LDCC reaction showed that the cytolytic ability of non-immune spleen cells was enhanced when 2 μg Con-A per ml was included during the cytotoxic assay (Fig. 1). Reports in the literature showed that the LDCC capability of alloimmune cells was 50 to 100 times greater, than that from non-immune cells, on allogeneic targets that were genetically dissimilar to the sensitizing allogeneic cell (Gately and Martz 1977). Thus, antigen specific alloreactivity was reportedly not enhanced, but lysis of other allogeneic targets was enhanced, by the presence of PHA or Con-A during the cytotoxic assay.

It is hypothesized that immune cell cultures are capable of an anti-autologous LDCC reaction. This hypothesis is based on three observations derived from the literature and experiments presented here: (1) both immune and non-immune cell cultures are capable of an LDCC response; immune cell cultures are also capable of an LDCC response against syngeneic target cells; (2) pre-incubation of immune spleen cells, alloimmunized either in vivo or in vitro, with super-mitogenic doses of Con-A results in immunosuppression; and (3) immune cell viability is reduced by incubation with Con-A via a mechanism that probably does not reflect a generalized toxicity of Con-A for lymphocytes.
Data presented here confirm the notion that immune cell cultures are capable of anti-autologous LDCC. Alloimmunized spleen cells lyse in the presence of 2, 10, or 100 µg Con-A per ml to a greater extent than do non-immune cells (Figs. 7 and 8). Colchicine, an inhibitor of the cytotoxic effector mechanism, blocks Con-A induced lysis (Fig. 9) suggesting that Con-A induces an anti-autologous cytotoxic response. Final demonstration of an anti-autologous LDCC response by immune cells was shown by the use of autologous immune cells as both effectors and targets in an LDCC reaction. Alloimmune spleen cells lysed both autologous alloimmune spleen cells, and syngeneic non-immune spleen cells, in the presence of 10 µg Con-A per ml (Fig. 10). Non-immune spleen cells were not capable of LDCC against syngeneic immune or non-immune cells.

During an anti-autologous LDCC assay, immune cells used as targets would self-lyse in the presence of Con-A, thus obliterating the effect that the addition of an effector cell would have on immune target cell lysis. Therefore the anti-autologous LDCC assay was performed under conditions that minimized any anti-autologous cytotoxic responses by the immune or non-immune target cells. Therefore, target cells were pre-treated with colchicine to inhibit target cell mediated target cell lysis. In addition, the anti-autologous LDCC reaction was performed at an effector to target cell ratio of 10:1. At this ratio, target cells were diluted out so that cell-cell contact would most likely occur between effector cells and target cells as opposed to target cells and themselves. Proof that Con-A mediated lysis was not due to a direct toxic effect on either immune or non-immune cells was
shown by little or no lysis of either target in the presence of Con-A plus non-immune effectors.

An attempt was made to distinguish the cells that serve as autologous targets from the cells that serve as autologous effectors. In approaching this problem, three points were considered: (1) autologous $^{51}$Cr release, anti-autologous LDCC, and anti-syngeneic LDCC was much greater in the alloimmune cell cultures than in the non-immune cell cultures; (2) autologous immune and syngeneic non-immune cells could serve equally well as targets; and (3) the correlation in the immune cultures of Con-A mediated suppression and anti-autologous cytotoxicity strongly suggested that alloimmune cytotoxic T cells could serve as target cells for anti-autologous LDCC. Thus, it was thought that the anti-autologous LDCC effector cell and the alloimmune cytotoxic T cell would be two distinct cells, with the latter serving as a target for the former.

