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COSTIMULATOR: CELLULAR ORIGINS, PHYSICAL PROPERTIES AND EFFECTS ON IMMUNE RESPONSES *IN VITRO*

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled COSTIMULATOR: CELLULAR ORIGINS, PHYSICAL PROPERTIES AND EFFECTS ON IMMUNE RESPONSES *IN VITRO* submitted by JENNIFER SHAW in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY.

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Abstract

Leukocytes participating in immunological responses to antigenic stimuli are functionally divisible into effector cells (producing antibody, or mediating killing), and regulatory cells which trigger, amplify, or suppress the development of effector functions. Regulatory and effector cells can communicate through soluble factors, termed lymphokines, which are released by antigen-stimulated leukocytes, and act on target receptors on other stimulated cells. The study of one such lymphokine, "costimulator", is the subject of this thesis.

Production of costimulator (a Con A-induced lymphokine) requires participation of two cell types: a T cell, and an adherent cell. The T cells bear the lymphocyte differentiation antigens Ly 1.1, Ly 5.1, and Ly 7.2, but not Ly 2.1 or Ly 6.1. T cells which are "helpers" in the CTL response have the same Ly phenotype. The adherent cell is probably a macrophage since its function can be supplied by macrophages cultured from bone-marrow, or a soluble macrophage-derived factor.

Costimulator appears to act as a second signal in immune induction of T cell precursors, the first signal being provided by antigens or mitogens. It allows the proliferation of thymocytes cultured at low cell density in response to Con A. It stimulates the generation of cytotoxic T lymphocytes (CTL) from thymocyte precursors, and restores

the response of splenic precursors which have been depleted of helper cells by treatment with anti-Ly 7.2 serum. Costimulator appears to replace helper cells in the antibody response of B cells to sheep erythrocytes, in this way resembling "T cell replacing factor". It also restores the CTL response of lymph node lymphocytes which have been depleted of adherent cells. Thus, costimulator is a product of interaction between adherent cells (macrophages) and T helper cells, and it replaces both of these in proliferative or immune responses by T cells.

Costimulator activity resides in a highly acidic (pI 3.7-4.5) protein or glycoprotein of 30,500 daltons and sedimentation coefficient 2.63S. It has a Stokes radius of 29.6, which is typical of a globular protein of 44,000 MW, possibly the result of an asymmetric shape. It does not bear Ia antigens, and is not H-2 restricted in any of its activities. These properties distinguish it from antigen-specific T cell factors, macrophage derived lymphokines, and "allogeneic effect factor".

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Abbreviations and Definitions

A cell:	cells adherent to nylon wool; macrophage
AEF:	allogeneic effect factor
AFC:	antibody forming cell
antigen:	protein or carbohydrate moieties which are immunogenic
B cell:	bone marrow-derived or "bursa-equivalent" lymphocyte
carrier:	portion of an antigen which is recognized by helper T cells
complement:	a complex series of serum proteins which cause lysis of cells which have antibody bound to them
Con A:	concanavalin A; a T cell mitogen
congenic:	mice which are identical at all but a very limited number of genetic loci
C region:	constant region of an immunoglobulin
CTL:	cytotoxic T lymphocytes
DNP:	dinitrophenyl; a hapten
dThd:	thymidine
FBS:	fetal bovine serum
hapten:	an antigenic determinant which is recognized by B lymphocytes
H-2:	mouse major histocompatibility genes
H chain:	immunoglobulin heavy chain
Ia:	I region associated
IEF:	isoelectric focusing
Ig:	immunoglobulin
IL1, IL2:	interleukin 1 and 2; soluble mediators secreted by leukocytes
I region:	region of the H-2 locus

KLH:	Keyhole limpet hemocyanin, an antigen
LAF:	lymphocyte activating factor; same as IL2
L chain:	immunoglobulin light chain
LPS:	bacterial lipopolysaccharide
Ly antigen:	lymphocyte differentiation marker; used to distinguish subsets of T and B lymphocytes
lymphokines:	soluble mediators secreted by leukocytes which exert their effects upon lymphocytes
2-Me:	2-mercaptoethanol
MHC:	major histocompatibility complex
mitogen:	substance which causes a large proportion of lymphocytes to undergo proliferation
MLC:	mixed leukocyte reaction
MLR:	mixed leukocyte reaction; refers to the proliferative response when allogeneic cells are cultured together
Mph:	macrophage; adherent cell
MW:	molecular weight
NMS:	normal mouse serum
NRS:	normal rabbit serum
NSF:	non-specific factor
NSM:	non-specific mediator
nu/nu:	congenitally athymic mice
PFC:	plaque forming cells in an antibody response
PHA:	phytohemagglutinin; a T cell mitogen
pI:	isoelectric point
PPD:	purified protein derivative of tuberculin
S.D.:	standard deviation
SRBC:	sheep red blood cells
T cells:	thymus-derived lymphocytes

TCGF:	T cell growth factor; same as IL2
Thy 1:	surface marker carried by all T cells
TL:	thymus leukemia antigen
TMF:	thymocyte mitogenic factor; same as IL2
TNP:	trinitrophenyl, a hapten
TRF:	T cell replacing factor; same as IL2
TSF:	thymocyte stimulating factor; same as IL2
V region:	variable region of an immunoglobulin

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I. Introduction

A. An Overview of the Immune System

Many diseases arise from aberrations of immune responsiveness. Either there is too little immunity, as is the case in the immunodeficiency states where common pathogens become life-threatening, or there is too much immunity as happens in autoimmune conditions such as multiple sclerosis and systemic lupus erythematosis. Inefficient or injurious immune responses can be related to failures of immune regulation - the capacity of the immune system to modulate it's own activity through amplification and reduction of effector systems. Knowledge of the ways in which this regulation is effected is crucial to successful pharmaceutical and immunological intervention in these diseases. The work described in this thesis is directed toward an understanding of the mechanisms governing immune responsiveness to foreign antigens.

Immune effector mechanisms are closely interrelated, but have been historically divided into humoral and cellular responses. Humoral responses are mediated through antibody secreted by B (bone-marrow derived) lymphocytes upon antigenic stimulation. Antibodies bind to pathogens, and their products, and aid in their removal either by direct toxicity through activation of serum complement, or by activation of phagocytic effector cells with receptors for antibody. Cellular responses are mediated by two major

classes of T (thymus-derived) cells. Cytotoxic T lymphocytes (CTL) act to kill somatic cells which express foreign antigens, such as viral proteins, on their membranes. Another class of T cell, responsible for delayed hypersensitivity (DTH), releases chemotactic factors in an area of foreign antigen infiltration so that phagocytic (antigen-nonspecific) cells are recruited to the area.

During the last decade it has been demonstrated that these effector systems are under the control of regulatory cells, which are usually T cells. T helper cells promote the proliferation and further differentiation of effector cell precursors whereas T suppressor cells inhibit them. The mechanism of communication between regulatory cells and effector cells is still a matter for speculation. It may occur through membrane to membrane contact, or alternatively, through the release of soluble mediators by the regulatory cells. Many soluble factors have been reported which are candidates for effector molecules in immune regulation (see section J). The physical and biological characterization of one such factor, costimulator, is the subject of this thesis.

The adaptive immune system, seen in its most intricate and developed form in vertebrates, performs the difficult task of aggressively and selectively destroying pathogens such as bacteria and viruses, while avoiding damage to the host. Essential for its efficiency in dealing with pathogens are several features which make the immune system perhaps

unique among biological systems: clonal restriction of cell-surface antigen receptors; diversity of receptor specificities; ability to discriminate between self and non-self; and "memory" for antigens encountered previously. A consideration of these properties of the immune system helps to explain why immune regulation is necessary and how it might act.

B. Clonal Restriction of Antigen Receptors

One of the basic tenets upon which modern immunology rests is the Clonal Selection Theory formulated by Burnet (1959). Stated simply, it predicts that a single lymphocyte (or clone derived from it) expresses only one of the many possible receptors for antigen that exist in the organism. Foreign antigens with binding affinity for a particular receptor would stimulate the lymphocyte bearing the receptor to proliferate. Lymphocytes bearing receptors non-complementary to the antigen would not be affected. In the case of antibody-secreting lymphocytes, the effector molecule would have the same specificity as the receptor. Thus antigen specificity of an immune response is a result of clonality.

Much experimental evidence now supports the Clonal Selection Theory. It was shown that only a small proportion (about 1/5000) of spleen cells bind ^{125}I labelled antigens such as BSA (Naor and Sulitzeanu, 1967), haemocyanin, or polymerized flagellin from Salmonella Adelaide (Byrt and

Ada, 1967). A figure of about $20/10^6$ antigen-binding cells was obtained for polymerized flagellin using ^3H-labelled antigen (Diener and Paetkau, 1972). More compelling evidence for clonal selection came from "antigen-suicide" experiments in which lymphocytes reactive to antigen (Salmonella type 1338 flagellin) could be specifically deleted from a population of spleen cells by exposing them to the antigen labelled to high specific activity with ^{125}I. The cells lost the ability to make antibody against Type 1338, but responded normally to a closely-related flagellin from Salmonella 871 (Ada and Byrt, 1969). The interpretation was that the cells bearing receptors specific for strain 1338 were selectively inactivated through radiation damage caused by the ^{125}I. Wigzell and colleagues demonstrated that passing a population of immunized cells over antigen-coated glass bead columns specifically removed antibody forming cells (AFC), precursors of AFC, and memory cells for that antigen, but not for other antigens (Wigzell, 1970). The binding to the antigen-coated beads was inhibited by pretreatment of the cells with anti-immunoglobulin (Ig) sera, suggesting that the specific interaction was mediated by Ig molecules on the membrane.

Because B lymphocytes appear to have a 100 fold greater capacity for binding antigen than T lymphocytes (Roelants and Askonas, 1971) it is likely that these demonstrations of clonal restriction detected only B cell receptors. However, clonal restriction of T cell receptors is supported by other

evidence from antigen suicide and binding experiments (reviewed in Goodman, 1977).

The antibody specificity of B cells and their progeny in stimulated clones generally remains constant (Askonas *et al*, 1972; Askonas and North, 1976; Klinman, 1972). Exceptions to this rule have been reported by Cunningham and Pilarski (reviewed in Cunningham, 1976). Changes in murine receptor specificities directed against heterologous erythrocyte antigens during the course of clonal proliferation were observed, and were thought to arise through somatic mutation.

C. Diversity of Antigen Receptors

B Cell Receptors

Given that receptors are specific for antigenic determinants, there must be many receptor specificities to account for the variety of antigens that can be reacted against with apparent specificity. Using isoelectric focusing gels to enumerate the Igs produced by CBA mice against a small antigenic determinant, Kreth and Williamson (1973) obtained evidence for 8000 or more distinct types. The total repertoire of Ig produced by an individual has been estimated to be 2×10^7 for CBA mice (Kreth and Williamson, 1973) and $1\text{-}5 \times 10^7$ for BALB/c mice (Klinman and Press, 1975; Kohler, 1976).

The structure of immunoglobulin (the B cell receptor), while outside the scope of this discussion, has been

determined in detail (reviewed in Secher, 1979). The basic 7-8 S Ig molecule consists of 2 identical heavy (H) chains of 40,000-70,000 MW, and 2 identical light (L) chains of 23,000 MW. Both H and L chains are comprised of a C-terminal constant (C) region and an N-terminal variable (V) region. The V regions of both H and L chains consist of about 110 amino acids. The C region is about 110 amino acids in the L chains, and 300 or more in the H chain, depending on the class. The V region provides the antigen binding site and is the only portion of the Ig molecule to come into contact with antigen (Hochman *et al.*, 1973). The C region performs such functions as binding to complement and to cell surfaces by Fc receptors. Ig molecules have been divided into 8 major classes based on the amino acid sequences, disulfide bond positions, and sizes of their H chains. In mice, the different H chain types give rise to IgG1, IgG2A, IgG2B, IgG3, IgA, IgM, IgE and IgD. Most of mouse light chains (95%) are of a single type, called kappa, but 2 additional types of chains, both called lambda (1 and 2) are also expressed.

The genes coding for H, lambda and kappa chains are arranged in 3 unlinked clusters in the genome. It is now known that the genes for C regions of both H and L chains are present in the genome in only 1 or a few copies (reviewed in Paetkau, 1975). The question as to how many V region genes exist has been under intensive investigation. The lambda 1 and 2 V genes appear to be only a few

(Tonegawa, 1976; Honjo *et al.*, 1976), but the kappa V genes, which are responsible for most of the light chain variability in the mouse, have been estimated to be around 100-300 in number, (Seidman *et al.*, 1978; Valbuena *et al.*, 1978). It is still a matter of controversy whether there are enough V genes present to account for the observed repertoire of Igs (reviewed in Rabbits, 1979). Some investigators believe that somatic mutation may act on a germ line gene pool to create new receptor specificities. The first data to support such a view came from experiments by Tonegawa (1976). Amino acid sequence data based on the known lambda light chain-bearing Igs predict that more than 25 different lambda V regions exist. However experiments using nucleic acid hybridization kinetics, with several lambda light chain mRNAs as probes, showed that only 1-3 lambda genes are present in genomic DNA which could account for the 25 or so different amino acid sequences. Hence the number of germ line V genes is too small to account for the observed amino acid sequence diversity.

No matter what the actual number of V genes is, there must be a mechanism for the selective association of any one of a pool of V genes with a single C gene. Dryer and Bennet (1965) first proposed that a C gene could become fused to any one of the V genes during lymphocyte differentiation by a process of DNA translocation. Direct evidence that segments of DNA coding for V and C are widely separated in embryonic DNA, but exist in close proximity to each other in

mature B lymphocytes has now been obtained (Hozumi and Tonegawa, 1976; Brack *et al.*, 1978). The gene rearrangement, thought to arise by somatic recombination, does not result in the contiguous fusion of V and C coding regions as expected. In differentiated (myeloma) DNA, there is an insertion sequence between V and C of 1200 bases for lambda (Brack *et al.*, 1978) and 2800 bases for kappa (Lenhard-Schuller *et al.*, 1978). The inserted sequence is thought to be removed at the RNA level.

A recent discovery has suggested a mechanism other than somatic mutation by which antibody diversity might be generated. The C terminal portion of the V region is encoded by a DNA sequence of 39 nucleotides called the J (joining) region, which lies near the C lambda gene in embryonic DNA (Brack *et al.*, 1978) but is directly linked with the V coding sequence in myeloma DNA (Bernard *et al.*, 1978). One J segment has been found in the region of the lambda C gene (Bernard *et al.*, 1978), and 5 such sequences near the kappa C gene (Sakano *et al.*, 1979; Max *et al.*, 1979). Thus the J regions actually code for the last 13 amino acids of the variable portion of light chains, while the N-terminal 95 amino acids are coded for by what was originally referred to as the V gene. The J sequence in lambda becomes joined to the 3' end of a V gene during somatic recombination in which the DNA between V and J is excised (Sakano *et al.*, 1979). A schematic representation of the current concept of kappa light chain gene arrangement is given in Figure 1. It has

been suggested that by varying the exact point of V/J recombination over a 3 nucleotide region, many of the known Kappa sequence variations occurring at amino acid 96 could be accounted for (Sakano *et al.*, 1979; Max *et al.*, 1979). Thus the V/J recombinational event may be an additional mechanism for generation of receptor diversity. It is interesting that amino acid 96 of the light chain has been found to be at or near the antigen binding site in a Kappa light chain-bearing Ig whose 3 dimensional structure is known (Padlan, 1976).

It should perhaps be pointed out that even within so-called genes, there are often a number of inserted, untranslated sequences, which may separate domains or functional genetic elements. Examples of this are an N-terminal leader sequence in light chain genes, and regions of DNA separating the hinge region and the three C region domains of mouse gamma 1 H chain DNA (Sakano *et al.*, 1979b).

Another interesting aspect of Ig gene expression is the phenomenon of "class switching". A single clone of antibody producing cells appears to maintain its receptor specificity throughout differentiation, but changes the class of antibody produced (reviewed in Hood, 1975). This implies that the originally selected VL and VH genes are invariantly expressed, but that the CH gene changes. Thus the clone would express the antigen-binding portion of the Ig in conjunction with the various heavy chain classes IgM, IgD, IgG, IgA, IgD and IgE, which are capable of different

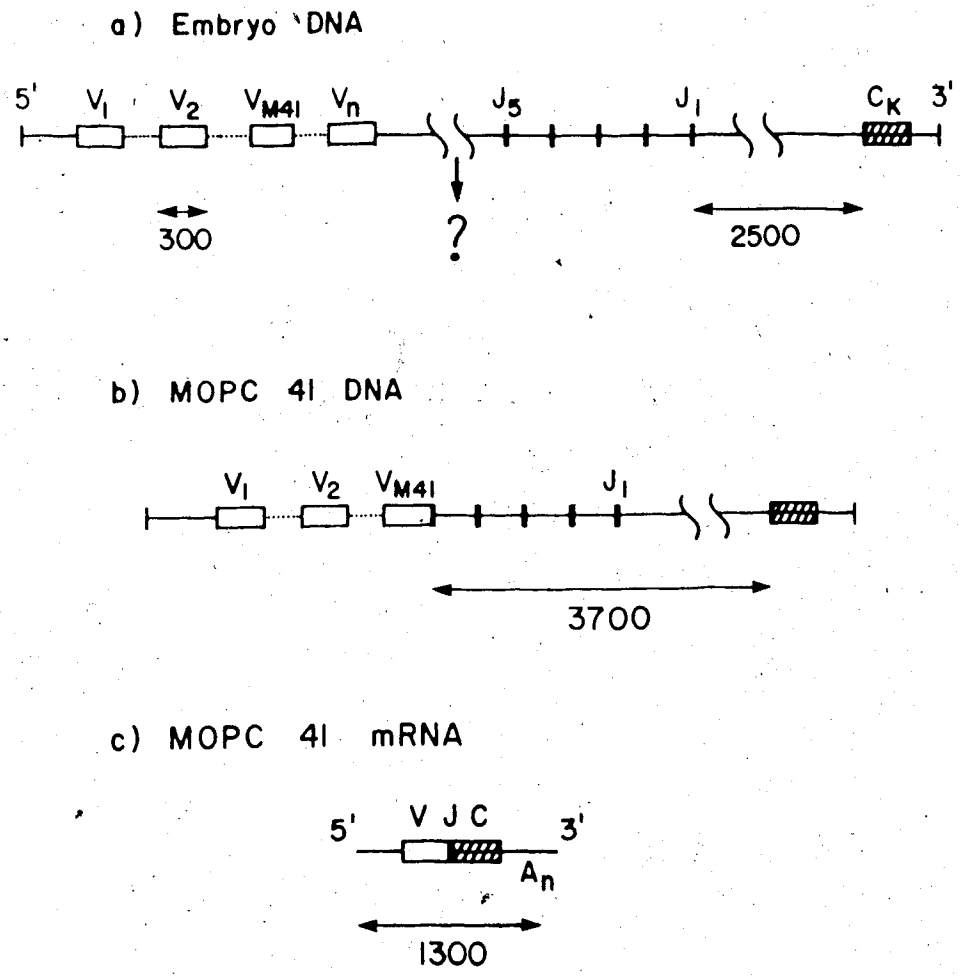


Figure 1. The arrangement of kappa light chain genes in embryonic DNA, MOPC 41 myeloma DNA, and mature MOPC 41 mRNA. Boxes represent coding sequences. Approximate distances are given in numbers of nucleotides. The arrangement of V genes with respect to each other and the C gene is hypothetical. The mRNA has non-coding regions at the 3' and 5' ends. V: variable region; Ck: constant region; J: joining segment. (Adapted from Seidman *et al.*, 1979).

effector functions. IgM is highly effective in binding complement and agglutination of bacteria. IgG also binds to complement and certain phagocytic effector cells, and can cross the placenta. IgA is secreted across mucous membranes, and IgE causes the degranulation of mast cells during allergic reactions. IgD is thought to function mainly as a surface receptor and is probably not secreted.

The evidence that cells producing Igs of different classes actually have the same V regions comes in part from analysis of human myeloma proteins. Most myelomas are thought to be monoclonal in origin, but some gave rise to Igs with different constant regions: IgM and IgG (Levin *et al.*, 1971), IgM and IgA (Seon *et al.*, 1973) or IgG and IgA (Wolfenstein-Todel *et al.*, 1974). It was found by peptide mapping that the light chain V and C regions were identical between the pairs of Igs, and peptides from the N terminals of the heavy chains were also similar, suggesting identical V regions (Wolfenstein-Todel *et al.*, 1974; Seon *et al.*, 1973). Antibodies against antigen-binding V region determinants also showed similarities between an IgM and IgG pair of myeloma proteins (Levin *et al.*, 1971)

By cloning mouse spleen cells in soft agar, it has recently been demonstrated that a single B cell precursor gives rise to progeny which secrete both IgM and IgG (Wabl *et al.*, 1978). This is taken as direct evidence that class switching takes place within a clone, rather than different clones having similar or identical V regions being committed

to IgM or IgG synthesis. The molecular mechanism by which cells produce Ig from a single V gene and 2 CH genes, both simultaneously and sequentially, has not been elucidated.

T Cell Receptors

Compared to the detailed genetic and structural information available about B cell receptors, there is little definitive work on T cell receptors. There is evidence that antigen specific receptors exist on T cell surfaces, that they are actually T cell products, and that they bind a wide spectrum of antigens (reviewed in Lindahl and Rajewsky, 1979). The fine specificity of the T cell receptor - its ability to discriminate between antigens which are closely related structurally - appears to be similar to that of B cells (Lindahl and Rajewsky, 1979). However, the repertoire of T cells, and the types of determinants recognized may be different (Benacerraf and Paul, 1977). Atkins and Ford (1975) found that about 10% of rat T lymphocytes respond *in vivo* to MHC antigens. Using *in vitro* techniques, others have arrived at figures ranging from 0.05-3% (Wilson *et al.*, 1968, Bach *et al.*, 1969). It has recently been demonstrated that about 1/1000 murine lymph node cells are precursors for cytotoxic T lymphocytes (CTL) against a given set of foreign MHC antigens (Teh *et al.*, 1977). The significance of high numbers of MHC - reactive T cells may relate to a requirement to recognize antigens in conjunction with self MHC antigens. This will be discussed in section F.

Attempts have been made to characterize and isolate T cell receptors, and to determine what, if any, similarities they share with B cell Ig receptors. In an elegant series of experiments (reviewed 1977a,b), Binz and Wigzell demonstrated that rat and mouse antibodies to allogeneic MHC antigens, and T cell receptors which bind the alloantigens, share idiotypic determinants. An idiotype is a determinant on an immunoglobulin which is unique to antibodies expressing a particular V gene. An anti-idiotypic antibody, then, detects V region determinants that may be part of an antigen binding site. Binz and Wigzell produced anti-idiotypic antibodies by injecting parental type (A) T lymphocytes into an F1 (AxB) animal. The resultant anti-idiotypic antibodies are almost entirely restricted to those reacting with the combining site of Igs directed against B antigens (or related antigens). The (AxB) F1, due to self-tolerance constraints, does not have such a combining site, but has all other antigens present on A cells. The anti-idiotypic antibodies raised in such a manner reacted with A anti-B antibodies *and* T cells directed against B antigens, using radioimmunoassay and affinity chromatography techniques (Binz and Wigzell 1975b). Thus it would appear that T and B cells directed against the same antigens share a V region determinant. It was found, however, that there were idiotypic determinants on B cells which were not present on T cells. Work by Ramseier and Lindenmann (1972) also indicated idiotypic cross reactions

between antibodies and receptors on T cells mediating cytotoxic responses to alloantigens.