To investigate the notion that the anti-autologous LDCC cell was separate from the alloimmune cytotoxic T cell, $H^{-2^b}$ anti-$H^{-2^k}$ cytotoxic lymphocytes were isolated on L cell monolayers. These killer-enriched cells, and the corresponding monolayer non-adherent killer-depleted cells, were tested for the ability to lyse in the presence of Con-A. If anti-autologous LDCC was due to a cell separate from the alloimmune cytotoxic T cell, then separation of anti-autologous LDCC effector cells from the autologous target cells (alloimmune cytotoxic T cells) would result in a reduction of autologous $^{51}$Cr release in the presence of Con-A for both separated populations.
The data presented here show that both killer-enriched and killer-depleted cells lysed in the presence of Con-A (Fig. 11). In addition, killer-enriched cells were suppressable with Con-A (Table 5). Thus, there may be any one (or combination) of four explanations as to why Con-A was capable of inducing presumptive anti-autologous cytotoxicity in both killer-enriched and killer-depleted cell populations: (1) the separation procedures used may not have sufficiently depleted or enriched the wanted cell subpopulations; (2) some anti-autologous LDCC cells may bind to the alloimmune cytotoxic T cell and therefore be present in the killer-enriched (as well as killer-depleted) cell population; (3) alloimmune cytotoxic T cells and anti-autologous LDCC cells may each be capable of lysing themselves in the presence of Con-A; and (4) the anti-autologous LDCC cell may also be capable of anti-allogeneic LDCC and be an immature alloimmune cytotoxic T cell (pre-killer cell). This latter explanation is favored because it most accurately accounts for the data presented here, as well as that from the literature.

Pre-killer cells have been reported to be both adherent (Bach, Bach and Sondell 1976) and non-adherent (Kamat and Henney 1975, 1976) to homologous allogeneic cell monolayers. It may be that the ability of pre-killer cells to bind to target cells is a function of maturation. Therefore adherence to target cells would be a quality of pre-killers which would depend upon their degree of differentiation; relatively mature pre-killers would be capable of target cell binding without cytotoxic capability.
The relatively mature pre-killers would be present in the killer-enriched cell population, and would be responsible for anti-autologous LDCC against mature killer cells. The relatively immature pre-killers would be found in the killer-depleted cell population, and would be responsible for anti-autologous LDCC against killer-depleted cells. The pre-killer cells would be capable of LDCC against allogeneic, syngeneic, and autologous cells (including themselves) and thus would be responsible for Con-A induced autologous lysis and suppression of alloreactivity. Similarities between the pre-killer cell and the LDCC cell have been speculated on previously (Rubens and Henney 1977).

The antigen specific alloimmune cytotoxic response was not enhanced with an optimum LDCC dose of Con-A (Fig. 12), in contrast to Con-A enhancement of killing by non-immune cells. This observation is in agreement with others (Bevan and Cohn 1975; Bonavida and Bradley 1976; Rubens and Henney 1977). Thus, mature cytotoxic T cells are not capable of using the LDCC mechanism for cytolysis of their respective targets. The fact that potent LDCC could be found in the killer-depleted group (which presumably contains pre-killer cells) argues in agreement with others that LDCC cells and mature allospecific cytotoxic T cells are two separate cells (Rubens and Henney 1977; Laux and Tunticharoen 1978; Davignon and Laux 1978; Tsoukas and Martz 1978).

Augmentation of specific cytotoxicity by pre-incubation of an alloimmune cell culture for 12 or 24 hours *in vitro* with a mitogenic dose of Con-A (Fig. 5) is in agreement with others (Falkoff and Dutton 1977). Data presented here show that the Con-A induced augmentation
of cytotoxicity probably is a result of stimulation of cells found in the killer-depleted group (Fig. 12). Of these cells, the pre-killer cell is the most likely candidate for Con-A induced maturation to mature cytotoxic cells. In addition, exposure of killer-depleted cells to homologous allogeneic cells in a 24 hour $^{51}$Cr release assay resulted in the generation of a cytotoxic response. This is in agreement with Kamat and Henney (1975) who showed maturation of pre-killers to killers by a 24 hour culture in vitro prior to testing for cytotoxic function.

Pre-incubation of immune cells with 2 $\mu$g Con-A per ml resulted in enhancement of cytotoxicity concomitant with a loss of cell viability (Fig. 6) and autologous $^{51}$Cr release (Fig. 8). Of the cells that are susceptible to Con-A mediated viability loss, mature cytotoxic cells must be included because killer-enriched cells are capable of autologous $^{51}$Cr release in the presence of 2 $\mu$g Con-A per ml (Fig. 11). Therefore, it can be concluded that an anti-autologous LDCC reaction was occurring from which mature cytotoxic cells were not spared. At the same time, a Con-A induced maturational event was adding functional killer cells to the existing killer cell pool. The maturation of effector cells was greater than the loss of effector cells due to anti-autologous LDCC and resulted in a net enhancement of the anti-allogeneic cytotoxic response due to increased numbers of mature cytotoxic effector cells.