A somewhat different approach to the analysis of T cell receptors was taken by Eichmann and Rajewsky (reviewed in 1977), who took advantage of the restricted numbers of Igs which are produced against some antigens. A single Ig, which is called the A5A idiotype, accounts for about 25% of all antibodies raised in A/J mice against Group A streptococcal carbohydrate (A-CHO). It can be identified by isoelectric focusing. Anti-idiotype antibodies raised against A5A, when injected into mice, stimulated both T and B cells reactive against A-CHO. Furthermore, all of the anti-A-CHO Ig found in the serum of such mice was of the A5A idiotype: other clones of lymphocytes reactive to A-CHO were not stimulated (Eichmann and Rajewsky, 1975). Anti-idiotype antibody of the IgG1 class stimulated helper T cells, while antibody of the IgG2 class stimulated suppressor T cells (Eichmann, 1975). (See Section H for functional T cell subsets).

Another series of experiments employed an idiotype called S117, which is carried by an Ig secreted by a mouse myeloma. This Ig also binds to A-CHO. The ability of inbred mouse strains to produce both the A5A and S117 idiotypes is known to be linked to the Ig-1 complex, which also controls the production of Ig H chains. Using a panel of 13 inbred mouse strains, Hammerling *et al.* (1976) demonstrated that strains which produced the A5A but not the S117 Ig also had helper T cells bearing the A5A idiotype, and not the S117

idiotype. Those strains which produce S117 but not A5A, had S117- but not A5A- bearing T cells. Thus there was a direct correlation between the production of a given idiotype by B cells and by helper T cells. These genetic studies led to the conclusion that helper T cells responsive to stimulation with anti-idiotype must express the gene encoding VH of the Ig to which the anti-idiotype was raised.

In other experiments, anti-idiotypic antibodies were separated according to reactivity with VH and VL. Stimulation of idiotype-bearing T cells *in vivo* could only be achieved with the anti-VH; anti-VL reactive antibody had no effect (Rajewsky and Eichmann, 1977). This suggests that the helper T cell receptor does not have VL determinants, or that they are inaccessible to antibody if present. A recent study in which anti-VH and anti-VL binding capacity of T cell subsets was compared showed that cells of the helper class reacted with anti-VH, a conclusion similar to Rajewsky and Eichmann's. However cells of other subclasses, bearing the Ly 2 cell surface antigen, reacted with anti-VL but not anti-VH anti-idiotypic antibody (Lonai *et al.*, 1978). Thus the V regions expressed by T cells may not be limited to the VH group, but may include VL products.

Attempts have been made to isolate the receptor itself. Binz and Wigzell (1977a,b) have isolated idiotype-bearing, alloantigen-binding molecules from medium in which purified T cells were cultured. The molecular weight was determined to be 150,000 on SDS gels, and the material gave a single

band of 75,000 after reduction. The putative T receptors did not bear detectable markers of any Ig constant region type, when antisera of appropriate specificity were employed. In another system, material removed from T cell surfaces by antigen-coated nylon fibers was found to share a major idiotype with serum Ig against the hapten 3-nitro-4-hydroxyphenylacetate (NP) produced by C57BL/6 mice. This "receptor" material also lacked serologically detectable determinants of Ig C regions (Lindahl and Rajewsky, 1979).

Results from these experiments using isolated receptors must be interpreted with caution, since there is no *direct* evidence that T cells, and not contaminating B cells, were t[redacted] of the material. However, it is considered likely th[redacted]l C region for the T cell receptor exists in the ge[redacted]erhaps in the same cluster as the Ig C region genes (Bi[redacted]d Wigzell, 1977a).

D. Se[redacted]/non-self Discrimination and Implication for Immune Regu[redacted]tion

A diverse and large repertoire of receptor speci[redacted]ici[redacted]ties is clearly of evolutionary advantage in eliminating pathogens from an organism. However, it is almost inconceivable that such diversity could arise without the generation of specificities that react with self antigens. These are potentially very harmful; autoimmunity can be lethal, as evidenced by such diseases as systemic

lupus erythematosis and multiple sclerosis. The mechanism by which tolerance to self antigens is achieved is still a matter of controversy. It is agreed, however, that self-reactive clones of cells arise in an individual, and that their elimination involves a learning process. This idea was put forward by Jerne (1971) who postulated that self-reactive lymphocytes are removed by the thymus. Strong arguments, both theoretical and experimental, that self-non-self discrimination is learned, and not encoded in the germ-line have been discussed by Bretscher (1977) and will not be reproduced here. It will suffice to say that if the genes for self-antigens are not linked to, and are selected for independently from, genes for lymphocyte receptors, it seems logical that the forbidden specificities cannot be "known" *a priori*. Of course any somatically-generated specificities must be dealt with via a learning process.

Seen in this light, it seems likely that evolution of a library of diverse receptors was dependent upon a mechanism for controlling self-reactivity. Consequently there is a need for the control of responsiveness by a means other than the presence or absence of antigen.

It is difficult to design a model in which self-reactive lymphocytes are rendered inactive, not only during ontogeny of the organism, but also as they arise from stem cells in the bone-marrow throughout the lifetime of the individual, while responsiveness to foreign antigens is

maintained. Two major schools of thought exist as to how self-tolerance is effected. One maintains that self-reactive clones are eliminated during an early stage of their differentiation by contact with (self) antigens under certain conditions (Bretscher and Cohn, 1970; Bretscher, 1975; Nossal and Schrader, 1975). The other supports the view that self reactive clones are controlled by active suppressor mechanisms which are antigen-specific. Suppression could take the form of suppressor cells, antigen-antibody complexes, or a network of anti-idiotypic antibodies (reviewed in Howard, 1979).

A comprehensive theory to explain self/non-self discrimination which is consistent with many experimental observations was put forward by Bretscher and Cohn (1970). The theory postulates that all antigen-reactive cells require two signals to be induced: one (Signal 1) provided by the binding of antigen to a cell surface receptor, and another (Signal 2) given by an auxiliary cell which also recognizes the antigen, but not necessarily the same determinant. Even the signal 2 producing cells require signal 2 for induction. However, precursor cells which receive only signal 1, and not signal 2, are irreversibly inactivated. The theory predicts that during ontogeny, the development of signal 2 production lags behind that of receptors on antigen-reactive cells. Thus anti-self lymphocytes are presented with self antigens in the absence of signal 2, and are destroyed. Eventually, enough signal 2

is produced (constitutively) to begin the process of induction to a few foreign antigens. By this time, the signal 2 producing cells specific for self antigens have been destroyed, so that when new self-reactive precursors arise, lacking a source of signal 2, they are also destroyed. The theory, which cannot be considered in any more detail here, also accounts for situations in which self tolerance is broken, and autoimmunity occurs (Bretscher, 1972).

Whether or not this mechanism operates in self tolerance, perhaps with other suppressor mechanisms acting as a backup system, remains to be proven. However, one of the main predictions of the theory - that cells require a second signal in addition to antigen for induction - has been supported by experimental evidence from many sources, some of which is reviewed in section G. The two signal model has been challenged by proponents of a one signal model, who suggest that signal 2 is the only signal experienced by the lymphocyte precursors, antigen binding (Bretscher's Signal 1) acting only to focus the source of signal 2 to the appropriate place (Coutinho, 1975). However there is no evidence that virgin precursor cells can be triggered in the absence of antigen binding to specific receptors (this excludes triggering by mitogens), even when signal 2 is present in saturating amounts in the form of soluble factors.

It has recently been suggested that the antigen binding

signal may induce the appearance of signal 2 receptors on cell surfaces (Smith *et al.*, 1979b). It could be postulated that if the cell does not receive signal 2 within a certain length of time thereafter, it becomes inactivated.

E. The Ontogeny and Physical Characteristics of T Cells, B Cells and Macrophages

Origin of Hematopoietic Stem Cells

All of the differentiated cell types which comprise the adult hematolymphoid system are derived from hematopoietic stem cells which develop in the yolk-sac blood islands of the embryo. This was first suggested by Moore and Metcalf, (1970) and has recently received direct experimental support from Weissman *et al.* (1978). Blood island cells from 8-10 day old mouse embryos were injected into embryos which differed from the donors in either a chromosomal marker or a cellular antigen. The marked donor cells were detected in the recipients as pluripotent stem cells after birth.

A single type of pluripotent stem cell gives rise to the lymphoid cell series (T and B cells), as well as the myeloid series: macrophages, monocytes, granulocytes, erythrocytes and megakaryocytes (Abramson *et al.*, 1977). These pluripotent stem cells are thought to migrate from the yolk sac blood islands to the fetal liver, which is the site of embryonic hematopoiesis, and then to the bone marrow, where they persist in adults (Metcalf and Moore, 1971). A type of stem cell restricted to the myeloid pathway of

differentiation has been found in the bone marrow. Such stem cells, when transferred to recipients in which they can later be detected, repopulate the myeloid, but not the lymphoid, compartment (Abrams *et al.*, 1977, Wu *et al.*, 1967). Some less conclusive evidence points to a restricted stem cell for T lymphocyte differentiation. So far none has been found for B lymphocytes. A stem cell, whether pluripotent or restricted in its potential, is defined by its ability to self-perpetuate, as well as to give rise to progeny which differentiate further. A schematic drawing of hematopoietic differentiative pathways from pluripotent stem cells is shown in Figure 2.

The Macrophage

The macrophage is derived from the myeloid restricted stem cell as shown in Figure 2, which is a simplified scheme omitting the various intermediates between the stem cell and the macrophage. Promonocytes, which are non-adherent and non-phagocytic (Domizig and Lohmann-Matthes, 1979) are produced in the bone marrow, and give rise to blood-borne monocytes, which are adherent, phagocytic, and the immediate precursors of macrophages. Promonocytes are capable of division, but the differentiation step from monocyte to the 3 fold larger macrophage can apparently occur without cell division, after lodging of a monocyte in tissue such as lung or lymphoid organs (Cline, 1975). The mature macrophages are adherent to glass (Cline, 1975) and nylon wool (Schwartz and Paul, 1975), and will phagocytose particulate matter such as

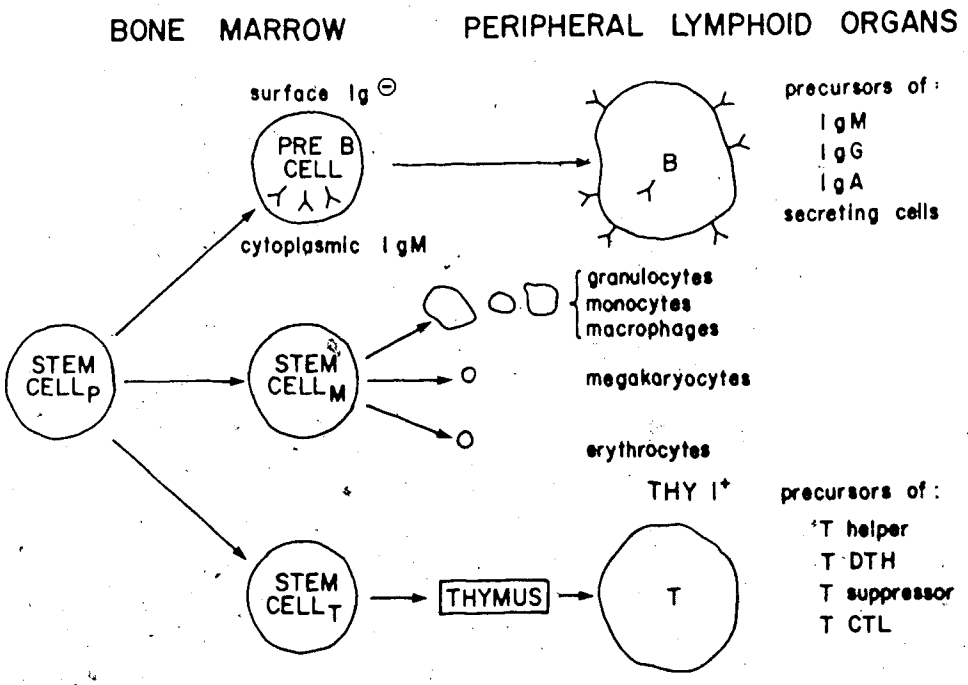


Figure 2. Schematic representation of the differentiation of lymphoid (T and B) and myeloid (granulocytes, monocytes and macrophages) leukocytes from a pluripotent stem cell (stem cell p). Stem cells restricted to the T and myeloid, but not to the B cell, differentiative pathways have been found in mouse bone marrow.

polystyrene beads. They can be physically distinguished from lymphocytes on the basis of lysosomal enzymes such as esterases and beta-glucuronidase (Cline, 1975). Macrophages have surface receptors for IgG (called FC receptors), and receptors for certain components of complement. These surface markers can be induced within 3 days if macrophages are grown *in vitro* under appropriate conditions from bone marrow precursors, presumably promonocytes (Rabellino *et al.*, 1978). Ia antigens (see section F) are present on some macrophages, mainly the smaller, and presumably less mature of the heterogenous group of cells which are collectively referred to as macrophages (reviewed by Lee, 1980).

The differentiation of macrophages and granulocytes from bone marrow precursors is promoted by a soluble factor(s), "colony stimulating factor" (CSF; Stanley *et al.*, 1976; Stanley and Heard, 1977., Burgess *et al.*, 1978). Macrophages can be grown in culture from stem cells if CSF is present (Rabellino *et al*, 1978). Its mode of action is not understood. CSF has been partially purified from several sources including human urine, mouse lung conditioned medium, and mouse L-cell conditioned medium (reviewed in Burgess *et al.*, 1978). CSF from the latter source is 70,000 daltons (consisting of 2 disulfide linked 35,000 dalton polypeptides), and of somewhat heterogeneous pI: 3.7-4.9 (Stanley and Heard, 1977). There may be multiple CSF species which are specific for different progenitor cells, eg. granulocyte and monocyte procursors (Burgess *et al.*, 1978).

Phagocytosis of foreign matter was, until recently, the only function ascribed to macrophages. It is now established that macrophages play a central role in regulation of immune responses. Macrophage functions have recently been reviewed (Lee, 1980; Immunological Rev., Vol. 40, 1978). Macrophages appear to be a requirement for virtually all *in vitro* immune responses: antibody responses to T cell dependent and so called T - independent antigens (Lee *et al.*, 1976); generation of CTL responses (Wagner *et al.*, 1972); proliferative secondary responses of T cells to soluble antigens (Lee, 1980), and proliferation of lymphocytes in response to Con A (Mills *et al.*, 1976). Macrophages also secrete factors which influence immune responses (see Section J). Macrophages can "present" antigens to T and B cells (Treves, 1978; Lee 1980). The antigen may be bound to the macrophage surface without alteration, however, it is more likely that the macrophage internalizes the antigen, processes it (perhaps by proteolytic cleavage into fragments) and then re-expresses it on its cell surface, possibly in conjunction with H-2 I region antigens (reviewed in Waldmann and Howard, 1979).

Macrophages taken from murine lymphoid organs are a heterogeneous group of cells in terms of both size and function. The heterogeneity may represent various stages along a single differentiative pathway, or subpopulations of cells of different lineages. The lack of known cell surface antigens unique to macrophages has hampered the study of

macrophage subsets, however a monoclonal antibody specific for macrophages has recently been produced, and will no doubt be of value in macrophage analysis (Springer *et al.*, 1979).

T Cell Ontogeny

Stem cells from the yolk sac and fetal liver in the embryo, and the bone marrow in the adult migrate to the thymus, the site of T cell maturation. The stem cells, prothymocytes, are presumably committed to become T cells before they leave the bone marrow. Thymic epithelium cells are thought to control the further differentiation of prothymocytes into "immature T cells", at least partly through the influence of factors (reviewed by Owen, 1979). Although several thymic factors have been described (reviewed by Goldstein, 1978), the only one which has been shown to be specific for prothymocytes (as opposed to B cells) is thymopoietin, which is secreted by thymic epithelial cells. Thymopoietin acts on prothymocytes *in vitro* to induce the appearance of cell surface markers characteristic of immature T cells: the TL (thymus leukemia) antigen, the Thy 1 antigen, and the lymphocyte differentiation antigens Ly 1,2,3, and 5 (Storrie *et al.*, 1976). These antigens are discussed below. Their expression occurs within 2 hours of exposure to thymopoietin, and requires both RNA and protein synthesis, but not DNA replication.

Thymopoietin from bovine thymus, which consists of 49

amino acids (MW 5560) has been purified and sequenced (reviewed in Goldstein, 1978). A segment of 13 amino acids (29-41), as well as the entire 49 amino acids have been synthesized, and found to have the biological activity of native thymopoietin.

The agents which induce further differentiation of immature thymocytes into immunocompetant T cells are unknown. At some point during T cell differentiation, expression of the TL antigen ceases, the Thy-1 antigen density decreases, and the density of some MHC antigens increases (Cantor and Boyse, 1977). Contact with the thymic epithelial cells may play a role in the maturation of antigen-sensitive T cells from prothymocytes. Thymic extracts (cell-free) have never been as effective as thymus grafts in reconstituting the immune responsiveness of athymic (nu/nu) mice (Kindred, 1979). Recent evidence suggests that contact with thymic epithelial cells may be crucial in "selecting" the receptor specificities of the maturing thymocytes that will be clonally amplified and released into the peripheral T cell pool (Zinkernagel *et al.*, 1978). The H-2 alleles (see section F) expressed by the thymic epithelial cells appear to determine, to some extent, the repertoire of antigens against which a mouse can develop immunity. This aspect of T cell recognition is more fully discussed in section F.

T Cell Surface Antigens and Subsets

The antigens discussed in this section, and the

production and characterization of antibody against them, have recently been reviewed by McKenzie and Potter (1979).

The Thy 1 antigen (formerly called theta; Rief and Allen, 1964) is expressed on all T cells, and is the marker most commonly used to experimentally distinguish T cells from other types of leukocytes. T cells can be specifically deleted from a population of cells by incubating them with an antiserum directed against Thy 1 followed by complement, which lyses the cells to which the antibody has bound. Immature thymocytes have a higher density of Thy 1 on their surfaces than peripheral T cells. The existence of two alleles of the antigen in mice has allowed the production of anti-Thy 1 alloantisera, and more recently a hybridoma antibody (Marshak-Rothstein *et al.*, 1979).

The TL (thymus leukemia antigen) is carried by some strains of mice, and is found only on the 90% of thymic lymphocytes which are immature. Peripheral T cells lack the antigen, which may be re-expressed in leukemic cells.

Alloantisera directed against alleles of antigens present on subpopulations of murine thymocytes have been important tools in the analysis of post-thymic T cell differentiation. The lymphocyte differentiation antigens (Ly antigens), first detected by Boyse *et al.* (1968) have been used to dissect the T cell pool into functional subsets. Eight Ly antigens (Ly 1-8) have been described so far (McKenzie and Potter, 1979); others may exist. Some appear to be glycoproteins, but none are well characterized. The

production of antisera against these membrane antigens involves multiple immunization of mice with lymphoid cells from donor mice which differ at the Ly locus in question. The donor and recipient mice can be congenic (differing mainly at the specific Ly locus), or they may differ at additional loci. The congenic combination produces the most specific antibody. For some antigens, it is necessary to use mouse strains differing at more loci to induce antisera of reasonable titre, which are then absorbed with the appropriate mouse tissues to render them specific. Two alleles for each of the Ly loci have been detected. Cantor and Boyse (1977) have proposed that the TL^+, Ly $1^+,2^+,3^+$ immature thymocytes give rise to T cells which are TL^-, Ly $1^+,2^+,3^+$, which in turn differentiate into Ly $1^+,2^-,3^-$ or Ly $1^-,2^+,3^+$ T cells, perhaps after antigenic stimulation. The peripheral T cell pool (non-thymic T cells found in spleen and lymph nodes) consist of: 50-55% Ly $1^+,2^+,3^+$ cells, 30-35% Ly $1^+,2^-,3^-$ cells, and 5-10% Ly $1^-2^+3^+$ cells.

Functional studies using antisera directed against products of the Ly 1.2 and 2.2 alleles indicated that CTL effector cells are Ly $1^-,2^+$, that CTL- and AFC- helper T cells are Ly $1^+,2^-$, and that suppressor cells are generally Ly $1^-,2^+$ (Cantor and Boyse, 1975a, b; 1976a). Some Ly $1^+,2^+$ suppressor cells have also been detected (Cantor and Boyse 1976b; Al-Adra *et al.*, 1980). Precursors of CTL effectors (before antigenic stimulation) are Ly $1^+,2^+$ for modified-self antigens, and Ly $1^+,2^-$ for alloantigens

(Cantor and Boyse, 1976b). Additional Ly antigens have subsequently been found on some of these T cell subsets. The Ly 5 antigen appears to be present on all T cells, and recently has been detected on some B cells (McKenzie and Potter). The Ly 6 antigen is associated with CTL effector cells, but not their precursors (Woody *et al.*, 1977), and with suppressor cells for CTL responses (L. Pilarski, personal communication). The Ly 7 antigen is present on some B cells (McKenzie *et al.*, 1977), but only recently has been detected on helper T cells for CTL responses against alloantigens (L. Pilarski, personal communication). The Ly 4 antigen is probably an antigen unique to B cells (McKenzie *et al.*, 1977). A close association between the Ly 2 and 3 antigens exists - they are always found together, but appear to be on separate molecules.

The use of antisera against some of these specificities to define the lymphocyte subpopulation responsible for costimulator production will be described in Chapter 4.

It should be pointed out that antisera against the Ly 1.1 antigen (in the appropriate mouse strains carrying the Ly 1.1, rather than the Ly 1.2 allele) removes the CTL effector, as well as most precursors. Thus the CTL effector is Ly 1.1^+, 2.1^+. In this respect, the distribution of the Ly 1.2 antigen appears to differ from the Ly 1.1 antigen (McKenzie and Potter, 1979). However, recent studies using a fluorescence activated cell sorter to detect the presence of the Ly 1.2 and 2.2 antigens on T cells, showed that there

was no Ly[redacted] population (reviewed in McKenzie and Potter, 1979). T[redacted] antigen may be present in lesser amounts on CTL ce[redacted] Ly 1.1, or the antisera used to detect the lat[redacted] ant[redacted] may be more potent. Weak antisera against Ly 1.2 [redacted]ve c[redacted]sistently been a problem.

B Lym[redacted]hocy[redacted]e Origin and Properties

[redacted]actors which influence the generation of immuno[redacted]petant (antigen-sensitive) B cells from pre-B cell precurs[redacted]s in the bone marrow are poorly understood. In birds, [redacted]cell differentiation probably takes place in a specializ[redacted]d organ, the Bursa of Fabricius. However, in mammals, no discrete organ has been located. Maturation of B cells is thought to occur in the secondary lymphoid organs: spleen, ly[redacted] nodes and Peyer's patches (small lymphoid modules o[redacted]uter walls of intestines). Except for a "bursa-poietin" in chickens, which is a low molecular weight, but otherwise uncharacterized factor, (Brand *et al.*, 1976), no B cell differentiating hormones have been described.

It is fairly well established that B cells first express IgM intracellularly, and then as a surface receptor (reviewed in Owen, 1979). It has been postulated that cells bearing *only* IgM as a surface receptor cannot be induced to secrete antibody, and that contact with antigen at that stage of development results in irreversible inactivation (Uhr *et al.*, 1978). The next step in differentiation is thought to be the appearance of IgD (in addition to IgM) as

a surface receptor, which allows the cell to secrete IgM if induced by antigen. Cells which have further differentiated to secrete IgG have predominantly IgD, or IgD and IgG on their surfaces. Memory cells which secrete IgG, IgA or IgE upon appropriate stimulation, have surface receptors of the same Ig class. All are thought to arise from the IgM-bearing early B cell, since specific removal of IgM^+ cells from neonatal mice by injection of anti-IgM antiserum suppresses the subsequent secretion of IgM, IgG and IgA (Lawton *et al.*, 1972).

F. The H-2 Complex and Immune Responsiveness

H-2 Gene Products

The major histocompatibility complex (MHC), referred to as H-2 in mice and HLA in humans, is as important in immunology as the Ig gene clusters. It appears to control the ability of individuals to respond to antigens, and is also involved in lymphoid cell interactions. The MHC complex codes for at least 2 groups of polymorphic cell membrane glycoproteins. It was first detected genetically as a region which caused rapid graft rejection between non-identical individuals. Other histocompatibility loci (called minor H loci) cause eventual, but less rapid, graft rejection. A schematic diagram of the H-2 complex is shown in Figure 3. The complex comprises about 1/1500 of the mouse genone (Barnstable, 1979).