Non-immune cells pre-incubated for 24 hours with 2, 10, or 20 $\mu$g Con-A per ml were suppressed in their LDCC capability (Fig. 3). In fact, LDCC was lower than control values for cytotoxicity. Control values for non-immune cytotoxicity were enhanced when cells were pre-treated for six or 24 hours with 2, 10, or 20 $\mu$g Con-A per ml.
Therefore, pre-incubation of non-immune cells with Con-A will stimulate cytotoxicity concomitant with a strong suppression of cytotoxicity if the stimulated cells are assayed for cytotoxicity in the presence of an optimal LDCC dose of Con-A (2 µg Con-A per ml). The same suppression was seen when unfractionated alloimmune cells were assayed for cytotoxicity in a 24 hour $^{51}$Cr release assay in the presence of 2 µg Con-A per ml (Fig. 12). Suppression was also seen when killer-depleted cell populations were pre-incubated with 2 or 10 µg Con-A per ml and assayed for cytotoxicity in a 24 hour $^{51}$Cr release assay in the presence of 2 µg Con-A per ml. Thus, it is possible that: (1) anti-autologous LDCC is responsible for the suppression; pre-incubation of non-immune and killer-depleted cells with Con-A (or in the case of immune cells, exposure to the sensitizing alloantigen in a 24 hour $^{51}$Cr release assay) causes pre-pre-killers to mature to pre-killers with anti-autologous LDCC capability causing suppression; and/or (2) suppressor cells have been induced which function in the presence of 2 µg Con-A per ml. The concept of suppression upon re-stimulation is in agreement with Soren and Maller (1979) who found that purified protein derivative (PPD)-stimulated lymphocytes re-stimulated with PPD showed a decreased PPD responsiveness. In any event, it can be concluded that pre-incubation of non-immune cells with Con-A affects functional changes in both non-immune cytotoxicity and LDCC.

In conclusion, the data reported here support the hypothesis that the LDCC cell and pre-killer cell are one and the same. Non-immune cell cultures are capable of only weak LDCC against allogeneic targets (Fig. 1), a reflection of the small numbers of pre-killers
present in the absence of alloimmunization. Once alloimmunized, the levels of both killers and pre-killers rise along with LDCC capability. The pre-killer cells will express their cytotoxic capability against any target cell in the presence of Con-A. As pre-killer cells increase in number, anti-autologous (or anti-syngeneic) cytotoxicity can be quantitated in the presence of Con-A by anti-autologous LDCC (Figs. 8, 9, and 10), viability loss (Fig. 6), and immunosuppression (Tables 2, 3, and 4; Fig. 5). Relatively mature pre-killer cells are capable of binding to homologous allogeneic cell monolayers, thus becoming responsible for the Con-A induced autologous lysis seen in the killer-enriched cell population (Fig. 11). Those pre-killers that do not bind to the monolayers are responsible for the Con-A induced lysis seen in the killer-depleted cell population (Fig. 11). The pre-killers present in the killer-depleted group are also capable of maturation to killers either by culture with a mitogenic dose of Con-A, or in vitro culture with the sensitizing alloantigen (Fig. 12).

The significance of these data lies in the further understanding of the lymphoid maturational events leading to production of a cytolytic response against a disease (such as viral or neoplastic). The lymphoid maturational events described after alloimmunization showed a potential for an anti-self immune response. This concept lends further credence to the evidence that autoimmunity may be preceded in some instances by a relatively normal immune response.

If the conclusion is correct that the LDCC cell and pre-killer cell are the same, then LDCC could be a powerful way of detecting pre-killer cells in lymphoid cell populations. Thus, a stage of
immunological maturation would be isolated in the absence of a mature immunological response. This could aid in resolving the steps involved in the generation of a cytolytic response, or the lack thereof. Thus, it would be possible to measure whether priming had occurred in the absence of mature cytolysis. For example, lack of a cytotoxic response against one's own tumor may be resolved as to whether the defect lies with either priming or cytolytic cell maturation by the absence or presence of anti-autologous or anti-allogeneic LDCC capability.
LIST OF REFERENCES