The K, D and I regions code for murine cell surface

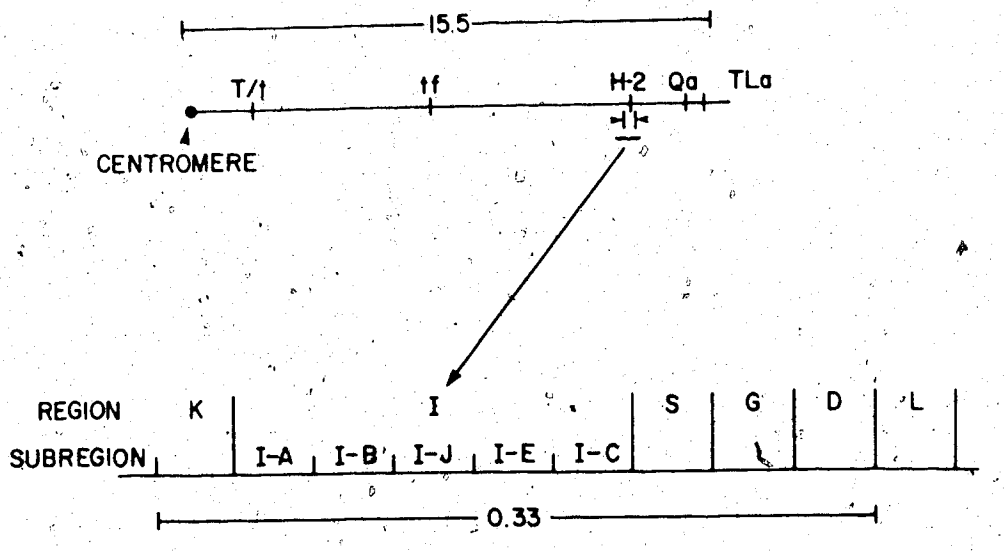


Figure 3. Genetic map of the murine major histocompatibility complex (H-2) on chromosome 17. Regions and subregions are defined by recombination. Approximate map distances are given in centimorgans. See text for explanation.

glycoproteins which have recently been under intensive investigation. Considerable structural and protein sequence information is available for K, D and I region products (Cunningham *et al.*, 1976; Uhr *et al.*, 1976; Silver *et al.*, 1976; Uhr *et al.*, 1979). The analagous human proteins, called HLA - A, B and D, respectively, are even more well characterized (Strominger *et al.*, 1976; Orr *et al.*, 1979; Snary and Crumpton, 1979). The sequence of the 271 N-terminal amino acids of an allelic product of HLA-B, called B7, has been reported (Orr *et al.*, 1979) as have partial sequences for HLA D and H-2 K, D and I proteins.

The murine K and D proteins, detectable on the membranes of all somatic cells, are the only known products of the K and D regions, which of course are large enough to code for many more proteins. A heterozygous individual expresses two K proteins and two D proteins. Two I region products have been isolated, which map to the I-A and the I-E/C subregions. Assuming that only the two I proteins exist, a heterozygous individual would carry 4 different I region proteins. Polymorphism at HLA-A and B is high: 19 and 26 serologically defined alleles, respectively, have been detected. Estimates based on specificities found in wild mice put the level of polymorphism for K and D proteins much higher - probably over 100. An allele of an H-2K, D or I protein is written as H-2K(k), D(k) or I(k), if the specificities designated "k" are carried by the mouse strain.

The K and D glycoproteins (reviewed in Crumpton and Snary, 1979) are 46,000 MW, and span the lipid bilayer. The serologically detectable antigenic portion of the molecules are thought to reside in the protein, and not the carbohydrate moieties. K and D (as well as the TL and Qa proteins, mentioned below and in Figure 3) exist in noncovalent association with beta-2-microglobulin, a 12,000 MW polypeptide, which is non-polymorphic and not a product of the H-2 complex. It has regions of homology with domain 3 of the Ig H chain C region. The beta-2-microglobulin does not penetrate the membrane and is not glycosylated.

Sequence studies have shown a great deal of homology between the K and D glycoproteins, which are as similar as the K or D allelic products are to each other. There also seems to be a high degree of homology between HLA and H-2 molecules. Orr *et al.*, (1979) have recently estimated that there is 72 and 74% homology between the murine H-2K protein and the HLA-A2 and the HLA-B7 proteins respectively. The differences between these molecules appear to be concentrated in 2 clusters.

The interesting observation that an 88 amino acid fragment of the HLA-B7 protein contains several regions of homology with Ig constant domains was recently made (Orr *et al*, 1979b). The existence of an Ig-like region in an MHC product suggests that the humoral and cellular aspects of the immune response may be evolutionarily related.

Glycoproteins coded for by the I region, unlike the K

and D proteins, are mainly expressed on leukocytes. The Ia (I region-associated) cell surface antigens (reviewed by Uhr *et al.*, 1979) were first recognized as products of the I region (Figure 3), which partially controls the capacity of an individual to mount an immune response against a particular antigen. Five I subregions have been distinguished through functional, serological and biochemical analyses. The Ia proteins were isolated using antisera raised in congenic mice which differed at the whole I region or only a subregion (Figure 3). The murine Ia proteins are composed of 2 polypeptides, alpha and beta, of approximately 34,000 and 26,000 daltons, respectively, which span the lipid bilayer. The alpha subunit of both the known Ia antigens is coded for by the I-A subregion, as is the beta subunit of the "A" antigen. The beta subunit of the "E/C" antigen is coded for in the I-E or I-C subregion. The murine Ia antigens are homologous to the human HLA-D antigens. Both are less polymorphic than the H-2K and D and HLA-A and B antigens. The "A" and "EC" antigens do not appear to have extensive regions of homology.

The H-2 complex also contains the S region, which has a locus coding for the 4th component of mouse complement, and the G region, which is associated with with a red blood cell antigen (Barnstable *et al.*, 1979). Some other antigens of interest are linked to H-2. One is the TLA region (McKenzie and Potter, 1979), which controls the expression of at least 2 TL antigens found on immature thymocytes and some leukemia

cells. Expression of TL seems to be inversely related to expression of H-2: as T cells begin to mature, they gain H-2 antigens, and lose TL antigens. The Qa region has 3 known loci, coding for antigens Qa-1, Qa-2 and Qa-3. These are expressed on thymocytes and some lymph node T cells. The Qa-1 antigen has been associated with suppressor cells, but little is known about Qa function. Both the Qa and TL antigens are associated with beta-2-microglobulin. Structural studies have shown that they have a size and subunit structure similar to the H-2 K and D glycoproteins (reviewed in McKenzie and Potter, 1979). It is considered likely that the H-2 K, D and the Qa and TLA genes arose from a single ancestral gene.

Involvement of K, D Proteins in Cytotoxic Reactions

The biological significance of the H-2 K and D proteins - their strong antigenicity, extreme polymorphism and widespread tissue distribution - have long remained a mystery. It now appears that the D and K proteins are involved in a recognition system designed to protect organisms against intracellular pathogens. Langman (1978) and Lafferty and Woolnough (1977) have proposed that the K and D proteins arose evolutionarily from a self-recognition system in primitive multicellular organisms, in which recognition of a self marker *prevented* a rudimentary immune mechanism from carrying out its destructive role.

Work by Doherty, Blanden and Zinkernagel (1976), and Shearer *et al.* (1976) (and others mentioned in these

references) provided a framework for understanding the biological significance of K and D antigens as being involved in the recognition of modified (abnormal) somatic cells by T lymphocytes. They found that CTL which recognize and kill virally-(Doherty *et al.*) or chemically-(Shearer *et al.*) modified cells do not do so unless the target cells share at least a K or a D allele with the modified cells which were used to induce the immunity. In other words, CTL generated *in vivo* when an H-2(n) mouse is infected with virus will not kill any virally infected target cell *in vitro* unless it expresses an H-2K(n) or an H-2D(n) allele, and of course, the correct viral antigens. This phenomenon is known as H-2 restriction of cytotoxic effector cells.

In further experiments, which focused on the induction phase of anti-viral immunity, it was demonstrated that the H-2 type of the killer cell *per se* was not the factor determining which modified H-2 specificities could stimulate anti-viral CTL (Zinkernagel *et al.*, 1978). Rather the H-2 type of the thymus reticulum in which the T cell differentiated determined the H-2 type of virally infected target against which the T cell could respond. A mouse of H-2 type A can be made chimeric by the injection of (AxB) bone marrow cells after lethal irradiation. The differentiated (AxB) T cells generated CTL against A + virus but not B + virus, even if transferred to an (AxB) F1 host where B + virus antigens were also available to the precursors. However, if the (A+B) bone marrow cells

differentiated in a type B mouse, they would generate CTL able to lyse only B + virus. (AxB) bone marrow cells which differentiated in an (AxB) host could lyse both A + virus and B + virus targets. Thymus grafting experiments demonstrated that it is the H-2 type of the thymus reticulum cells in which the thymocytes differentiate that dictates their preference for A + virus or B + virus. In another system, it was shown that such H-2 restriction is not absolute, and can be overcome to some extent by priming mice with the "non-preferred" H-2 type + antigen. (Matzinger and Mirkwood, 1978). To summarize, the precursor T cells learn "self", probably in the thymus, and preferentially recognize altered-self.

CTL recognize the H-2 proteins and viral antigens on virally infected cells, during both the induction and effector phases of the immune response. Two models could explain such restriction (reviewed by Langman, 1978). One proposes that viral products associate with H-2 K and D on cell membranes, creating new antigenic units. These antigens are the determinants recognized by the T cell receptor, and would not be present if *different* alleles of K and D associated with the same virus. Alternatively, the involvement of K and D proteins could be part of a self recognition system which is necessary to activate a "destructive signal". In this model, the viral antigens could be recognized independently from the K and D by T cell receptors. There is no definitive experimental evidence to

distinguish between these models. Regardless of which model is correct, the central involvement of K and D proteins in an immune mechanism for the destruction of virally infected cells suggests a reason for their existence on all somatic cells.

Involvement of the I Region in Immune Response

One of the most fruitful observations in immunogenetics was that within a species, inbred individuals with some genotypes respond to a particular antigen by synthesizing antibody, while others do not. This phenomenon of genetic restriction of immune responsiveness was discovered by Gorer and Schutze (1938), who found that resistance of inbred mice to *Salmonella* infection correlated with their ability to produce antibody against the bacteria. More recently, synthetic polypeptide antigens of defined structure and relatively few determinants were used to investigate the genetics of immune responses. Benacerraf *et al.*, (reviewed 1967) were the first to show that responsiveness to the synthetic antigens poly-L-lysine or polyglutamyl-lysine in guinea pigs was inherited as a single autosomal dominant gene, which they called the PLL gene. The ability to respond to another antigen, GA (a copolymer of L-glutamic acid and L-alanine) was linked to the PLL gene, but shown to be separated from it by recombination (reviewed in Barnstable *et al.*, 1979).

Polypeptide antigens of simple structure were subsequently used in mice to define immune response (Ir)

genes. One of the earliest examples of such experiments was carried out using the antigens (T, G)-A--L, (H, G)-A--L and (Phe, G)-A--L, which are copolymers of tyrosine (T), histidine (H), or phenylalanine (Phe) and glutamic acid (G), attached to a lysine backbone by alanine side chains. In a series of papers (reviewed in Katz, 1977, Chapter 12), McDevitt and co-workers demonstrated that responsiveness to these antigens in inbred mice was determined by their H-2 haplotype. Using recombinant mice, they mapped the gene(s) to a region distinct from K and D, but between them, which was called the I region. Responsiveness to all of the above antigens were not controlled by a single gene. High responders to (T, G)-A--L, such as C57BL/10 (H-2b) were low responders to (H, G)-A--L, and high responders to (Phe, G)-A--L. In contrast, mice which carried the H-2(k) haplotype were high responders to (H, G)-A--L and (Phe, G)-A--L, but responded poorly to (T, G)-A--L.

This aspect of immune responsiveness is independent of the Ig genes, and is manifest at the helper T cell level (Katz, 1977). Thus if a non-responder to an antigen such as DNP-PLL is given that antigen coupled to an immunogenic carrier such as ovalbumin, the animal becomes a responder and produces antibody against DNP-PLL.

Subsequently, responsiveness to many antigens has been mapped to the I-A, B and C regions (Figure 3). For some antigens, however, 2 complementing genes are required in a responder mouse. In at least one case, they have been mapped

to the I-A and I-C regions (reviewed in Katz, 1977, chapter 12). (The complementing genes required may be associated with the Ia glycoprotein, whose 2 subunits map in the I-A and I-E/C subregions).

The responder/non responder field was made even more complex by the discovery that non-responsiveness can map to the I-J region, and be associated with specific suppressor cells for an antigen. This has been particularly well documented for the synthetic antigen GAT by Kapp and coworkers (reviewed by Katz, 1977, chapter 12; Kapp *et al.*, 1974).

Clearly, the control of immune responsiveness by genes in the I region is far from understood. However, some interesting possibilities exist as to why the presence of a particular I region allele might be important for stimulating an immune response. It will become evident that the models to explain the role of I region gene products follow some of the same lines of reasoning as those put forward to explain the role of K and D proteins in cytotoxic responses. One possibility is that Ia proteins *themselves* associate with soluble antigens (or perhaps processed fragments of antigen), probably on a macrophage cell membrane. The Ia glycoprotein-antigen complex would display the antigen in a form which is immunogenic to T or B cells. (This would be analogous to the postulated association of viral products from within a cell with K or D proteins on a macrophage membrane.) If an individual's set of Ia antigens

failed to include an Ia protein specificity that would bind to a particular antigen, it would be a non-responder to that antigen (suggested by Miller and Vadas, 1976; Paul *et al.*, 1976 and others). Although there has never been any direct proof that an Ia antigen (coded for by I-A or I-E/C subregions) is the product of an immune response (Ir) gene, this theory makes such an association.

Another theory to explain immune response genes arises from the idea that germ line gene-encoded T cell receptors for antigen are actually receptors for the H-2 gene products of the species (Jerne, 1971). Von Boehmer *et al.* (1978) have modified this idea. In their hypothesis, the T cell receptors recognize not all the H-2 products of the species, but only those of self. According to the von Boehmer model, all T cell receptors would arise from anti-self I, K or D receptors by somatic mutation and selection in the thymus. Therefore the ability to respond to an antigen would depend on the specificity of the anti-self receptor before it mutated away from being anti-self. Non responders would have an anti-self Ia receptor that could not easily mutate to be anti-(T, G)-A--L, for example.

Genes in the I region also determine the ability of cells to interact with each other during an immune response. In general, I region identity is required for helper cells and suppressor cells to communicate with their target cells (reviewed in Katz, 1977 chapter 12). This I region restriction can be viewed as analogous to the K/D

restriction found for cytotoxic cells and virally infected target cells. The requirement for I region identity is not difficult to reconcile if the antigenic determinant recognized by a T or B cell on a macrophage cell surface is a new determinant created by the association of antigen with a particular Ia protein. Association of the same antigen with a different Ia protein may not create the same determinant.

It should be pointed out that I region restriction is not absolute, but appears to reflect antigens seen during priming. Rosenthal, Shevach and co-workers were the first to discover the effect of priming on I restriction in T cell - macrophage interactions (Paul *et al.*, 1976). If (A x B) guinea pigs were first exposed to antigen on A macrophages, they would develop a secondary response to the antigen presented on A but not B macrophages. However, if their first exposure to the antigen was in association with B macrophages, then a secondary response could be induced with antigen-pulsed B, but not A, macrophages.

G. Cellular Interactions in AFC Responses

T Cell-Macrophage Interactions

It has already been stated that macrophages are required for most immune responses (section E) *in vitro*. The experimental evidence for this has largely been obtained by depleting macrophages from lymphocyte populations so that an *in vitro* immune response is impaired, and then adding back

macrophages of various types (Lee, 1980). In general, the small Ia$^+$ macrophages are most efficient in presenting antigen to T cells (Lee, 1980). This would be expected if antigen must be associated with I region products to be effectively immunogenic. This idea is supported by experiments in which the stimulation of T cells by antigen - pulsed macrophages was specifically blocked by antibody against Ia antigens on the macrophages (Lyons *et al.*, 1979). Macrophages are also known to secrete soluble factors which have an immunostimulatory effect *in vitro* on T, and perhaps B lymphocytes (section I).

T-B cell Interactions

The first indication that interaction between two types of antigen-specific cells is required during the induction of an immune response came from *in vivo* experiments using lethally irradiated mice (whose lymphocytes could not divide) as recipients for various populations of syngeneic leukocytes (Claman *et al.*, 1966). The irradiated mice served essentially as "living test tubes," into which bone marrow cells or thymus cells, and SRBC, the antigen, were injected. Bone marrow or thymus cells injected alone did not enable the hosts to mount an AFC response against SRBC, but when injected together, they synergized to give a good response. It was subsequently established by other investigators that the antibody producing cells were contained in the bone [redacted]tment, and that the thymus cells served an [redacted] (reviewed in Katz, 1977, chapter 10).

Mitchell and Miller (1968) repopulated neonatally thymectomized mice with allogeneic T cells, and demonstrated, using antisera directed against the donor or host H-2 antigens, that the AFC were of host (B cell) origin.

The inability of nu/nu (athymic) mice to produce antibody against SRBC and virtually all other antigens (reviewed in Kindred, 1979) further substantiated the role of T lymphocytes in AFC generation. Thymus grafted nude mice are capable of AFC responses.

The development of culture techniques for AFC generation *in vitro* allowed the direct demonstration that cells bearing the Thy 1 marker were required for AFC responses against SRBC. A glass- or plastic-adherent cell, called an A cell (probably a macrophage) was also a requirement (Mosier and Coppleson, 1968).

The classical hapten-carrier experiments further clarified the role of T cells in AFC responses as being antigen specific. Haptens are small antigenic moieties such as DNP (dinitro-phenol) or NIP (4-hydroxy-5-iodo-3-nitrophenacetyl) which cannot elicit an AFC response *in vivo* if administered in free form. However, antibody against them is readily obtained if they are coupled to an immunogenic "carrier", a larger molecule such as BSA or ovalbumin (Ova). Mitchison (1971) and co-workers determined that the "carrier effect" was due to helper T cells which recognized the carrier portion of the complex.

Spleen cells from mice which had been immunized to NIP-Ova were injected into irradiated syngeneic recipients, which were then challenged with NIP-Ova or NIP-BSA. The NIP-Ova, but not the NIP-BSA challenged mice gave a secondary antibody response to the hapten, NIP. However, if spleen cells from BSA - immunized donors were mixed with the NIP-Ova primed cells, then challenge with NIP-BSA gave a positive response. This was interpreted to mean that specific recognition of the carrier (in this case BSA) was required.

In a similar system Raff (1970) demonstrated that the active carrier-primed cell was Thy 1-bearing. It was generally found that the hapten and the carrier had to be physically linked on the challenge antigen. Presentation of NIP-BSA, for example, together with free Ova in NIP-Ova primed mice would not be as effective as challenge with NIP-Ova. The requirement for linked determinants was thought to reflect a necessity for B cells and primed T cells to come into close contact for the T cells to deliver an inductive signal to the B cell (Mitchison, 1971).

It has already been pointed out that helper cells for antibody responses are of the Ly 1^+2^- type (section E). This has been shown for several antigens (reviewed in Cantor and Boyse, 1976b): SRBC, KLH, BSA and HGG (human gamma globulin) to name a few. The Ly 1^+2^- identity of the helper cells was established by treating a population of lymphocytes with anti-Ly 1 or anti-Ly 2 antiserum and complement. The

resultant decline in AFC response by anti-Ly 1 treated cells could be reversed if anti-Ly 2 treated cells were added to cultures.

Congenic mice were employed in cell transfer experiments which demonstrated that helper T cells and B cells cooperating in an AFC response require common H-2 (possibly I region) determinants (Sprent, 1978a; 1978b). It is uncertain how much of this requirement is expressed at the level of the macrophage (Shiozawa *et al.*, 1979), which has usually been of the same H-2 type as the B cell in experimental systems, and whether the H-2 restriction reflects clonal expansion of helper T cells which "see" antigen associated with a particular I region product. Again, as was the case with H-2K and D restriction, the possibility exists that I - encoded proteins function in a purely physiological role involving cell triggering, and do not bind to antigens.

The T cell requirement for antibody production is not absolute. A small amount of IgM is produced, even by nu/nu mice in the absence of T cells. The so-called "T-independent" antigens, which are usually polymers or have repeating determinants (such as polymerized flagellar protein of Salmonella, levan, dextran, pneumococcal polysaccharide type III; Coutinho, 1975), elicit an IgM response in the relative absence of T cells. However, the switch to IgG secretion has always been found to be T-dependent. It was recently demonstrated that several B

cell-derived human tumor lines which normally secrete IgM, but not IgG *in vitro*, could be induced to synthesize IgG by the addition to cultures of normal human T cells (Kishimoto *et al.*, 1978). Macrophage factors enhanced the effect of the T cells.

Antibody responses are also under negative regulation. T cells which supress AFC responses to many antigens are Ly 1^-2^+, although their precursors are probably Ly 1^+2^+ before antigenic stimulation (Cantor and Boyse, 1976a). Gershon and Kondo (1971) using an *in vivo* adoptive transfer system, were the first to obtain evidence for suppressor T cells. Spleen cells from mice deliberately made unresponsive to SRBC, when mixed with normal thymocytes and bone marrow cells, suppressed an anti-SRBC response in an adoptive host. *In vivo* generation of suppressor cells was also noted by Tada and Takemori (1974) who adoptively transferred antigen-specific suppressor cells from DNP-KLH primed donors. The suppressor cells were shown to be T cells by their sensitivity to treatment with anti-Thy 1 serum and complement before transfer.

The AFC response to KLH is controlled by suppressor T cells which appear to act on helper T cells rather than directly on B cells (Tada and Taniguchi, 1976; Murphy *et al.*, 1976). Suppressor cells have also been found for GAT (Kapp *et al.*, 1978), SRBC, and TNP coupled to SRBC (Cantor and Boyse, 1976a). The GAT and KLH suppressor T cells carry H-2 I-J subregion determinants (Murphy *et al.*, 1976), which

is also true of several antigen-specific suppressor factors to be discussed in section J. The fractionation of regulatory T cells into helper and suppressor populations using anti-Ly 1 and anti-Ly 2 antisera favors the conclusion that two separate cell populations exist, and that suppression is not merely a result of too many helper cells (Jandinski *et al.*, 1976).

H. Cytotoxic T Cell Responses

Generation and Assay of CTL

Cytotoxic T lymphocytes (CTL) were first observed in the allograft situation. Cerottini and Brunner (1970) were the first to demonstrate that donor T cells found in the spleens of lethally irradiated allogeneic recipients were cytotoxic *in vitro* for target cells of the same H-2 type as the host mice. CTL could also be found in the spleens of normal mice which had been immunized with allogeneic tissue, for example organ, skin or lymphoid tissue grafts.

Cytotoxic activity of CTL is measured *in vitro* by incubating them with an appropriate target cell which has been internally labelled with the isotope ^{51}Cr. The mechanism of CTL inflicted Killing of target cells is not understood, but involves alterations in membrane permeability in a complement independent, divalent cation dependent, cell to cell interaction (Cerottini and Brunner, 1977). The net effect measured in the assay is ^{51}Cr-leakage out of damaged target cells during a 4-6 hr period.

The biological significance of the CTL effector mechanism remained a mystery until fairly recently, because its only manifestation seemed to be against allogeneic cells, which cannot be considered a selective evolutionary force. Studies by Doherty (reviewed in Doherty *et al.*, 1976) on the pathogenesis of LCMV (lymphocytic choriomeningitis virus) infection led to the discovery that lymphocytes from LCMV infected mice were able to kill LCMV infected syngeneic target cells *in vitro*, in a manner analogous to the allogeneic CTL system. The anti-viral CTL, which were found not only in the spleens, but in the cerebro-spinal fluid of LCMV infected mice, were indistinguishable from anti-allogeneic CTL in terms of physical properties.

Doherty's results led to other experiments in which CTL were identified in mice infected with various other viruses, such as ectromelia, Sendai, and vaccinia (reviewed in Doherty *et al.*, 1976), and an intracellular bacterial pathogen, Listeria monocytogenes (Blanden, 1974). Furthermore, Shearer *et al.* (1976) found that CTL could be generated against syngeneic lymphocytes which had been haptenated with TNP to produce "altered-self" cells. CTL responses were also detected in female mice immunized with the male H-Y antigen (Simpson and Gordon, 1977), and other minor H antigens (Bevan, 1976). The CTL to all of these antigens were H-2 K and D restricted as discussed in Section F. These findings led to the idea that the CTL mechanism had evolved to deal with intracellular viruses and bacteria,

whose products become associated with cell membrane proteins. CTL might also be effective in removing cells which display abnormal cell membrane antigens, such as tumor cells.

Despite its biological role, the phenomenon of CTL generation and its control has been most extensively studied in the allograft situation, both *in vivo* and *in vitro*. A great deal of the motivation for these studies came from the obvious clinical benefits of being able to prevent graft rejections.

In practical terms, CTL are generated *in vitro* by incubating "responder" cells of one genotype with "stimulator" cells of another genotype (or chemically or virally modified syngeneic cells). The stimulator cell population is usually irradiated or treated with mitomycin C to prevent cell division, so that a one-way reaction is observed. Two types of responses can be measured in such cultures. 1) a strong proliferative response (MLR response) occurs about 3 days after initiation of culture. 2) at about 5 days, cytotoxic T cells detectable in an *in vitro* assay are at their peak.

It is now well established that the bulk of the cells proliferating in the MLR are Ly 1^+2^- (Cantor and Boyse, 1975b), and that they are distinct from, and do not give rise to, the Ly 1^-2^+ CTL effector cell. The MLR proliferation is largely directed against I region differences (reviewed in Katz, 1977, Bach *et al.*, 1972). Use

of target cells from congenic mice has allowed the observation that most of the CTL generated against histoincompatible cells are directed against K and D region products. That CTL can, in lesser numbers, be generated specifically against I region products was demonstrated using strain combinations that differ only in the I region (Teh *et al.*, 1978). The most recent evidence suggests that CTL precursors (H.-S. Teh, personal communication) and anti-viral CTL effectors (McKenzie *et al.*, 1977) do not bear Ia antigens.

Properties of CTL Stimulator Cells

Although it was initially proposed that stimulator cells for CTL must be Ia^+ (reviewed in Katz), this does not now appear to be an absolute requirement (Talmage *et al.*, 1977). However, at least two properties are essential for a CTL stimulator cell: it must be of leukocyte origin, and it must be viable and metabolically active. Glutaraldehyde fixation, ultraviolet (UV) irradiation and actinomycin D treatment (which prevents RNA synthesis) all destroy the immunogenicity of a stimulator cell (Lafferty and Woolnough, 1977). Fibroblasts, and non-leukocyte derived tumor cells, likewise cannot promote a CTL response. Lafferty interpreted these observations as evidence that two signals are required to generate an anti-allogeneic CTL response: the first signal is provided by alloantigens, and the second is an inductive signal of another type provided by the stimulator cell. The ability to transmit the second signal is limited

to lymphocytes or monocyte/macrophages.

Other experiments have shown actinomycin D treated stimulator cells to be capable of promoting a response if they are present in much higher numbers (Wagner, 1973). An alternative explanation for their relative lack of immunogenicity, then, might be that actinomycin D treatment caused a diminution of the density of alloantigens on the cell membrane, or a shortage of alloantigen for macrophage processing.

The ability of lymphocytes, but not non-lymphoid tissue, to mediate graft rejection in a transplantation situation was demonstrated by Lafferty and Woolnough (1977). They cultured guinea pig thyroid tissue *in vitro* for sufficient time to allow the lymphocytes within it to die. In some cases, thyroid donors were treated with anti-lymphocyte serum to reduce the number of reactive T cells in the prospective graft. The thyroid graft, depleted of lymphocytes, was then successfully transplanted to an allogeneic guinea pig under conditions where uncultured grafts were rejected. Furthermore, if a second untreated (lymphocyte containing) graft was placed in a "successful" thyroid recipient, *both* grafts were subsequently rejected. Thus the "passenger leukocytes" in the second graft caused an allograft response which was effective in rejection of non-leukocyte tissue in the first graft.

This system has obvious implications for organ transplantation. With respect to the mechanism of CTL

induction, it suggests that auxiliary cells may be involved. The inability of glutaraldehyde fixed (metabolically inactive) cells to induce CTL can be overcome by the addition to cultures of primed antigen-specific helper cells (Pilarski, 1979), or by the soluble factor costimulator (Shaw *et al.*, 1978b; Lafferty and Woolnough, 1977; results in chapter V). These results, and others presented in section H suggest that CTL generation requires interaction between 2 classes of T cells. In addition to T cells, macrophages appear to be required for *in vitro* CTL responses, although they can be either of responder or stimulator genotype (Wagner *et al*, 1972).

I. Evidence for T Cell-T Cell Collaboration

Cantor and Asofsky (1970) first reported synergy between two types of T cells, obtained from thymus and lymph nodes, in the mediation of graft versus host disease (GVHD). GVHD is an *in vivo* correlate of MLR, in which donor cells of type A are injected into a neonatal (AxB)F1. The A cells proliferate in the neonatal spleen; the intensity of the reaction in quantitated by measuring its weight. Synergy between 2 cell populations, called T1 (from the thymus) and T2 (from lymph node) occurs in the generation of CTL *In vitro* (Wagner, 1973). By mixing AKR and CBA lymph node cells and thymocytes (both H-2(k) but possessing different alleles of Thy 1 which served as markers distinguishing between functional cell types), it was determined that CTL

precursors were derived from the lymph node cells, and that thymocytes amplified the response. The amplification by thymocytes is puzzling, in view of the recent data demonstrating that thymocytes contain very few mature helper cells (Pilarski, 1977). However, the thymocytes may serve as a source of helper precursors.

Using antisera directed against Ly antigens, Cantor and Boyse (1975b) demonstrated that Ly 1^-2^+ spleen cells are precursors for anti-allogeneic CTL effectors, and that the response is amplified by Ly 1^+2^- cells. The removal of Ia^+ cells from the stimulator population decreased the amplification effect of the Ly 1^+ cells, suggesting that these cells are stimulated by I region differences. Synergy between cells recognizing K/D and I differences was also found in experiments using congenic mice (Schendel and Bach, 1974). Low CTL responses were obtained when responder and stimulator differed only at the K region, or only at the I region. However, when 2 types of stimulators (K and I different) were mixed with responders in the cultures, good responses were obtained.

It should be pointed out that the requirement for both K/D and I region differences is not absolute (reviewed in Katz, 1977, chapter 10; H.-S. Teh personal communication) and is not found in all systems. Helper T cells which recognize K/D differences have been reported (Swain and Panfili, 1979), and interestingly, are Ly 1^-2^+ (unlike helper T cells which recognize I region differences, which

are Ly 1^+2^-). This suggests that the Ly phenotype of T cells may bear more relationship to the type of antigens they recognize than to their function *per se*.

Perhaps the most direct evidence for a helper T cell requirement in allogeneic CTL responses comes from experiments in which thymocytes were used as CTL precursors (Pilarski, 1977). Thymocytes are poor CTL responders, not because CTL precursors are lacking, but because the thymus apparently contains few differentiated helper T cells compared to the peripheral lymphoid organs. Helper T cells generated *in vivo* or *in vitro* (Baum and Pilarski, 1978) which are alloantigen specific, allow the efficient generation of CTL *in vitro* from thymocyte precursors. Analysis of the T helper effector cells revealed that they are Ly $1^+,2^-,4^-,5^+,6^-$ and 7^+ (Pilarski *et al.*, 1980).

Using bone marrow irradiation chimeras, Zinkernagel *et al.*, found I region-restricted helper T cells to be necessary for the generation of a CTL response *in vivo* against virally infected cells. Doherty, however, using a similar system failed to detect a requirement for helper cells, at least helpers which were I-restricted (Bennink and Doherty, 1978).

The role of soluble factors in the generation of CTL responses will be considered in Section J. In chapter IV, results are presented which indicate that the soluble factor costimulator can at least partially replace the helper T cell requirement in CTL generation. The properties of the

costimulator producing cell is compared to that of the helper T cell.

Specific T cell suppression of CTL responses has been described in several systems (reviewed in Katz, 1977). One of the obvious ways in which a T cell could appear to suppress a response *in vitro* would be to remove the antigen (stimulator cells) before the response was fully initiated. Recent work by Al-Adra and Pilarski (1978) shows that killing of antigen is not the only way in which suppressors can act, since the level of suppression was not related to the numbers of stimulator cells in a culture. Thus there appear to be suppressor T cells which are distinct from cytotoxic T cells, although the 2 types of cells have been reported to have the same Ly phenotype (Ly $1^-,2^+$; Cantor and Boyse, 1976b).

Al-Adra, Pilarski, and McKenzie (personal communication) found 2 types of inhibitory cells in CTL generating cultures. One, which was present at 3 days of culture was Ly $1^+,2^+$, while another, present after 5 days of culture, was Ly $1^-,2^+$. These cell types were both different from helper cells generated in the same system (Ly 1^+2^-), demonstrating that suppression is mediated by distinct subclasses of T cells, and is not simply an artifact seen if too many helper cells are added. The suppressor populations were not cytotoxic for the cells used as antigen in the cultures, and thus are also distinct from cytotoxic T lymphocytes. The "target" of the suppressor cell has not

been defined.

Antigen-specifc CTL suppressor cells have been detected in DBA/2 mice bearing a syngeneic mastocytoma tumor, P815 (Takei *et al.*, 1977). Tumor bearing mice exhibit a brief period of reactivity to the tumor, which can only be detected *in vitro* after culturing the DBA lymphocytes with P815. Thereafter, the reactivity of the mice to P815 declines, concomitant with the appearance of the suppressor cells, which can be assayed *in vitro*.

J. The Role of Soluble Factors in Immune Regulation

Soluble Factors as Mediators of Cellular Interactions

There is ample evidence that most immune reponses involve the participation of multiple cell types (sections G and I). The question arises as to how one cell type exerts its effect on another. A "signal" might be passed between cells during close membrane contact, such as that which occurs during killing of target cells by CTL (Cerottini and Brunner, 1977) or regulatory cells, upon stimulation, might secrete active products in a manner analogous to Ig synthesis by B cells, which would bind to receptors on effector cell precursors. The products might be antigen specific, reflecting the specificity of T cell receptors, or they might be non-specific inductive agents, normally released close to the appropriate target cell due to an antigen bridge between them.

Another possibility is that an inductive signal from a

regulatory T cell (whether antigen-specific or non-specific) is passed to a target B or T cell by a macrophage bearing antigen on its surface. T and B cells are anatomically separated in the spleen, a major site of immune induction. The participation of a third cell type of high mobility would increase the liklihood of two infrequent antigen specific cells meeting one another.

Double chamber experiments of Feldmann and Basten (1972) suggested that carrier-primed T cells could provide an inductive signal to hapten primed B cells if the two populations were physically separated by a cell-impermeable membrane. Thus in a system where all requisite cell types were present, cell to cell contact did not appear mandatory for AFC induction. This at least indicated that soluble factors might be released from leukocytes during a normal immune response.

In the last decade, many laboratories have described soluble factors secreted by or extracted from stimulated leukocytes. These factors have been reported in murine, rat, guinea pig and human systems. They have been found to exert their effects in several *in vitro* assays: AFC responses, CTL responses, synergy with mitogens such as PHA or Con A, and direct mitogenesis with no other deliberately added stimulants. Factors with helper activity have been shown to be either antigen specific and H-2 restricted, or non-specific, depending on the agents and methods used to induce them. The same has been found with soluble factors

which suppress immune responses. Many of the factor activities are detected in crude tissue culture supernatants; others have been partially purified.

In order to analyze the somewhat bewildering array of soluble factors, I have grouped them into 3 categories according to their properties and the way they were derived: 1) antigen-specific and H-2 related factors 2) apparently antigen-nonspecific, T cell derived factors and 3) antigen-nonspecific macrophage derived factors. Groups 2 and 3 were suggested at the 2nd International Lymphokine Workshop in 1979, where it was decided that many mitogen-induced T cell factors described by different groups are probably identical, and that the same was true of certain macrophage factors. For the most part, the discussion of these factors is restricted to the murine system, both because it is the most well characterized, and it is the most relevant to the experimental results presented in this thesis.

Factors with Specificity for Antigen and/or I-Region Determinants

Several antigen-specific helper factors for AFC responses have been described (see Table 1, factors 1-4). In general, the factors are obtained from lymphoid tissue of antigen primed mice, either by allowing the cells to secrete the factor in tissue culture, or by extracting the cells taken directly from the mice. Such factors either replace the requirement for helper T cells in AFC responses, or

Table 1.

Antigen specific and/or Ia+ helper and suppressor factors

Factor	Means of Production[1]	Assay in which[2] Activity is detected	Properties[4]	References
1. TAF(T cell-Augmenting factor)	SPC or Thy, KLH primed; Extract; Ly 1+,2- T cell dependent.	AFC: Increases 2° anti-DNP-KLH response. Requires I-A identical T cells to act.	Binds KLH; Ia+; MW: 30-60,000.	Tokuhisa et al., 1978.
2. T cell factor for (T,G)-A--L	KLH primed T cells cultured with (T,G)-A--L. Secreted.	In vivo: Replaces T cells in anti-(T,G)-A--L response; Not H-2 restricted.	Binds (T,G)-A--L; Shares idiotype with Ig against (T,G)-A--L; Ia+.	Taussig et al., 1974, 1975; Mozes and Haimovich, 1979.
3. Carrier-specific helper factor	Primed T cells against RGG, HGG, BSA or chicken RBC; Extract.	AFC: Increases 1° response against hapten carrier complexes; Replaces T cell requirement; H-2 restricted.	Binds antigen; Ia+; pI: 3 peaks 2.5, 4.0 and 4.6;	Shiozawa et al., 1977, 1979.
4. (T,G)-A--L and GAT helper factors (Hf)	Unprimed SpC cultured with antigens for 24 hours; Secreted.	AFC: Increases 1° response to (T,G)-A--L and GAT.	(T,G)-A--L Hf is I-A+; GAT Hf is I-J+; MW: 50-60,000.	Howie and Feldmann, 1977; Howie et al., 1979.
5. P815-specific helper factor	Extract of Spc from tumor-bearing DBA/2 mice.	CTL: Increases 1° response of DBA/2 SpC against P815. (not L1210)	Binds P815 membranes; Ia+; MW: 50,000.	Kilburn et al., 1979.
6. Restricted AEF (Allogeneic effect Factor)	2° MLC of Ia- responder cells against T stimulators; Secreted.	AFC: Increases 2° anti-hapten responses; Replaces T cell function; H-2 restricted.	Ia+ (Stimulator haplotype). Binds Con A and lentil lectin; MW: 40-50,000.	Delovitch and McDevitt, 1978; Delovitch et al., 1978; Delovitch and Sohn, 1979.

7. GRF (Genetically related factor)	Peritoneal Mph (T^-,B^-) cultured with KLH or (T,G)-A--L; Secreted.	AFC: Replaces Mph requirement for helper T cell induction; I-A restricted; Acts on Ly 1^+2^+ cell.	MW: 55,000 Ia^+; contains fragments of immunogen.	Erb and Feldmann, 1975; Erb et al., 1976, 1979.
8. T suppressor factor for KLH	KLH primed Thy; Extract; Requires Ly 1^-2^+ T cell.	AFC: Suppresses response to DNP-KLH; Acts via T cell. H-2 restricted	$I\text{-}J^+$; Shares idiotype with Ig against KLH; MW: 35-55,000.	Tada et al., 1975; 1976. Taniguchi et al., 1976, 1979.
9. GAT-TSF (T suppressor factor)	GAT primed SpC or Thy; Extract; Requires T cell.	AFC: Suppresses anti-GAT MBSA response in vivo or in vitro: (induces suppressor cells); H-2 restricted.	Binds GAT; $I\text{-}J^+$; Ig^-; Shares iodiotype with Ig against GAT.	Kapp et al., 1976. Germain et al., 1979.
10. P815-specific suppressor factor	Thy of 8 day P815-bearing mice; Extract.	CTL: Suppresses anti-P815 response of DBA/2 SpC.	MW: 40-60,000 PI: 4.6-4.9.	Takei et al., 1978.
11. Anti-SRBC suppressor factor	Secreted by hybridoma (BW5147 fused with SRBC-primed SpC).	AFC: Suppresses 1°,2° anti-SRBC and TNP-SRBC responses.	Binds to SRBC; Binds B (not T) cells; MW: 200,000.	Taussig, 1979.
12. KLH suppressor factor	Secreted by hybridoma (BW5147 fused with KLH primed SpC).	AFC: Suppresses 1°, 2° TNP-KLH response; Requires T cells to act; Not H-2 restricted.	$I\text{-}J^+$; Shares idiotype with Ig against KLH.	Kontiainen et al., 1978.

[1]SpC: spleen cells; Thy: thymocytes; LNC: lymph node cells; Mph: macrophages; Primed: previously exposed to the antigen (in vivo); 1°: primary response; 2°: secondary response. T^-; B^-: depleted of T or B cells.
[2]KLH: keyhole limpet hemocyanin; RGG, HGG: rabbit and human gamma globulin. See list of abbreviations for other antigens.
[3]Assays are in vitro unless stated otherwise.
[4] All MW determinations by gel filtration.
[5]Ia^+, $I\text{-}A^+$ or $I\text{-}J^+$ etc: having determinants detectable by antisera against antigens coded for in the H-2 I region or a specific subregion.

augment the response of T cell-containing cell populations. Helper factors for the antigens (T,G)-A--L (Taussig *et al.*, 1975; Howie *et al.*, 1979), KLH (Tokuhisa *et al* 1978), RGG, HGG, BSA and chicken red blood cells (Shiozawa *et al.*, 1977; 1979) can be absorbed by antisera directed against products of the I-A subregion of the H-2 complex, and by the antigen used to induce them. The helper factor to GAT, however, is absorbed by anti-I-J and not anti-I-A antibodies (Howie *et al.*, 1979).

With the exception of the factor described by Taussig *et al.* (1975), all have activity which is restricted to lymphocytes of the same H-2 or I type as the cells from which the factor was produced. The lack of H-2 restriction of Taussig's factor is puzzling, but may be related to the *in vivo* assay used to detect it. This factor bears idiotypes which cross react with antibodies against (T,G)-A--L, suggesting that it may represent a T cell receptor which has been secreted (Mozes and Haimovich, 1979). Its production is dependent upon Ly $1^+,2^-$ cells and it may be the product of a helper T cell.

None of these factors have been extensively characterized biochemically. The molecular weights, estimated by gel filtration, fall into the range of 40,000-60,000. The factor activity reported by Shiozawa *et al.* (1979) appears to be fairly heterogeneous. Antigen-specific helper activity for RGG is spread over a wide size range upon G-100 chromotography, and isoelectric

focusing resolves 2 peaks of activity, at pI 4.6 and 2.5, . The heterogeneity may represent actual differences in types of molecules, or degradation of the factor as a result of an extraction procedure in which proteolytic enzymes might be released.

Kilburn (1979) has reported an antigen-specific helper factor for a CTL response. It was extracted from the spleens of DBA/2J mice bearing a syngeneic (P815) tumor, which had not yet metastasized to the spleen. The factor enhanced the *in vitro* generation of CTL against this tumor about 5 fold. It did not stimulate the generation of CTL against L1210, another DBA/2J tumor, nor did it enhance CTL generation against DBA/2J spleen cells from C57BL/6 precursors. It could be adsorbed onto P815 membranes bound to Sepharose, and then eluted with high salt.

Two other "specific" helper factors have been reported which appear to be different from those that are both I-region-restricted and antigen specific. One is allogeneic effect factor (AEF; Table 1). It is secreted in mixed lymphocyte culture (MLC) by spleen cells which have been primed *in vivo* with stimulator cells from the same strain of mice. If the stimulator cells in the MLC are depleted of T cells, and the responder cells depleted of Ia^+ cells, the factor produced is only active in enhancing the AFC response of cells having I-A or I-B region identity with the stimulator cells, and is called "restricted AEF" (Delovitch *et al.*, 1978). The factor has determinants coded for in the

I-A or I-B subregion of H-2. It can be produced if the stimulator and responder strain differ only in the I-J subregion, in which case the factor activity is restricted to mouse strains identical to the stimulator cell at *I-J* (Delovitch and Sohn, 1979). AEF is not specific for any particular soluble antigen, and appears to be effective in inducing secondary AFC responses in the absence of T cells. It is considered likely that AEF is actually an *antigen specific factor for H-2 I region alloantigens*, and is able to induce AFC responses in B cells by binding to their Ia antigens and giving a "second signal" (T.L. Delovitch, personal communication). This would happen in the same way as an antigen-specific factor against SRBC might bind to a B cells whose Ig receptors were bound to SRBC. AEF has no helper activity in CTL (L. Pilarski, J. Shaw, and T.L. Delovitch, unpublished). This would be expected if CTL helper cells and CTL precursor cells were Ia^-, or had Ia antigens of a different sort from those present on (stimulator) B cells. AEF, like other antigen-specific factors, is about 40-50,000 MW, as estimated by gel filtration. A factor which may be similar has been reported to have antigen-specific helper activity *in vivo* for AFC against histocompatibility antigens in nude mice (Kindred and Corley, 1977). The factor is secreted *in vitro* after a 10 day MLC reaction.

An Ia^+ AEF has also been obtained from primary MLCs of only 24 hours duration (Armerding *et al.*, 1977). However,

Delovitch and coworkers are unable to detect AEF activity in MLC supernatants unless the responder cell populations have been primed.

The second type of unusual factor is GRF (genetically related factor) (Table 1) which, unlike the others, appears to be the product of a macrophage. It contains both Ia determinants, and fragments of the antigen used to induce it (Erb and Feldman, 1975; Erb *et al.*, 1976). It is possible that it represents "processed antigen" from macrophage surfaces, which is known to be more immunogenic than soluble antigen (Unanue, 1978). That would explain its ability to replace macrophages in AFC induction, while still requiring T cells to exert its effect.

Several suppressor factors (8-11 in Table 1) are also specific for their inducing antigens, and bear *I-J* (or H-2)-encoded determinants. None are adsorbed by anti-immunoglobulin. Suppressor factors to KLH (Taniguchi *et al.*, 1979; Kontiainen *et al.*, 1978) and to GAT (Germain *et al.*, 1979) have idiotypes which cross react with antibodies directed against the same antigens.

In addition to factors which suppress AFC responses, factors which supress contact sensitivity to picryl chloride (Green *et al.*, 1977) and 2,4-dinitrofluorobenzene (Moorhead *et al.*, 1977) have been reported. Another factor, extractable from the thymus of P815 bearing-mice, specifically suppresses the *in vitro* CTL response of DBA/2J mice against the syngeneic tumor (Takei *et al.*, 1978).

One of the difficulties in trying to characterize these factors is a paucity of material. An obvious approach is to produce hybridomas between tumor cell lines and lymphocytes which secrete the factors. Such hybridomas have been produced for factors which suppress antibody responses to KLH (Taniguchi *et al.*, 1979; Kontiainen *et al.*, 1978) and SRBC (Taussig *et al.*, 1979). The hybridomas have all been produced by the fusion of antigen-primed spleen cells with BW5147, an AKR (H-2k) thymoma, using the fusion and selection techniques pioneered by Kohler and Milstein (1975; 1976). It is not necessary for the spleen cells to be of the same H-2 type as the tumor cell line. BW5147 itself does not express the Ly 1.2 antigen on its membrane, but expression of that antigen, as well as the Ly 1.1 antigen (the allele carried by CBA cells) was observed on the fusion product.

The Use of Polyclonal Stimulants to Study Immune Responses

The term "mitogen", which is frequently used in the discussion of non-specific factors, refers to agents which induce lymphocytes non-specifically, regardless of their receptor specificities (Greaves and Janossy, 1972). The normal requirement for binding to a T or B cell receptor for antigen is somehow bypassed; the mitogens are thought to bind to other cell membrane receptors. A particular mitogen stimulates a large, but indeterminant number of lymphocytes, different mitogens stimulating different subpopulations. Some commonly used T cell mitogens are concanavalin A (Con A) and phytohemagglutinin (PHA) both plant lectins.

Lipopolysaccharide (LPS) from E. Coli is a mitogen for B cells. Pokeweed mitogen (PWM) stimulates both T and B lymphocytes.

Within a few days of culture with such mitogens, lymphocytes respond by proliferating, which can be quantitated by measuring the rate of DNA synthesis. Differentiation into effector cells, such as AFC or CTL, may subsequently occur.

In some instances it is advantageous to use mitogens rather than specific antigens to study the induction of immunity. Phenomena involving large numbers of cells can be readily detected, whereas antigen stimulation would affect too small a proportion of lymphocytes to measure. One example of the usefulness of mitogens in investigating immune induction is the use of LPS to measure the effect of stimulation of B cells on their intracellular levels of cyclic AMP and cyclic GMP (Watson, 1974).

Factors which Synergize with Antigens or Mitogens, Appear to be Non-Specific, and are induced by T cell stimulants

It is somewhat difficult to discuss this area without anticipating the results, since some of the closely related work of others was done after previously published work which is to be presented in this thesis (Shaw *et al.*, 1978a,b). However it is probably useful to discuss some of the properties of this group of factors, which includes costimulator, in order to place the results in a more understandable context. Other comparisons between

costimulator and various soluble factors will be discussed in the appropriate places throughout the thesis.

Some representatives of the group of factors designated Interleukin 2 (IL2) are listed in Table 2. The assignment of various factors to this group is somewhat arbitrary, due to a lack of information concerning the properties and activities of some. The group may turn out to be more heterogeneous than is now suspected. However, an overall view of the IL2 factors shows them to have one or more of the properties mentioned below.

The numbers given in parenthesis refer to the number of a factor in the first column of Table 2. References appear in the table.

The factors are secreted into culture medium by spleen or lymph node cells within 16-24 hours of stimulation with an inducing agent. They are induced by T cell mitogens such as Con A (1-6,8,9) and PHA (4,5,9), but not by the B cell mitogen LPS (1,4,5). In some cases factor has been produced by culturing antigen primed spleen cells with KLH *in vitro* for 24 hours (6,7,9). An apparently identical factor is secreted during the early stages of an MLC reaction in which two unprimed spleen cell populations differ in the entire H-2 region (1,3,4,5,6,8,9).

Production of the factor depends upon T cells being present in the stimulated cell population (1-9). Experiments using metabolic inhibitors indicate that DNA synthesis is not required for factor production, but protein synthesis

Table 2.

Some T cell-dependent, non-specific immunostimulatory lymphokines.

Factor	Means of induction[1]	Assay used to detect activity[2]	Properties[3]	References
1. Costimulator	Con A or MLC. Requires $Ly1^+$, 2^- T cells + Mph.	a) Mitogenesis: Thy. response to Con A, PHA b) CTL: Anti-allo MHC and anti-modified self c) AFC: Anti-SRBC (factor added Day0)	MW: 30,500; PI: 3.7-5.0; Not H-2 restricted; Ia^-.	Paetkau et al., 1976; Shaw et al., 1978a, b; Watson et al., 1979a.
2. TRF (T cell-replacing factor)	Con A.	a) Same as #1; b) Maintenance of CTL in continuous culture.	MW: 30-40,000; pI: 2 peaks 4.1, 4.9.	Watson, 1973; Watson et al., 1979a, b and c.
3. TRF (T cell-replacing factor)	Con A or MLC.	AFC: Anti SRBC (factor added Day2).	MW: 3 peaks range 25-45,000; pI: 2 peaks 5.1, 6.9; Ia^-.	Schimpl and Wecker, 1973, Muller et al., 1978 Hubner et al., 1978.
4. TCGF (T cell growth factor)	Con A, PHA, PWM or MLC. Requires T cells and Mph.	a) Maintenance of CTL in coninuous culture b) Same as # 1 and 2	Same as #2.	Gillis and Smith, 1977; Gillis et al., 1978a, b; Smith et al., 1979a, b; Watson et al., 1979c.
5. TMF (thymocyte mitogenic factor); also called KCHF (Killer cell helper factor) and TRF	Con A or MLC.	a) Mitogenesis: Thy. response to PHA b) CTL: Anti-allo-MHC c) AFC: Anti-SRBC	MW: 35-38,000.	Farrar et al., 1978; Simon et al., 1979.
6. NSM non-antigen - specific mediator	KLH primed SPC cultured with KLH; Con A or MLC.	AFC: Anti-SRBC	MW: 30-40,000; Ia^-.	Hunter and Kettman, 1974; Marrack and Kappler, 1975, 1977; Harwell et al., 1976.
7. NSF non-specific factor	KLH primed SpC cultured with KLH.	AFC: Anti-SRBC (not anti-KLH). (factor added Day0)	MW: 35,000; Ia^-; Does not bind KLH.	Waldmann and Munro, 1974; Waldmann, 1975, 1977; Waldmann et al., 1976.

8.	SCIF (secondary cytotoxic T-cell inducing factor)	Con A or MLC; Requires Ly 1+2- T cell.	CTL: Anti-allo-MHC, secondary response.	MW: 35-38,000.	Wagner and Rollinghoff, 1978.
9.	TSF (thymocyte stimulating factor)	Same as 7; PHA; MLC. Requires T cells, not B cells.	Mitogenesis: Thy. response to PHA.	MW: 30,000.	DiSabato et al., 1975, 1978; Chen and DiSabato, 1976.

1. Abbreviations as in Table 1. PWM: pokeweed mitogen.
2. Allo MHC: MHC antigens expressed on allogeneic cells.
3. Except for 1, MW determinations done by gel filtration.

is. Whether antigen-nonspecific, as they first appeared to be, or specific, but polyclonal as some investigators now suspect (J. Watson, personal communication; Bernabe *et al.*, 1979), none of the factors are H-2 restricted in their biological activities. All which have been examined for the presence of Ia determinants lack them (1,3,6,7).

Factor activity has been detected in several biological assays, including mitogen, AFC and CTL responses. Factors may induce thymocytes to divide in the absence of any other deliberate stimulation (direct mitogenesis; 1,5). The direct mitogenesis, however, is usually very low in comparison to the synergistic effect (up to 50 fold) of the factors in the response of thymocytes to Con A and PHA (1,2,5,9). The proliferative response of T cells is thought to be dependent upon IL2 type factors, and thymocytes are deficient in the particular T cell or macrophage type which is active in producing the factor (Paetkau *et al.*, 1976; Shaw *et al.*, 1978b). This synergy between factor and Con A is the basis for the routine assay used to detect costimulator (chapter III).

Several of the factors (2,3,6,7 as well as Dutton *et al.*, (1971), Britton *et al.*, (1972), and Sjoberg *et al.*, (1972), were first detected as the ability to enhance, or to replace the requirement for T cells in a primary *in vitro* AFC response against heterologous erthyrocytes, usually SRBC. Spleen cells from nu/nu mice, or normal spleen cells treated with anti-Thy 1.1 and complement (both deficient in

helper T cells) respond poorly to SRBC. The response can be restored by the addition to cultures of helper T cells primed to SRBC, or the so-called T cell replacing factor, TRF (2,3,6,7).

Some investigators report that TRF is most active if added after 2 days of a 4 day culture (3) while others add it on day 0 or 1 for maximal effect (2,7). This discrepancy has recently been resolved by Watson and colleagues, who find that preparations of IL2 contain another activity, called "late acting factor" which is most effective if given near the end of an AFC response. Late acting factor has a lower pI than IL2, and can be separated from it by isoelectric focusing (J. Watson, personal communication). Purified IL2 is most effective if present from the beginning of culture (Hoffmann and Watson, 1979).

There is some evidence that the T cell-replacing activity of these factors does not extend to responses against all types of antigens. While active against haptens such as DNP which are coupled to erythrocytes before immunization, the factors do not work for soluble antigens, such as KLH, or if the hapten is presented on a soluble carrier (Waldmann, 1975). It has been proposed that for soluble, non-particulate antigens, a carrier-specific T cell (or T cell factor such as those in Table 1) may be an additional requirement (Waldmann and Munro, 1975).

Some factors were initially detected primarily as enhancing activities in CTL responses (4,8). Others are

reported to have CTL stimulating activity in addition to mitogen/synergy and TRF activities (1,2,5). The thymocyte has proved to be a useful assay system for the effect of IL2 in CTL generation. Thymocytes, which are helper cell deficient but contain CTL precursors, respond poorly to alloantigens (PilarsKi, 1977). The addition of helper cells to cultures allows them to generate a primary CTL response, as does the addition of IL2 factors (1,5).

The factors are also capable of generating secondary CTL responses (8), and ultimately, of maintaining the continuous growth of effector CTL in culture for over a year (4).

Lafferty and co-workers have shown that IL2 factors can replace the requirement for a lymphoid, metabolically active stimulator cell in CTL generation (Lafferty and Woolnough, 1977; Talmage *et al.*, 1977; Lafferty *et al.*, 1978). Other conditions under which IL2 factors promote CTL responses, and the significance of such experiments are discussed in chapters V and VI.

The molecular properties of IL2 are just beginning to be understood. The material is produced [redacted]ry small amounts in chemical terms, although it is highly active biologically. It has not been purified to homogeneity. Therefore all experiments having to do with "molecular properties" rely on biological activity, and not on protein or other material detectable by physical methods, as a probe for structure. Gel filtration experiments show IL2 factors

to be between 30,000 and 50,000 daltons (1-9). The activity resides in a heterogenous population of acidic molecules, having pI in the range of 4-5 (1,2). The factors are sensitive to proteolytic enzymes, and may be glycoproteins. Experiments examining the molecular properties of costimulator are presented in chapter III, where the specific results of other investigators are discussed.

Macrophage - Derived Lymphokines

A group of macrophage-derived factors, designated Interleukin 1 (IL1) and usually referred to as lymphocyte activating factor (LAF), is represented in Table 3. Like the T cell-dependent factors, they are antigen-nonspecific. There is, however, no requirement for T cells for their production, since they can be produced by marophage tumor cell lines such as P388-D1 (Lachman *et al.*, 1977; Mizel, 1979) and RAW264 (Hoffmann *et al*, 1979). They are generally produced by stimulating macrophage tumor cells or normal mouse peritoneal cells with LPS in culture for at least 24 hours. The factors are secreted into the medium. It can also be elicited by phorbol myristic acetate (PMA; Mizel *et al.*, 1978c). T cells apparently enhance LAF production from the P388-D1 cell line up to 10 fold (Mizel *et al.*, 1978a).

The biological assay most frequently used to detect LAF is direct mitogensis, using mouse thymocytes cultured at relatively high cell density: $5\text{-}15 \times 10^6$/ml. The level of stimulation of cell division is considerably lower than that observed using IL2 synergy with Con A or PHA. Recently,

Table 3.

Lymphocyte-activating factors secreted by macrophages in culture.

Factor	Production[1]	Assay in which activity is detected	Properties	References[2]
1. LAF (Lymphocyte activating factor)	Adherent peritoneal cells + LPS, 24 hrs.	Direct Thy mitogenesis (5×10^6/ml).	MW: 18,000; pI: 4.8; S20,W: 2.0.	Economou and Shin, 1978.
2. LAF	P388-D1 cells: + PMA, 144 hrs; or, + T cells, 96 hrs; or, + LPS, 72 hrs.	Direct Thy mitogenesis; Synergy with PHA in Thy, mitogenesis (15×10^6/ml).	MW: 12-16,000; pI: 5.0-5.4.	Mizel et al., 1978a, b and c; Mizel, 1979; Mizel and Rosenstreich, 1979.
3. LAF	WEHI 3, J774, and P388-D1 cells +LPS, 48 hrs.	Direct Thy Mitogenesis (15×10^6/ml).	MW: 50-80,000; pI: 4.6-5.0	Lachman et al., 1978 a,b.
4. TRF-M (T cell replacing factor from macrophages)	Adherent peritoneal cells from C. parvum primed mice + LPS, 20hr; or PU5-1.8, RAW 264 cells + LPS	Increases 1° AFC to SRBC or TNP-mouse RBC in T-,A- Spc.	MW: 150,000 or 15,000; Synergestic with TRF (Watson et al., Table 2).	Hoffmann et al., 1979; Hoffmann and Watson, 1979.

1. Except for #1, MW was determined by gel filtration. PMA: phorbol mystric acetate. P388-D1, J774, WEHI 3, PU5-1.8 and RAW 264 are macrophage tumor cell lines.
2. Other abbreviations as in Table 1.
Similar factors have been reported by Gery and Handschumacher (1974), Beller and Unanue (1977), and Okada et al. (1978).

another assay for IL1 has been suggested: it acts as a T cell replacing factor in the AFC response of splenic lymphocytes which have been depleted of both T cells and A cells (Hoffmann *et al.*, 1979; Hoffmann & Watson, 1979). This assay distinguishes functionally between IL1 and IL2, which does not act as a T cell replacing factor in the absence of A cells. IL1 apparently does not induce cell division in B cells, but may be involved with their terminal differentiation. IL1 synergized with TRF in the anti-SRBC response. Results were best if IL1 was added on day 2, and TRF on day 0. (Hoffmann and Watson, 1979). This may explain the synergy observed in another *in vitro* system between TRF and LPS itself (Jacobs, 1979).

LAF may also be involved in the induction of IL2 (Smith *et al.*, 1979a; results in chapter II).

The molecular properties of LPS induced LAF have been most thoroughly studied by Economou and Shin (1978), who found the molecular weight to be 18,000, and the isoelectric point to be 4.8. Others have reported a similar pI (4.6-5.4 range; Lachman *et al.*, 1977; Mizel, 1979), and molecular weight estimates from gel filtration ranging from 12-16,000 (Mizel, 1979; Hoffmann *et al.*, 1979). Higher molecular weight forms of LAF have also been reported (Lachman *et al.*, 1977; Mizel and Rosenstreich, 1979), and may be precursors of the protein which is normally secreted upon LPS stimulation. The higher molecular weight forms (220,000, 50,000, 39,000 and 26,000) reported by Mizel and

Rosenstreich were found in cell-sonicates, and may represent intracellular forms of the factor which are modified prior to secretion.

LAF which are probably similar have been reported by Gery and Handschumacher (1974) and Unanue and co-workers (Beller and Unanue, 1977; Unanue *et al.*, 1976).

Non Murine Lymphokines

Mitogenic or synergistic factors have been described in non-murine systems, mainly human. They have been detected in culture medium from human MLCs. (Kasakura *et al.*, 1965; Gordon and MacLean, 1965; Rubin *et al*, 1974; Farrar *et al.*, 1977; Koopman *et al.*, 1977; Simon *et al.*, 1977; Uotila *et al.*, 1978; Chiorazzi *et al.*, 1979). Other groups have reported human lymphokines released after mitogen stimulation of peripheral blood leukocytes using Con A (Mackler *et al.*, 1972; DiSabato *et al.*, 1977b), PHA (Larsson *et al.*, 1979; DiSabato *et al.*, 1977b; Gery *et al.*, 1971) and LPS (Gery *et al*; Blyden and Handschumacher, 1977; Lachman *et al.*, 1977). A human LAF could also be obtained from human acute monocytic and myelomonocytic leukemia cell-conditioned medium (Lachman *et al.*, 1978). The most well characterized of those factors are listed in Table 4, with some of their properties. Three groups have found lymphokines of 13-15,000 MW, 2 of which have a pI of 6.5-6.8 (Smith *et al.*, 1979b; Lachman *et al.*, 1977 and Farrar *et al.*, 1977). Larrson *et al.* (1979) have reported a factor which appears to be physically different (MW 40-50,000, pI 8). The assay system

Table 4.

Some lymphokines generated from human peripheral blood[1] leukocytes (PBL).

INDUCING AGENT	ASSAY	PROPERTIES	REFERENCES
PHA	Mitogenic for T cell-enriched human PBL.	MW: 40-50,000; pI: 8.0-8.5.	Larrson et al., 1979.
PHA	Assayed on mouse cells: a) Con A / synergy (Thy) b) CTL c) CTL continuous culture d) AFC.	MW: 15,000; pI: 6.5.	Smith et al., 1979b.
LPS	Assayed on mouse Thy: Synergy with Con A or PHA; Direct mitogenesis.	MW: a) 13,000 b) 85,000; pI: 6.8 (a).	Blyden and Handschumacher, 1977; Lachman et al., 1977a.
MLC	Assayed on mouse spleen, Increase AFC of T-depleted cells to SRBC	MW: a) 10-15,000 b) 20-25,000 c) 40-≤77,000; a and c act synergistically.	Farrar et al., 1977.

[1]Abbreviations as in Table 1.

used to detect it employed human PBL, rather than mouse leukocytes, which the other authors used.

Lymphokines have been detected after stimulation of rabbit (Gery and Waksman, 1972), rat (Hoessli *et al.*, 1977; Gery and Waksman, 1972) dog, monkey (Blyden and Handschumacher, 1977) and guinea pig (Gately *et al.*, 1975) leukocytes. The factor from guinea pig cells is the most thoroughly characterized non-murine lymphokine. It is secreted during secondary stimulation (*in vitro*) of ovalbumin primed guinea pig lymph node cells. Its molecular weight is 20,000.

K. Aims and Rationale of Work Presented in this Thesis

At the time this project was undertaken (1976) several groups had reported soluble factors with various biological activities in *in vitro* immune responses, a number of which have already been mentioned. Some of the initial work on costimulator had been done by V. Paetkau, G. Mills and colleagues (see Introduction to chapter 3). However most "factors" were actually activities in tissue culture media in which lymphoid cells had been cultured. Little definitive biochemical work had been done on immunostimulatory lymphokines.

Most crude supernatants, especially those induced by strong mitogens, probably contained several factors. For example, the supernatant of Con A stimulated cells is now known to contain a) IL2 activity (section J), b) interferon

(anti-viral) activity (Simon *et al.*, 1979), c) a small amount of 'LAF' activity (Farrar *et al.*, 1978), d) a cytotoxic lymphokine known as lymphotoxin (Hiserodt *et al.*, 1979, and references therein) and e) a factor which induces macrophages to destroy bacterial parasites (Buchmuller and Mauel, 1979). All except the latter factor have now been distinguished from each other using biochemical criteria.

Another problem has been the diversity of immunological assays used by different groups to assay factors which may have some of the same activities. It was recently reported by Watson and Smith (1979) that purified TRF, initially detected as having activity in AFC responses, also has TCGF activity, which allows CTL to proliferate continuously in culture. On the other hand, factors which appear to be active in the same assays, such as TRF and TRF-M (Hoffmann and Watson, 1979), may be products of different cell types and have different molecular properties.

The central problem in this field, then, it to define a lymphokine in molecular terms, and to determine how its synthesis is controlled, and how it acts in the immunoregulatory network. The ultimate goal of this work is to ascertain how these factors and the cells which secrete them interact *in vivo* with target cell surface receptors, and how they exert their effects at a molecular level.

The objective of the work to be described in the following three chapters was to analyze the physical and biological properties of a particular lymphokine,

costimulator. Chapter III deals with the purification procedures used and the molecular properties . Chapter IV summarizes studies on the cellular interactions required to produce the factor, and the cell type which actually secretes it. The biological systems in which costimulator has activity, and its possible biological role in immune responses is considered in chapter V.

II. Materials and Methods

A. Animals and Tumor Cell Lines

All mice were maintained at the University of Alberta Health Sciences animal center. Breeding stock for the inbred strains of mice listed was originally obtained from the Jackson Laboratories, Bar Harbor, Maine.

Strain	*H-2 haplotype*
CBA/J	k
CBA/CaJ	k
BALB/cCr	d
(BALB/cCr x DBA/2J)F1	d
(C3H/HeJ x DBA/2J)F1	k/d
(CBA/CaJ x DBA/2J)F1	k/d
(CBA/J x DBA/2J)F1	k/d
(CBA/CaJ x BALB/cCr)F1	k/d
DBA/2J	d
C57Bl/10J	b

Outbred (Swiss or ICR) mice were sometimes used for costimulator preparation.

BALB/cCr (nu/nu) mice were obtained from the Basel Institute for Immunology, Switzerland. Mice were used between 5 and 16 weeks of age. Within an experiment they were of the same sex.

Tumor cell lines P815 (H-2d mastocytoma) and EL4 (H-2b lymphoma) were maintained by passage in tissue culture.

B. Tissue Culture Medium and Conditions

Minimal essential medium (MEM), RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Flow Laboratories, Rockville, Md. RPMI 1640 was supplemented with 5 x 10^{-5}M 2-mercaptoethanol, 20 mM sodium bicarbonate, 0.34 mM pyruvate and 0.02M Hepes (Sigma, St. Louis, Mo., Cat# H-3375) pH 7.3, and made up to 950 ml (medium RHM). For 9.5% serum-containing medium (RHFM), 100 ml heat inactivated (56°C for 30 min) FBS was added. MEM was prepared in a similar manner (serum-free, MHM; with 9% FBS, MHFM), with 1 x 10^{-5} M 2-mercaptoethhanol, 36 mM sodium bicarbonate, 0.43 mM pyruvate and 0.025 M Hepes. Both media contained 40 microgram/ml gentamycin sulfate (Garamycin, Schering Corp. Ltd., Pointe Claire, Quebec), and 50 microgram/ml Penicillin G potassium (10 x 10^4 I.U./950ml, Ayerst Laboratories, Montreal, Quebec). Media were sterilized by Millipore filtration. RPMI 1640-containing cultures were incubated in a humidified atmosphere of 5% CO2 in air at 37°C; 10% CO2 was used with MEM. The two types of tissue culture media gave similar results, although responses tended to be higher using RPMI.

C. Preparation of Spleen, Thymus, Lymph Node and Peritoneal Cells

Mice were killed by cervical dislocation. Solid lymphoid organs were removed into chilled (4°C) tissue culture medium, minced with small scissors and teased

through a stainless steel grid. Clumps were allowed to settle out for 5-10 min, and the supernatant transferred to a fresh 17 x 100 mm plastic tube. The cells were pelleted by centrifugation at 750 x g in an IEC bench centrifuge at room temperature for 8 min. Cell pellets were resuspended in an appropriate medium. and viability determined with 0.1% w/v eosin Y. To obtain peritoneal cells, untreated mice were killed and injected intraperitoneally with 5 ml of cold PBS (without Ca^{2+} or Mg^{2+}). The abdomen was massaged and the fluid removed through a small incision using a Pasteur pipette. Cells were washed 3 times and irradiated (2000 rad with a Cs^{137} source) before use.

D. Assay for Costimulator

Thymic lymphocytes cultured under dilute conditions (<1 x 10^6/ml) respond poorly to Con A unless exogenous costimulator is added to the medium (Paetkau *et al.*, 1976). This system provides a quantitative assay for costimulator, since over a certain range of factor concentration, there is a direct relationship between the amount of costimulator added and the rate of cell proliferation in response to Con A (see Fig 5). For the standard assay, CBA/J thymocytes were seeded in round-bottomed Linbro 96-well microtitre trays (Flow) at a density of 0.5 x 10^6/ml in RHFM or MHFM. The total volume of 0.1 - 0.2 ml included the costimulator to be tested and Con A at a concentration of 3 microgram/ml. Cultures were incubated for 67-72 hours at 37°C, and pulse

labelled during the final 3-5 hours by adding 0.01 ml of 50 or 100 micromolar ^{3}H-dThd with a specific activity of 300 - 750 cpm/pmole, to make the contents of the cultures 5 micromolar in added dThd. ^{3}H-dThd (20ci/m mole) was purchased from New England Nuclear, and diluted with the appropriate amount of unlabelled carrier. Cultures were harvested onto glass fiber filters using a Titertek multiple sample harvester (Flow). The harvesting medium was 0.9% saline or distilled H2O, which gave the same results as washing with 5% TCA. Filters were dried and assayed for radioactivity in a liquid scintillation system. The results are expressed as pmole dThd incorporated per 10^6 starting cells per hour, to facilitate comparison between experiments. Even where it isn't indicated in figures and tables, the label "costimulator" or "costimulator activity" refers to pmoles dThd/10^6 cells/hr.

E. Large-Scale Preparation of Costimulator for General Experimental Use

Spleen cells from CBA/J, CBA/CaJ, BALB/cCr or Swiss mice were cultured at a density of 10-12 x 10^6 cells/ml in serum-free medium (RHM or MHM) containing 1.5 microgram/ml Con A (Calbiochem., San Diego, Ca. Cat. #234567). Cultures (200-250 ml) were set up in tissue culture bottles (Blake, surface area 250 cm^2), and incubated for 18-22 hours at 37°C. The cells were pelleted at 750 x g and discarded. The supernatant was either lyophilized, or protein containing

the factor was precipitated with (NH4)2SO4 at 80-90% saturation. The latter technique was easier to handle on a large scale, although the activity recovered after freeze drying was equivalent. The required amount of ammonium sulfate was added slowly to the culture supernatant with gentle stirring. After 15 hours at 4°C, the precipitate was collected by centrifugation for 15 minutes at 10,000 x g and redissolved in a small volume of H2O. The partial purification of costimulator by gel chromatography and isoelectric focusing is described in Results. Factor which was used in routine biological experiments, however, was usually subjected to G-100 Sephadex chromatography to give what is referred to in the text as fraction 3, followed by ion exchange chromatography on DEAE - Sephacel, the product of which is termed fraction 4 costimulator. Ion exchange chromatography gave a higher recovery of activity than isoelectric focusing, which made it more practical, although the degree of purity achieved with this method was at least 10 fold lower.

F. Gel Chromatography of Costimulator

G-25 and G-100 Sephadex were obtained from Pharmacia Fine Chemicals, Upsala, Sweden. BioRad P-100 was from Biorad. Columns were equilibrated with Buffer A: 0.05 M NaCl and 0.01 M Hepes pH 7.3. The void volume (Vo) and the total available volume (Vt) of G-100 and P-100 columns were determined with ^{3}H-labelled T7 DNA and ^{14}C-dThd

respectively. The protein markers used to calibrate the columns were bovine serum albumin (BSA,MW 67,000, Sigma cat. #A-4503), ovalbumin, chymotrypsinogen A, and RNase A (MW 44,000, 23,200 and 13,700 respectively, all from a Pharmacia calibration kit).

G. Ion-Exchange Chromatography

Preswollen DEAE-Sephacel resin (Pharmacia) was equilibrated with Buffer B: 0.05M NaCl, 0.05M Hepes, pH 7.3 and 0.2mM EDTA, and transferred to a column with 8 ml packed bed volume. Fraction 3 (G-100 purified) costimulator was applied to the column, which was then washed with Buffer B and eluted with a linear salt gradient from 0.05 to 0.35M NaCl. Fractions of peak activity (see Figure 35) were routinely pooled as fraction 4 costimulator. This method was developed by Barry Caplan, and performed routinely by Cliff Gibbs.

H. Unit of Costimulator Activity

A unit/ml of activity is defined as the concentration of costimulator required to obtain 1/3 of the maximal stimulation in the thymocyte proliferation assay. The maximal response was determined by adding a saturating amount of fraction 3 or 4 costimulator. Thus a preparation capable of 33% maximal stimulation at 1/40 dilution would have 40 units of activity/ml.

I. Sucrose Gradient Centrifugation

Linear gradients containing Buffer A and extending from 5 to 20% in sucrose were prepared in polyallomer tubes for the Spinco SW 50.1 (swinging bucket) rotor. Samples containing marker proteins and costimulator were layered on gradients in 0.1 - 0.2 ml volume. Sedimentation was carried out for 18 hours at 48,000 RPM at 4°C. Fractions (0.1ml) were collected and assayed for costimulator activity, or absorbance at 280 nm (in the case of marker containing gradients). A plot of S versus distance migrated was found to be linear over the range of interest. Values of S20,w for marker proteins taken from the data quoted in Sober (1968) were: BSA, 4.41S; ovalbumin, 3.6S; chymotrypsinogen A, 2.58S, and cytochrome c (bovine heart) 1.83S.

J. Isoelectric Focusing

Experiments were performed using a 110-ml preparative isoelectric focusing column (LKB, model 8100-1), assembled and operated according to the instruction manual. Sucrose or glycerol gradients were used for stabilization, as described in the instruction manual, with the sample incorporated into the gradient solutions. Ampholytes (Ampholine, LKB) were used at a final concentration of 1%. In experiments in which the pH range was 2.5 - 5.0, Ampholines with a pH range of 2.5 - 4 and 3.5 - 5 were mixed in a 1:1 ratio. Focusing was carried out in the cold room for 16 - 36 hours. At the beginning of the run, 460 volts were applied, which gave an

initial current of 9 mA. After 4 hours, when the current had fallen to approximately 3 mA, the voltage was increased to 600 - 700. Fifty-five 2 ml fractions were collected and their pH was measured. Aliquots of fractions were taken to assay for costimulator. For further processing of material, fractions were neutralized with 1M Tris Base to pH 7-8 and stored on ice until results of the assay were available. The appropriate fractions were pooled and dialyzed to remove glycerol or sucrose and ampholytes.

K. Protein Assays

Protein was determined by the Coomassie method (Bradford, 1976). Protein samples of 0.1 or 0.2 ml in Buffer A were mixed with 0.5 or 1.0 ml, respectively, of Coomassie reagent. The A595 was read within 1 hour, in glass cuvettes. A standard curve over the range of 2-20 microgram protein was made using BSA . The Coomassie reagent was prepared by mixing 100 mg Coomassie Brilliant Blue G-250 (Serva, Heidelberg) with 50 ml of 100% ethanol. One-hundred ml of 85% H_3PO_4 were added, and the volume made up to 1000 ml with double distilled H_2O.

L. Costimulator Generation on a Small Scale

For analysis of types of cells involved in costimulator production, spleen or mesenteric lymph node cells were cultured at a density of 10×10^6/ml in 96-well V-bottom microtitre trays (Linbro, supplied by Flow), containing 0.1

ml RHM, MHM or RHFM per well. Con A was present at 1.5 microgram/ml in serum-free cultures, and at 4.0 microgram/ml with RHFM. After 21-24 hours at 37°C, 0.075 ml of supernatant from each of 3 or 4 replicate cultures were removed using an Eppendorf pipette, pooled, and frozen at -20°C until assayed. Costimulator generation in 1.0 ml cultures was carried out in glass or plastic 17 x 100 mm test tubes (Canlab). Unless otherwise indicated, the conditions and cell density were the same as for microcultures.

M. Antisera

Rabbit anti-mouse brain serum (Cedarline Laboratories, cat. #T51) was the source of anti-Thy 1.2. Antisera directed against Ly and Ia antigens were prepared by Dr. I.F.C. McKenzie and tested for specificity and cytotoxic titre using the appropriate mouse strains. The antisera were shipped on dry ice and stored at -80°C until use. The mouse strains used for preparation of the antisera are given in Table 5.

Table 5. Generation of Antisera to Ly and Ia Antigens.

Antiserum	*Donor*	*Recipient*
466 anti-Ly 1.1	B6.Ly-1(a)	(129 X B10.C-H-3(c)) F1
458 anti-Ly 1.1	B6.Ly-1(a)	(B10.AKM X 129) F1
678 anti-Ly 2.1	CE	C57BR/cd
782 anti-Ly 4.1	CXBK	(B6-C-H-2(d) X CXBG) F1

918 anti-Ly 5.1	A.SW	(DA X SJL) F1
922 anti-Ly 6.1	C3H	(C3H.B6 X B6) F1
754 anti-Ly 7.2	CXBK	(B6.C-H-2d X CXBG) F1
701 anti-Ia(k)	A.TL	(A.TH X B10.S) F1

The production of these antisera has been described (McKenzie and Potter, 1979).

N. Use of Antisera

Sera were diluted in L-15 medium (Gibco), pH 7.0, containing 0.1% gelatin and Millipore filter-sterilized. The spleen cells to be treated were resuspended in the diluted antiserum at a concentration of 20 x 10^6/ml and incubated for 30 minutes in a 37°C water bath. Cells were pelleted by centrifugation at room temperature, immediately resuspended in filter sterilized complement, and incubated for 45 minutes at 37°C. The cells were then washed at least twice. The number of viable cells remaining after treatment was determined by eosin dye exclusion. In the experiment reported in Fig. 29 only, treated cells were adjusted to equal the number of cells in untreated or NMS treated controls. Otherwise the number of viable treated cells added to cultures was that which remained after treatment with antiserum and complement.

The source of complement was agarose adsorbed, 1/6 diluted, guinea pig serum (Gibco, cat. #919) in the case of treatment with anti-Thy 1.2, and selected normal rabbit

serum diluted 1/5 with all other antisera. Agarose adsorption was done on guinea pig serum which had been diluted 1/3 with saline. 100 mg agarose (Indubiose, Gallard-Schlesinger Chemical, Carle Place, N.Y.) was slowly added to 30 ml of complement with gentle magnetic stirring. The mixture was kept on ice and stirred for 45 minutes, and the agarose removed by centrifugation and Millipore filtration. Complement was stored at -80°C and was only thawed once.

O. Generation and Assay of CTL

Spleen or lymph node cells were cultured for 5 days in RHFM or MHFM with Gamma-irradiated (2000 rad) stimulator cells. Cultures were either in microtitre trays (round or V bottom) containing 0.20-0.22 ml, or in 12 x 75 mm plastic tubes containing 1.0 ml. For the assay, 4 serial 1/3 dilutions were made in V-bottom microtitre trays for each of 4 replicate cultures, and target cells labelled with ^{51}Cr (New England Nuclear) were added to the wells. P815 targets were used at 3 x 10^4/well, and EL4 targets at 2 x 10^4/well. After incubation for 4.5-6 hours, half the supernatant (0.1ml) was removed and the amount of radioactivity released by target cells determined by liquid scintillation or gamma counting. The fraction of ^{51}Cr specifically released (F) was calculated as follows:

$$F = \frac{\text{test culture cpm - background release cpm}}{\text{total lysis cpm - background release cpm}}$$

The total cpm was determined by adding 1/2000 (v/v) "Zap - Isoton" (Coulter Electronics, Hialeah, Fla.). For a few experiments, the data are expressed simply as % specific ^{51}Cr release (F X 100). In most cases the data have been processed to give a parameter, KA (Killer Activity), which is proportional to the number of cytotoxic lymphocytes in a culture. The parameter KA is essentially derived from the relationship suggested by Miller and Dunkley (1974): $F=1-\exp(-N \bullet KA \bullet t)$ where N = the number of viable cells in a culture being assayed, t = time of assay in hours, and KA is a complex parameter proportional to the number of cytotoxic lymphocytes. KA is equivalent to the "alpha" parameter defined by Miller and Dunkley, except that N is the number of starting cells represented in a given assay well, and not the number of viable cells on the day of assay. A computer program devised by Dr. V. Paetkau was used to obtain values for KA, using linear regression analysis. Figure 34 shows ^{51}Cr release data from a typical experiment, together with values for KA derived from the data. The limit of sensitivity was taken to be 2% specific release (F = 0.02); lower values of F are given as < KA corresponding to 2% release.

P. Purification of T Cells on Nylon Wool

Mesenteric lymph node cells were first filtered through glass wool (packed in a 10 ml syringe up to the 5 ml mark), which removed most dead, but no more than 5% of viable

cells. Cells were then put on nylon wool columns, incubated at 37°C, and eluted as described by Julius *et al.* (1973) or according to a modification of that technique (Schwartz and Paul, 1976). Columns for 10^8 or fewer cells contained 0.6-1.0 g nylon wool (Fenwall Laboratories, Morton Grove, Ill., code number 4c 2906); up to 4×10^8 cells were applied to columns containing 2.5 g nylon wool. The medium used was MHM or RHM with 5% FBS. Cells recovered from the column were > 95% Thy 1.2 bearing, and represented 30 - 46% of the starting cell number.

Q. UV Irradiation of Cells

Spleen cells were irradiated in 32 mm diameter petri dishes containing 10×10^6 viable cells in 1 ml. The dish, lid off, was placed under a UV germicidal lamp (General Electric, 15 watt bulb). The distances from the UV source and the times of exposure are given in the Figure legends.

R. Glutaraldehyde Fixation of Cells

Cells were resuspended in 1 ml RHM or MHM; an equal volume of glutaraldhyde-containing medium was added. Cells were incubated for 3 minutes at room temperature, washed 3 times to remove glutaraldehyde, and resuspended in serum-containing medium.

S. Gamma Irradiation of Cells

Cells were irradiated in a ^{137}Cs source (Gamma cell 40, Atomic Energy of Canada Ltd.) in 17 x 100 mm plastic tubes at room temperature. The dose rate was approximately 100 rad/min. Unless otherwise indicated, the dose of irradiation given to cells was 2000 rad.

T. Responses to PHA and LPS

Cells were cultured in round bottom microtitre trays in MHFM or RHFM. PHA (Wellcome Laboratories) or LPS (Lipopolysaccharide w from *E. coli* strain 026:B6, Difco, cat. #3121-25) were added at the appropriate concentrations, with or without costimulator. Cultures were harvested and proliferative responses determined exactly as described for the response to Con A in Section D.

U. Generation and Assay of Antibody Secreting Cells

The culture system described by Mishell and Dutton (1969) was used. Spleen cells were cultured in 10 x 35 mm dishes (Costar) for 4 days (approximately 96 hours) with 0.02% v/v thrice washed SRBC, in a total volume of 1 ml. The dishes were rocked on a Bellco rocking platform at a rate of 6 - 7 oscillations per minute. Quadruplicate cultures were fed daily with 0.1 ml of a nutritional cocktail containing the following: a) 35 ml MEM (prepared by dissolving 9.8 g MEM/950 ml H2O), b) 5 ml of essential amino acids (50x, Gibco) c) 2.5 ml of nonessential amino acids (100 x, Gibco),

d) 2.5 ml of 200 mM glutamine (Gibco), e) 7.5 ml of a solution containing 7.5% w/v NaHCO3 and 6.7% w/v dextrose. FBS was added to a final concentration of 30%. The cocktail was aliquoted and stored at -20°C until use. Antibody secreting cells were assayed by the plaque assay of Cunningham and Szenberg (1968). For each culture, an assay mixture (0.17 ml) was prepared containing an aliquot of cells, 3% v/v SRBC and 0.05% guinea pig serum (complement) in MEM. The mixture was pipetted into a glass slide chamber. Plaques were counted after 1 hour incubation at 37°C.

V. TNP-Modification of cells

TNP modification of spleen or P815 cells was carried out by resuspending them in 10 ml PBS containing 2.25 mg/ml TNP-picryl sulfonic acid (2,4,6, Sigma cat. number P5878) which had been adjusted to pH 6.8 with 1M NaOH. The cells were incubated for 10 min at 37°C in a water bath, and washed 3 times before culturing. Cells used as targets in CTL assays were labelled with ^{51}Cr before TNP-modification.

III. Partial Purification and Molecular Characterization of Costimulator

A. Introduction

The lymphokine activity called costimulator was first detected in tissue culture medium in which murine thymocytes had been cultured at high cell density ($8x10^6$/ml) with the mitogenic lectin Concanavalin A (Con A) for about 24 hr (Paetkau, Mills, Gerhart and Monticone, 1976). At low cell density ($2x10^6$/ml) murine thymocytes gave a low proliferative response to Con A, whereas when cultured at high cell density, they responded well. The criteria of responsiveness were the rate of DNA synthesis at 68-72 hours of culture, and the increase in cell number.

If "conditioned" medium taken from the high cell density cultures after 24 hr was added to low cell density cultures, they, too, gave a strong proliferative response at 72 hr. Control experiments showed that the conditioned medium did not promote a response unless a mitogen was also present. Con A was necessary in the high density cultures to induce production of the active ingredient in the medium.

The activity in the medium which synergized with Con A to allow the proliferation of thymocytes cultured under dilute conditions in response to Con A was referred to as costimulator. It appeared to be a requirement for mitogen-induced proliferation of thymocytes, not only to Con A, but to PHA, another T cell mitogen. It was postulated

that thymocytes cultured at low cell density did not proliferate because the level of costimulator in the tissue culture medium was limiting. This idea was supported by the finding that the more mature splenic T cells, which respond to Con A under extremely dilute conditions ($\leq 10^4$/ml), produce costimulator in much greater amounts than thymocytes.

Since the T lymphocyte response to mitogens is a convenient model of the proliferative phase of immune induction, it was considered relevant to further investigate the nature of costimulator activity, and how it acts to promote the proliferation of thymocytes.

The initial experiments were directed toward purifying costimulator activity from the tissue culture medium , using standard biochemical techniques such as gel filtration and isoelectric focusing. An experimental value for the molecular weight of costimulator activity was determined using the method described by Siegel and Monty (1966) for proteins in impure systems. This involved determining: a) the partial specific volume (∇), which was done by Dr. Paetkau (Shaw *et al.*, 1978a), b) a value for S20,w, and c) the hydrodynamic radius, a, from gel filtration on calibrated columns. Molecular weights estimated from gel chromatography alone are accurate only for proteins of the same symmetry and hydration as those used for the calibration. Although molecular weight estimates by this method are considered to be accurate within 10% for globular

proteins, they can be much less reliable for asymmetric or carbohydrate-containing proteins (Andrews, 1964).

Other physical properties of costimulator, such as its thermal stability, sensitivity to protease, isoelectric point, and adherence to anti-Ia immunoadsorbants were also investigated.

B. Results

Source of Costimulator used in These Experiments

The optimal conditions for costimulator production are discussed in Chapter IV. The material used in these physical studies was generated in bulk cultures from mouse spleen cells stimulated with Con A for 18-24 hrs as described in section C of Chapter II (Materials and Methods), and illustrated in Figure 4. The cell-free supernatant from such cultures was concentrated, and usually run over a G-25 Sephadex column to remove salts, Con A, and some low molecular weight inhibitory material before proceeding with any of the steps discussed below. The costimulator activity eluted in the void volume of the G-25 column, with the bulk of the protein (data not shown). It was concentrated by lyophilization and redissolved in the smallest possible volume of distilled water. This is referred to as fraction 2 costimulator.

The Thymocyte Proliferation Assay is a Quantitative Assay for Costimulator

The routine assay for costimulator used throughout

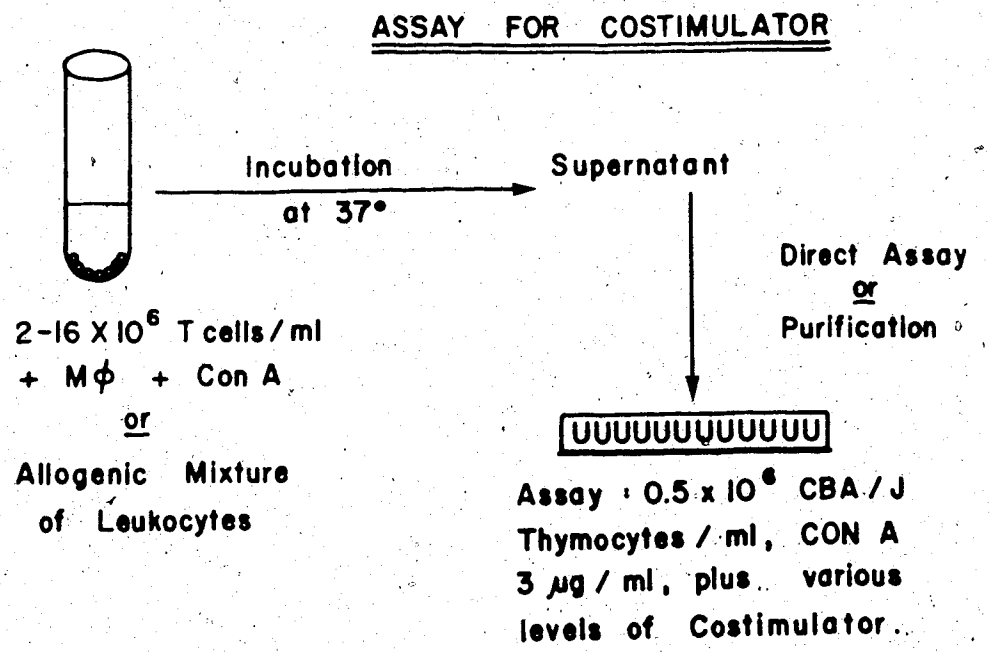


Figure 4. Schematic diagram of production and assay of costimulator.

purification studies is based on the inability of dilute mouse thymocytes to proliferate in response to Con A unless exogenous costimulator is added to the medium. The assay is fully described in Materials and Methods, section D. Briefly, CBA thymocytes were cultured at 0.5×10^6/ml in medium containing Con A for about 72 hrs in microtitre wells to which were added the samples of costimulator to be assayed (Figure 4). The cultures were pulsed with ^{3}H-dThd during the final 3-5 hrs to provide a quantitative estimate of the rate of DNA synthesis in the test thymocyte population (Gerhart *et al.*, 1976). The rate of DNA synthesis at 72 hrs correlated with an actual increase in cell number relative to that originally seeded in cultures (Mills *et al.*, 1976). This assay will be referred to as the thymocyte proliferation assay.

This system provides a quantitative assay for the amount of costimulator in a given preparation. Figure 5 shows that over a certain range, there is a direct correlation between the amount of costimulator added to the assay (dilution) and the level of activity observed. In a typical dilution curve (Figure 5) a maximal level of thymocyte proliferation is observed at some dilution of costimulator, in this case 1/40. If more costimulator is added, the activity appears to be reduced. This inhibitory effect is even more pronounced in Figure 6, which shows dilution curves for costimulator preparations at various stages of purification, here plotted on a logarithmic scale.

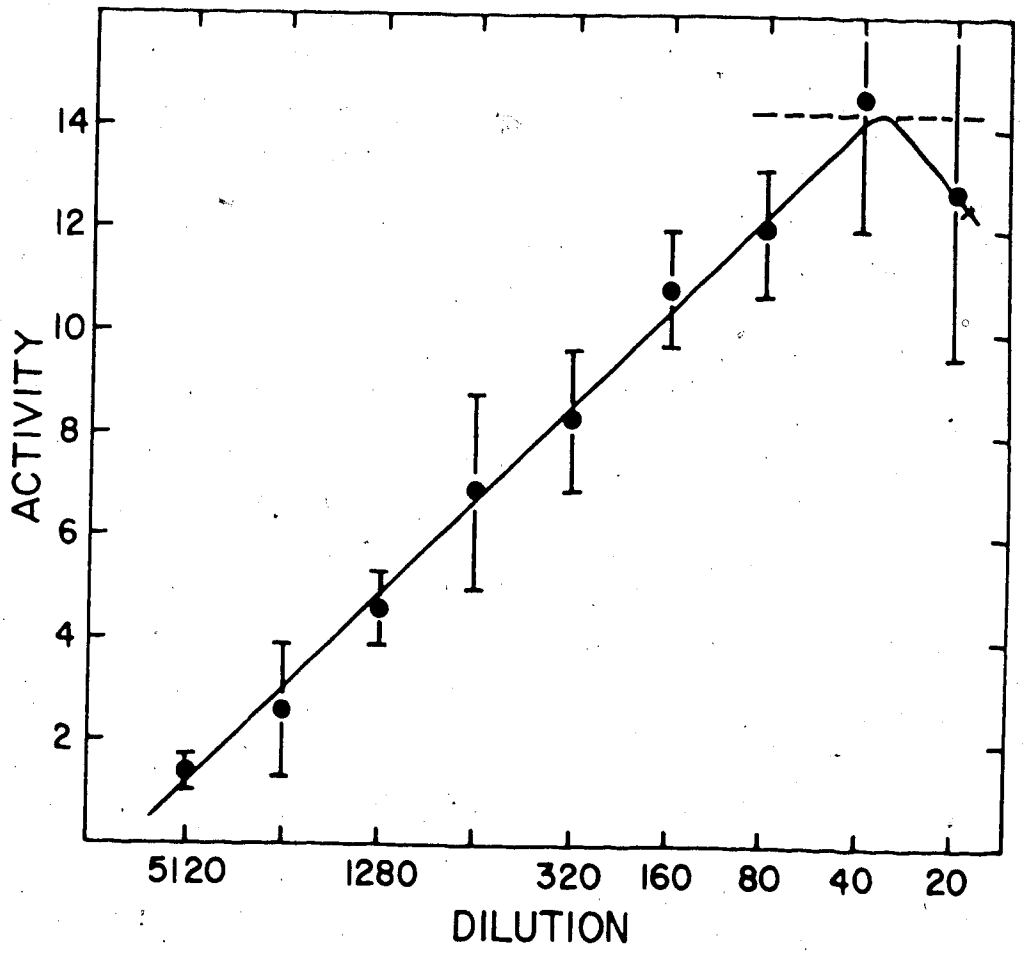


Figure 5. Assay for costimulator activity.
Fraction 3 costimulator from CBA/J mice was used to stimulate mitogenesis in cultures containing $1x10^5$ CBA/J thymocytes and 3 microgram/ml Con A as described in Materials and Methods. Dilution of costimulator ranged from 1/20 to 1/5120. Costimulator activity (on the ordinate) is expressed as pmoles dThd incorporated/10^6 starting cells/hr. Each point represents the mean of 6 replicate cultures; error bars indicate standard deviations.

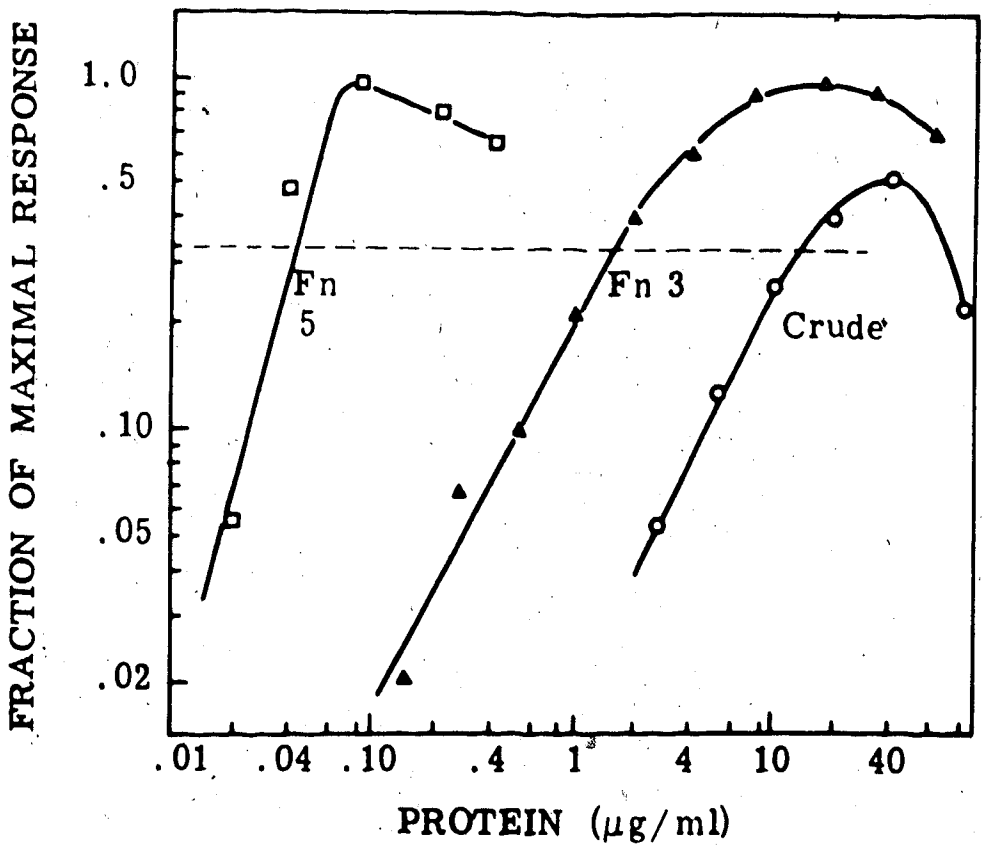


Figure 6. Titration of costimulator at various stages of purification in the thymocyte proliferation assay. The protein contents of the dilutions of crude, fraction 3, and fraction 5 used in the assay are shown on the abscissa. The maximal response was determined by adding a saturating amount of a standard preparation of fraction 3 costimulator. A unit of activity is defined as the concentration of factor giving 1/3 of the maximal response. Points represent the means of 6 replicate cultures.

The inhibition is not completely abated by further purification. It may be due to contaminating inhibitory factors, or the addition of excessive amounts of costimulator itself.

Using this quantitative assay, it was possible to monitor the recovery of costimulator activity after every purification step, and thus to estimate specific activities. As mentioned in Chapter I, all physical studies are based on biological activity as a marker, since no protein has been physically isolated and shown to possess costimulator activity. The unit of activity (Materials and Methods), is defined as the amount of costimulator which will induce 1/3 of the maximal response in 1 ml of cultured thymocytes. The fraction 3 costimulator titrated in Figure 5 contains about 1280 units/ml activity.

Gel Chromatography

The material recovered from G-25 Sephadex chromatography was applied to a G-100 Sephadex column (Figure 7), which had been calibrated with protein markers. Fractions were assayed for activity, and absorbance (A280). Most UV absorbing material was eluted in the void volume, but the biological activity emerged from the column at Kd (distribution coefficient)=0.22-0.23. Comparison with protein markers showed the activity to reside in molecules of slightly higher Stokes radius than ovalbumin (O; Figure 7) which has a molecular weight of 44,000. The peak fractions from a G-100 columns were pooled to give material

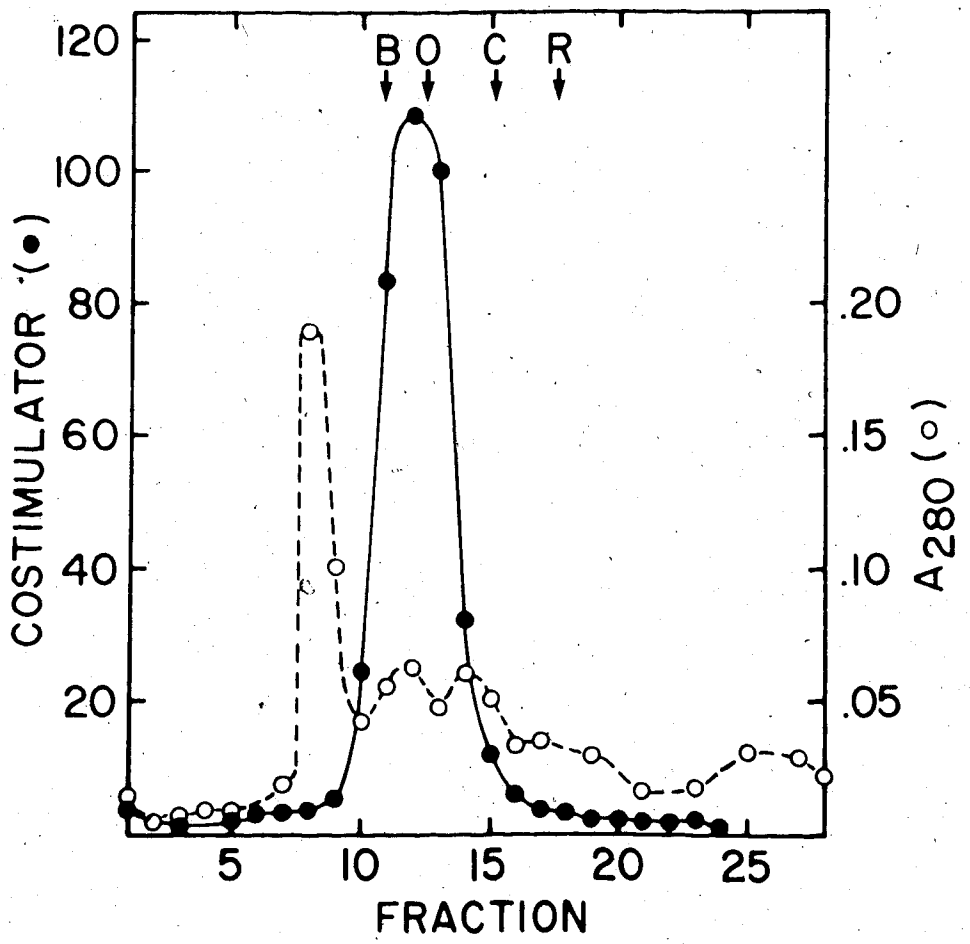


Figure 7. Chromatography of fraction 2 costimulator on G-100 Sephadex. Pooled peak fractions from a G-25 Sephadex column (fraction 2) were concentrated and applied to a 1.5 x 90 cm column of G-100 Sephadex which was equilibrated with Buffer A at 4°C. Five ml fractions were collected at a rate of 16 ml/hr, and analyzed for absorbance (A280;○), and costimulator activity at 1/30 dilution (•). This dilution gave responses for peak fractions which were on the linear portion of the dilution curve. The peak of absorbance corresponds with the void volume of the column (Vo). The total volume available for fluid in the column, (Vt), was estimated by the position of ^{14}C-dThd (fraction 26). The elution positions of protein markers are indicated by arrows in the figure. B: bovine serum albumin; O: ovalbumin; C:chymotrypsinogen A; R: ribonuclease A. Fractions 11-13 were pooled as fraction 3.

referred to as fraction 3 costimulator.

It was considered possible that costimulator might adsorb to Sephadex beads, especially if it were a glycoprotein. Therefore, the material was also analyzed on another gel filtration medium, Biorad P-100, which consists of polyacrylamide beads (Figure 8). The Kd using P100 was about 0.11, but again, the position of the peak of biological activity was close to that of ovalbumin, this time appearing to be slightly smaller. Thus the observed hydrodynamic radius was not an anomaly due to interactions with the Sephadex matrix.

Isoelectric Focusing

Isoelectric focusing was carried out on fraction 3 costimulator to determine its isoelectric point, and as a further purification step. Costimulator samples were applied to a 110-ml sucrose (or glycerol in some experiments) gradient containing ampholytes designed to give pH gradients over various ranges as described in Materials and Methods. Initially, a pH range of 3.0-10.0 was employed (Figure 9). Fractions were assayed for pH and for activity in the thymocyte proliferation assay before dialysis. The activity banded at around pH 4.

Subsequently, costimulator was focused over pH ranges 2.5-5.0 (Figure 10) or 3.0-6.0 (Figure 11). The results shown in Figure 10 indicate a heterogeneity in pI, with a characteristic peak of activity at around pH 3.8, and activity trailing to nearly pH 4.7. The trailing material

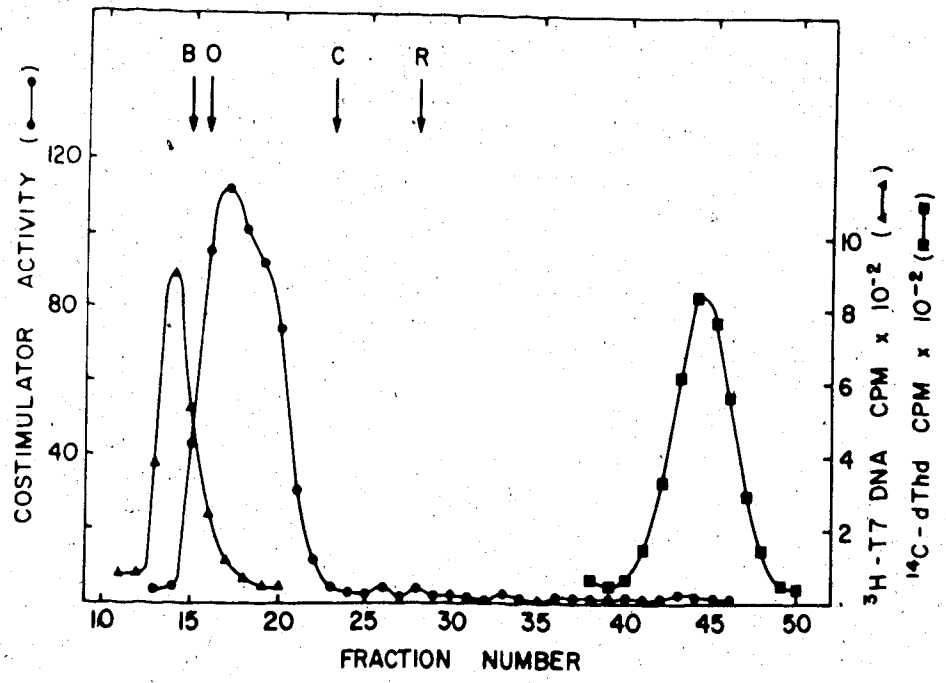


Figure 8. Chromatography of fraction 2 costimulator on BioGel P-100. Costimulator was applied to a 1.5 x 90 cm column of P-100. The conditions were the same as in Figure 7. Vo and Vt were determined using ^{3}H-T7 DNA and ^{14}C-dThd respectively, as markers. Fractions were 2 ml. The position of protein markers used to calibrate the column, B (bovine serum albumin), O (ovalbumin), C (chymotrypsinogen A) and R (ribonuclease A), are indicated by arrows.

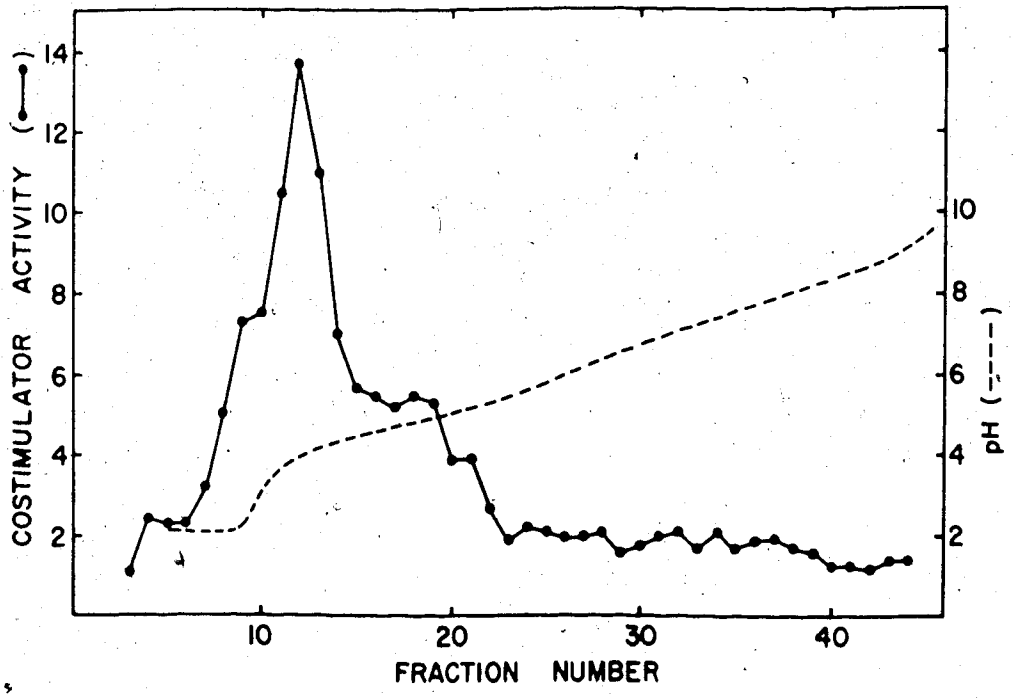


Figure 9. Isoelectric focusing of fraction 3 costimulator over a pH range of 3-10. A preparative isoelectric focusing column was filled with a 100 ml sucrose gradient containing the appropriate ampholytes for a pH range of 3-10; and a 20 ml sample of CBA/CaJ fraction 3 costimulator (12,000 units of activity). Focusing was carried out for 36 hrs at 300 volts. Two-ml fractions were collected and analyzed for pH, and costimulator activity in the thymocyte proliferation assay. The profile shown represents a 1/40 dilution of the fractions. The background activity (no added costimulator) was 2.0. The maximal level in the assay was 21.

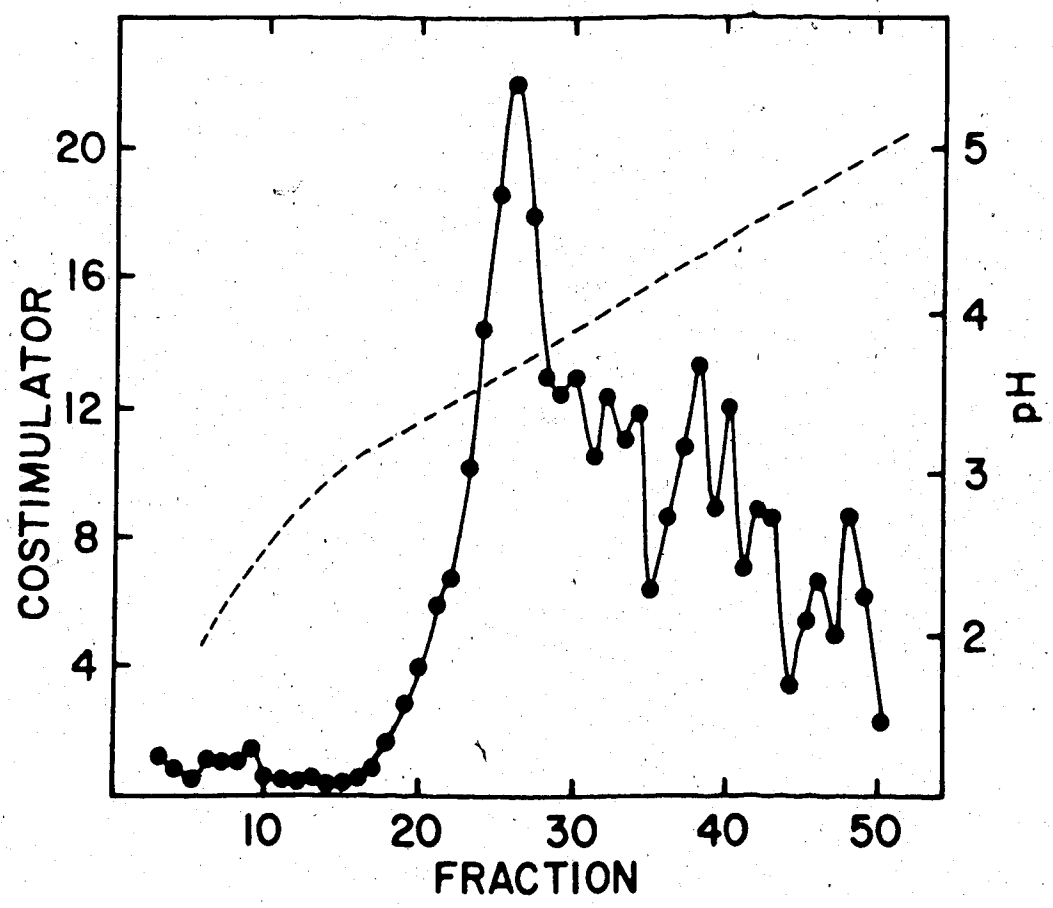


Figure 10. Isoelectric focusing of fraction 3 costimulator over the pH range of 2.5-5. Preparative isoelectric focusing was done as described in the legend to Figure 9, except that focusing was done at 460 volts, and the pH range was 2.5-5.0. The sample was 12,000 units of CBA/CaJ fraction 3 costimulator. Two-ml fractions were assayed for pH, and for costimulator activity in the thymocyte proliferation assay at 1/200 dilution. The background activity, with no costimulator added, was 0.5. Fractions 22-29 were pooled, dialyzed against Buffer A, lyophilized, and dissolved in water as fraction 4 costimulator. pH (---); costimulator activity (•).

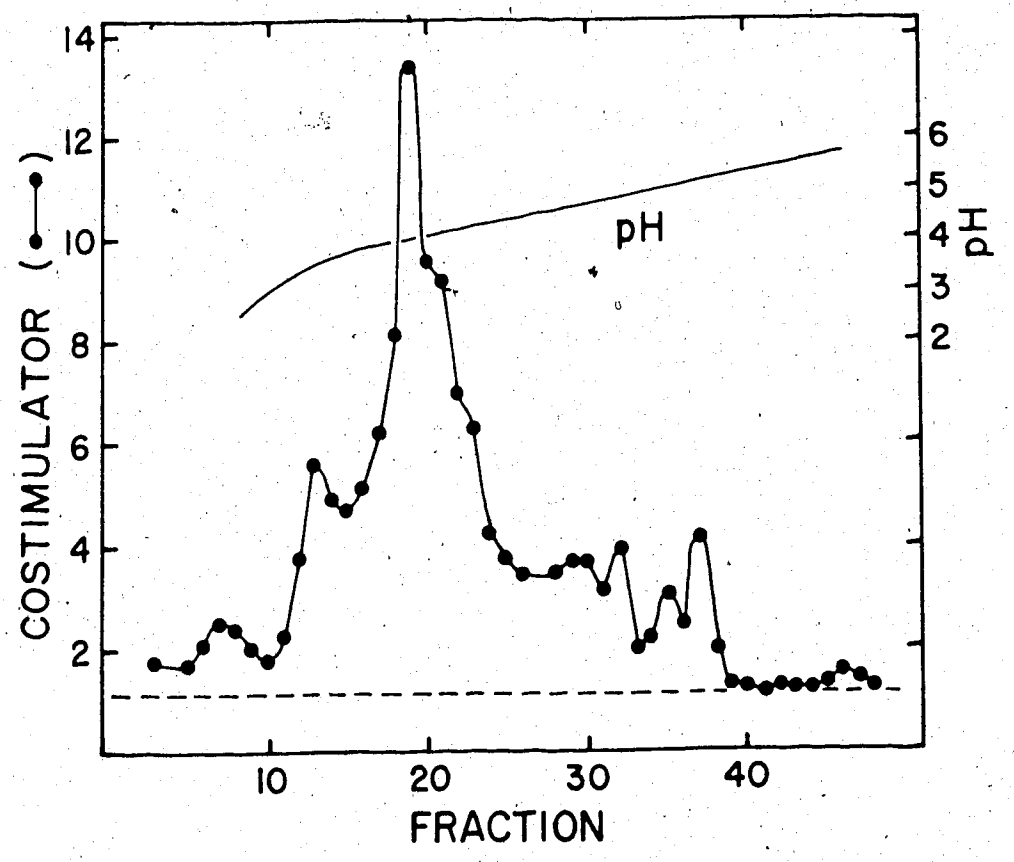


Figure 11. Isoelectric focusing of fraction 3 costimulator over the pH range of 3.0-6.0. Focusing and assay of fractions in the thymocyte proliferation assay were done exactly as described in Figure 9. Costimulator activity at 1/40 dilution (●).

was more evident in some experiments than others (compare Figures 10 and 11).

The patterns in Figures 10 and 11 were obtained by focusing in sucrose gradients, for 36 hours at 4°C. The conditions were varied slightly (Figure 12), to determine whether better recovery of input activity was obtainable. It can be seen that there is not much difference in the activity profiles observed whether the focusing was carried out for 36 (a) or 16 (b) hrs. The use of glycerol (Figure 12) rather than sucrose (Figures 10 and 11) for the support medium also seemed to have little effect.

Figure 12 also shows that most of the protein is separated from the activity using IEF. There is no protein peak corresponding to the activity peak. A total of 15 mg protein were applied in both a and b, containing approximately 3200 units of activity. Two pools of activity, A (low pI material) and B (high pI material) were made from profiles a and b. The pooled material was titrated in the thymocyte proliferation assay and the total recoveries calculated:

a, pool A 490 units (15%)

a, pool B 1048 units (33%)

b, pool A 400 units (12.5%)

b, pool B 1000 units (31%)

Thus it can be concluded that 45-50% of the input activity is recoverable, and that 12-15% of this is in the main (3.8-4.0) peak.

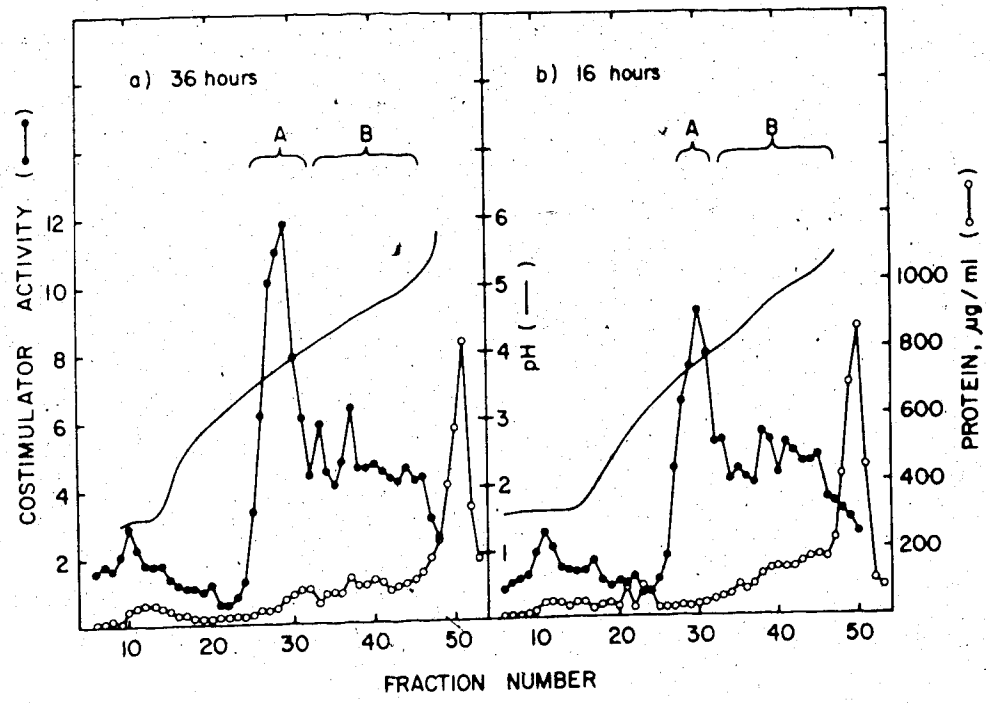


Fig. 1. Isoelectric focusing of fraction 3 costimulator over a pH range of 2.5-5.0 for (a) 36 or (b) 16 hours. A preparative isoelectric focusing column was filled with a 100 ml 0-50% glycerol gradient, containing the appropriate ampholytes for pH range of 2.5-5.0, and the sample, 3200 units of fraction 3 costimulator from CBA/CaJ mice (15 mg protein). Two successive runs were carried out, identical except for the length of time. Focusing was carried out as described in Materials and Methods. Two-ml fractions were collected and assayed for costimulator activity in the thymocyte proliferation assay at 1/100 dilution. Background activities in the assays (no added costimulator) were 1.4 (a) and 1.6 (b). Protein in the fractions was determined using the Coomassie dye method (Materials and Methods).

When the results of the assays were available, fractions were pooled according to their pI. From a, two pools were made: A (fractions 25-32) and B (fractions 33-46). Two pools were also made from b: A (28-32) and B (33-47). Enough purified BSA was added to the pools to give a final concentration of 30 microgram/ml, and they were dialyzed against Buffer A for 48 hrs to remove ampholytes. The four pools were titrated in the thymocyte proliferation assay to estimate recoveries (see text).

Pools A (low pI), and B (high pI) in Figure 12 were refocused, in order to determine whether the main peak and trailing material run true. The low and high pI peaks refocused in their original positions upon refocusing Figure 13) indicating that the pI observed is a stable property of the molecules, and not an artifact of incomplete equilibration of the pH gradient or degradation during focusing.

Sephadex G-100 Chromatography of IEF Purified Costimulator

Fraction 4 costimulator was obtained by pooling the main peak fractions of an IEF column. Fractions 22-29 from the profile in Figure 10 were pooled, and rerun on a second calibrated small G-100 column (Figure 14). This step was performed to remove ampholytes (MW ≤ 2000) and inhibitory material from the preparation, which had not been dialyzed. A second G-100 step gave better recovery than dialysis, probably because the protein solution was very dilute (about 5 microgram/ml). The material eluted in a peak at Kd=0.23. The peak was sharper than the primary G-100 profile (Figure 7), perhaps because heterogeneous forms of active molecules were removed during the isoelectric focusing procedure. Fractions 13-15 were pooled as fraction 5 costimulator.

Summary of Purification Procedures

A summary of the purification steps used, the recovery after each step, and the specific activity of the pooled fractions is given in Table 6. The most effective purification step in terms of increasing the specific

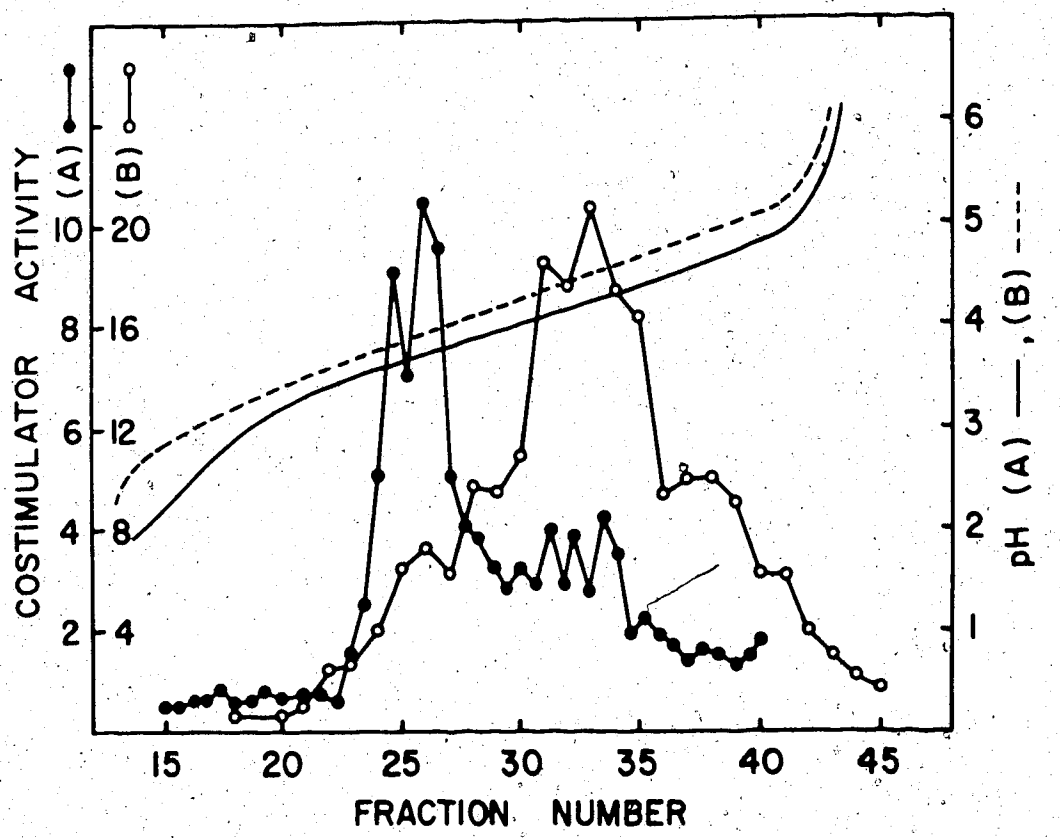


Figure 13. Refocusing of "low pI" and "high pI" costimulator activity. Pools A (low pI) and pools B (high pI) from a and b of Figure 12 were combined. Each was used as a sample in a separate isoelectric focusing column. The pH range was 2.5-5.0, and focusing was carried out for 18 hrs in glycerol gradients as described in Materials and Methods. Sample A contained 800 units, and sample B contained about 2000 units of activity. Fractions were assayed for activity in the thymocyte proliferation assay at 1/50 dilution. Profiles obtained for samples A and B are superimposed. Background activities (no added costimulator) were 2.6 (A) and 0.9 (B).

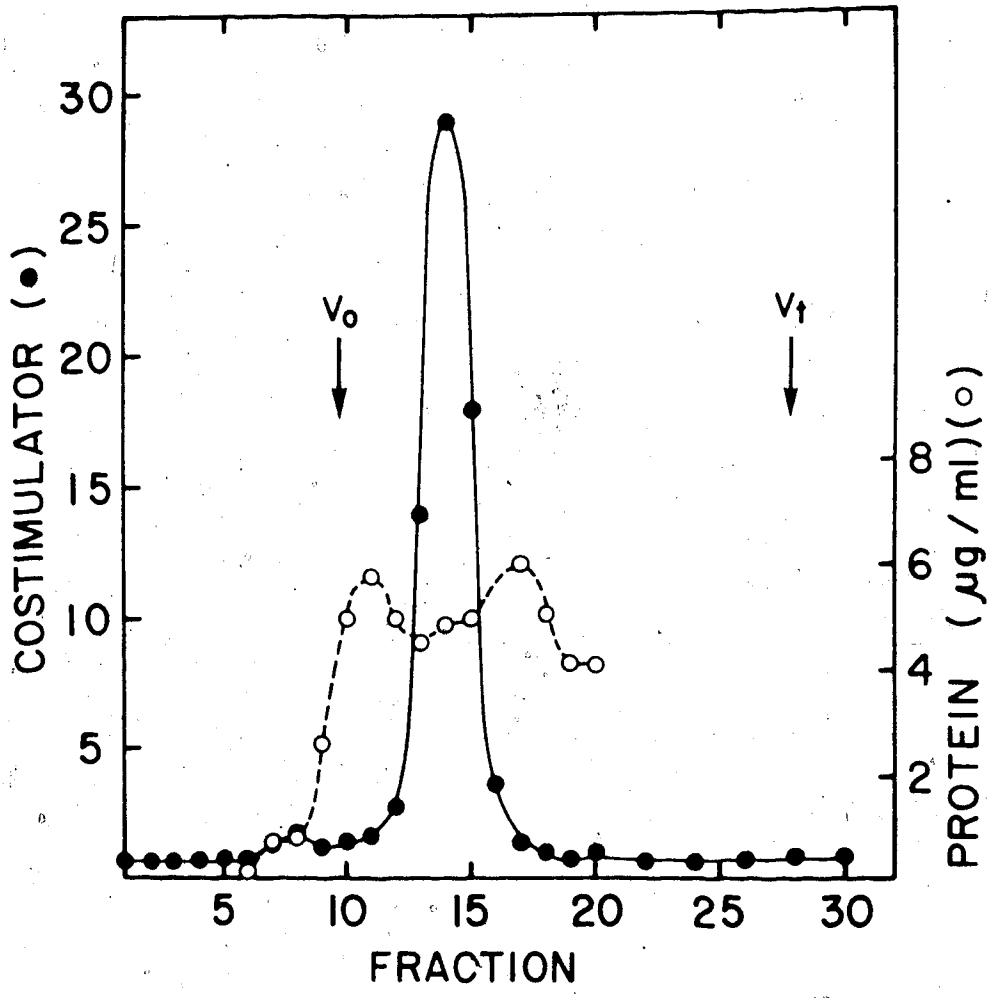


Figure 14. Chromatography of fraction 4 costimulator on G-100 Sephadex. Fraction 4 was prepared as described in Figure 10 and chromatographed on a 0.9 x 90 cm G-100 column as described in Figure 7. The flow rate was 8 ml/hr, and the fraction size was 2 ml. Fractions were analyzed for protein using the Commassie dye method (Materials and Methods), and for costimulator activity at 1/100 dilution in the thymocyte proliferation assay. The positions of excluded (Vo) and included (Vt) markers were determined as described in Figure 8, and are indicated in the figure. The background activity in the thymocyte proliferation assay was 0.6.

activity is IEF, but it is at this step that the greatest loss in activity occurs. Fraction 5 costimulator contains about 5 microgram/ml protein (Figure 14). As with IEF profiles (Figure 10) there was no protein peak coincident with the peak of activity.

Since the amount of activity in the G-100 fractions (Figure 14) did not directly correlate with their protein content, it must be concluded that an indeterminant portion of the protein in fractions 12-15 is not costimulator. However, a lower limit can be estimated for the specific activity. The costimulator assay detects a minimum of 1/10 unit of activity (Figure 6), and costimulator fraction 5 contains up to 300 units/5 microgram protein (if only the peak fraction in Figure 14 is considered). Therefore costimulator activity can be detected at a protein concentration as low as 1.7 ng/ml. Assuming a molecular weight of 30,500 for costimulator (see below), whis means that the factor can be detected at concentrations lower than 10^{-10}M.

The titration curves for costimulator at each stage of purification are shown in Figure 6. Fraction 4 costimulator, the pooled peak fractions from IEF, was not titrated, as it contains ampholytes which inhibit the assay unless diluted at least 100 fold. Figure 6 shows that all fractions are inhibitory at high concentration. However, crude material, even at its peak dilution (1/40), appears to have only about half of the maximum activity attainable in the assay with

Table 6.

Summary of costimulator purification and recoveries[1]

Procedure	Units of activity per 10^9 spleen cells[2]	Specific activity (units per microgram protein)
Crude (medium from Con A-stimulated spleen cells)	5160	.09
↓ Concentrate 20x by lyophilization		
(fraction 1)	1150	.23
↓ G-25 chromatography (data not shown)		
(fraction 2)	2170	.52
↓ G-100 chromatography (Figure 7)		
(fraction 3)	1300	1.38
↓ Isoelectric focusing pH range 2.5-5.0 (Figure 10)		
(fraction 4)	ND	ND
↓ G-100 chromatography (Figure 14)		
(fraction 5)	87	≥30

[1] See text for explanation
[2] Units of activity at each stage was determined by titration in the thymocyte proliferation assay
[3] Protein was measured by the Coomassie method (Materials and Methods).

the more purified fractions. This may result from the presence of other inhibitory material, which is probably of low molecular weight, since it is removed in the G-25 step. Thus it is difficult to estimate the true amount of costimulator activity in a crude preparation.

Sedimentation Coefficient of Costimulator

The sedimentation coefficient of fraction 3 costimulator was determined to be 2.63S (Figure 15). Costimulator and various mixtures of standard protein markers of known s value were sedimented through 5-20% sucrose gradients. Graphs of s value versus distance sedimented were constructed from the positions of marker proteins in the gradients. The s value for costimulator was estimated from these.

Estimation of Molecular Weight of Costimulator

The molecular weight was calculated according to the relationship suggested by Seigel and Monty (1966): $M = 6(pi)Nnas/1-\nu p$, where M = molecular weight, N = Avogadro's number, a = stokes radius, s = sedimentation coefficient, ν = partial specific volume, and p = density of the medium. A value for the Stokes radius (a) was experimentally obtained from gel filtration experiments described above. The Stokes radius of BSA, ovalbumin and chymotrypsinogen A determined a line whose equation was: $a \times 10^{8} = 67.15-61.56(Kd)^{1\ 3}$ (Siegel and Monty, 1966). The value of a for costimulator was estimated from this line by least squares fitting to be

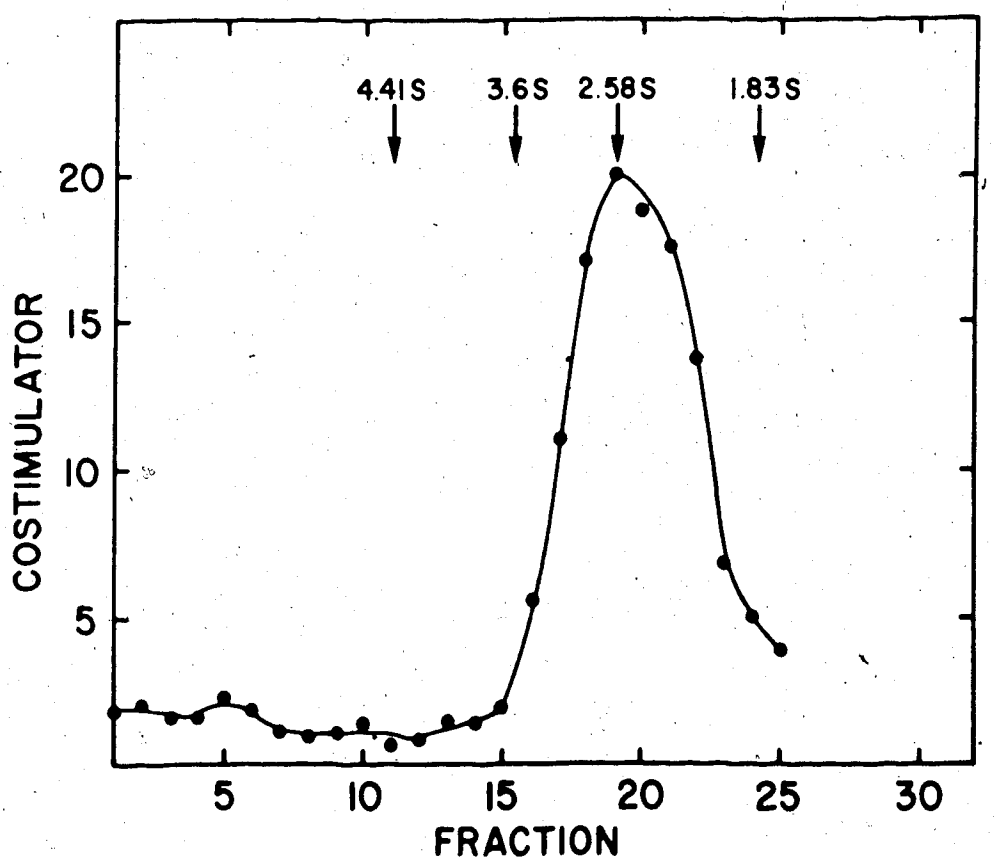


Figure 15. Sedimentation of fraction 3 costimulator in sucrose gradients. Costimulator and marker proteins were sedimented through 5-20% sucrose gradients as described in Materials and Methods for 18 hr at 48,000 rpm and 4°C. The sample was 80 units of CBA/J fraction 3 costimulator. The S values in the figure correspond to markers BSA (4.41S), ovalbumin (3.6S), chymotrypsinogen A (2.58S) and cytochrome c (1.83S). The position of marker proteins was determined by absorbance (A280). Fractions (0.1ml each) were analyzed for costimulator activity in the thymocyte proliferation assay at 1/50 dilution. The background activity in the assay was 1.2.

29.6×10^{-8}, when an average value for Kd=0.227 was employed.

The experimentally determined value for the partial specific volume (∇) used in the calculation of MW was ∇ = 0.71 ± 0.04 cm^3/g, obtained by centrifuging costimulator to equilibrium in a CsCl gradient. This work was done by Dr. V. Paetkau, and is fully described in Shaw *et al.*, (1978a).

The value obtained for M was 30,500± 4600. The rather high probable error for M is mainly due to the uncertainty in ∇ (Shaw *et al.*, 1978a). The molecular weight of costimulator is surprisingly low for a protein of Stokes radius 29.6, the expected Stokes radius of a globular protein of MW 45,000. From the data presented here, the diffusion coefficient was calculated to be 7.25×10^{-7} $cm^2 sec^{-1}$, and the frictional coefficient, f/fo to be 1.45. The physical properties of costimulator are summarized in Table 7.

Heat and Trypsin Sensitivity of Fraction 3 Costimulator

Costimulator activity was fairly heat resistant, requiring a temperature of 90°C to destroy at least half of the activity in 10 min (Table 8). Crude costimulator was more sensitive to heat, possibly due to the presence of proteases which had leaked from dead cells. Incubation with a low concentration of trypsin destroyed the activity completely (Table 9) suggesting that costimulator activity resides in protein-containing molecules.

Costimulator Activity is not Removed by Anti-Ia Immunoadsorbants

Table 7.

Physical properties of costimulator

Experimentally derived:	
Hydrodynamic radius, a[1]	$29.6 \pm 0.15 \times 10^{-8}$ cm
Sedimentation coefficient, $S_{20,W}$[2]	$2.63 \pm 0.15 \times 10^{-13}$ sec.
Partial specific volume, ν[3]	$.71 \pm 0.04$ cm^3/g
Calculated::	
Molecular weight,[4] M	30,500±4,600
Diffusion coefficient,[5] $D_{20,W}$	$7.25 \pm x0.10 \times 10^{-7}$ cm/sec^2
Frictional coefficient,[6] f/fo	1.45

[1] Uncertainty in a is based on the reliability of the standard curve (±0.4); experimental variation in a was ±0.3

[2] Uncertainty in S (±0.15S) is based on the error in predicting the S value of marker proteins from the distance sedimented in gradients.

[3] Determination described in Shaw *et al.*, 1978a

[4] $M = 6\pi N n a s/(1-\nu\rho)$

[5] $D = RT/(6\pi i N n a)$

[6] $f/fo = a/3\nu M/4\pi i N)^{1\ 3}$

Table 9.

Sensitivity of fraction 3 costimulator to trypsin[1]

Treatment	Activity (pmoles ^{3}H-dThd incorporated/ 10^6 cells/hr)
Incubation for 100 min. (no trypsin)	99
No incubation (4°C) control for trypsin)[2]	102
Trypsin 1 microgram/ml	
0 time	89
5 min	54
10 min	31
30 min	13
60 min	11
100 min	9

[1] Fraction 3 costimulator (135 units/ml) was incubated at 37°C with or without trypsin (184 units/mg; Worthington Biochemical Corp., Freehold, N.J.) for the lengths of time indicated. Samples were assayed at 67-fold dilution in the thymocyte proliferation assay, which gave control values on the linear portion of the dilution curve. Activity in the assay was 5 in the absence of costimulator.

[2] An equivalent amount of trypsin was added to the assay wells before costimulator, to control for the effect of trypsin in the culture system.

Table 8.

Heat sensitivity of fraction 3 costimulator[1]

Temperature of 10 minute incubation (degrees C)	Dilution in Assay	Activity (pmoles ^{3}H-dThd incorporated/ 10^{6} cells/hr)
4 (control)	1/100	66
40	1/100	60
50	1/100	62
60	1/100	57
70	1/100	47
80	1/100	42
90	1/100	19
4	1/50	100
4	1/200	28
4	1/400	10
no costimulator		4

[1] Fraction 3 costimulator (about 150 units/ml) was heated as indicated and assayed in the thymocyte proliferation assay. The activity of unheated costimulator at 1/100 and 1/200 shows that the assay was performed on the linear portion of the dilution curve.

Fraction 3 costimulator prepared from CBA (H-2k) spleen cells was passed through anti-Ia(k) and anti-Ia(s) columns to assess whether the factor has I region determinants. Material in the unbound fractions from each of the columns had activity in the thymocyte proliferation assay which did not differ significantly from that of control fraction 3 (Figure 16). If costimulator contained Ia determinants, the expected result would be retention of activity by the anti-Ia(k), but not the anti-Ia(s) column. The same columns used in this analysis were previously shown to remove Ia(k)-positive allogeneic effect factor (AEF; Delovitch *et al.*, 1978 and T.L. Delovitch, unpublished results).

C. Discussion

The Physical Properties of Costimulator

Data have been presented on some of the molecular characteristics of the lymphokine costimulator. The activity resides in molecule(s) of about 30,500 daltons, and a pI 3.7-4.5. Its trypsin sensitivity indicates that it contains protein. Its partial specific volume, 0.71, is consistent with it being a protein or a glycoprotein (Shaw *et al.*, 1978a). Fetuin, a glycoprotein containing 23% carbohydrate has a $\bar{v}$ of 0.70, while BSA, which contains little or no carbohydrate, has a $\bar{v}$ of 0.73. It was not possible to confirm that costimulator is a glycoprotein by any other means. Some glycoproteins, such as AEF, stick to a concanavalin A - Sepharose affinity adsorbant if they

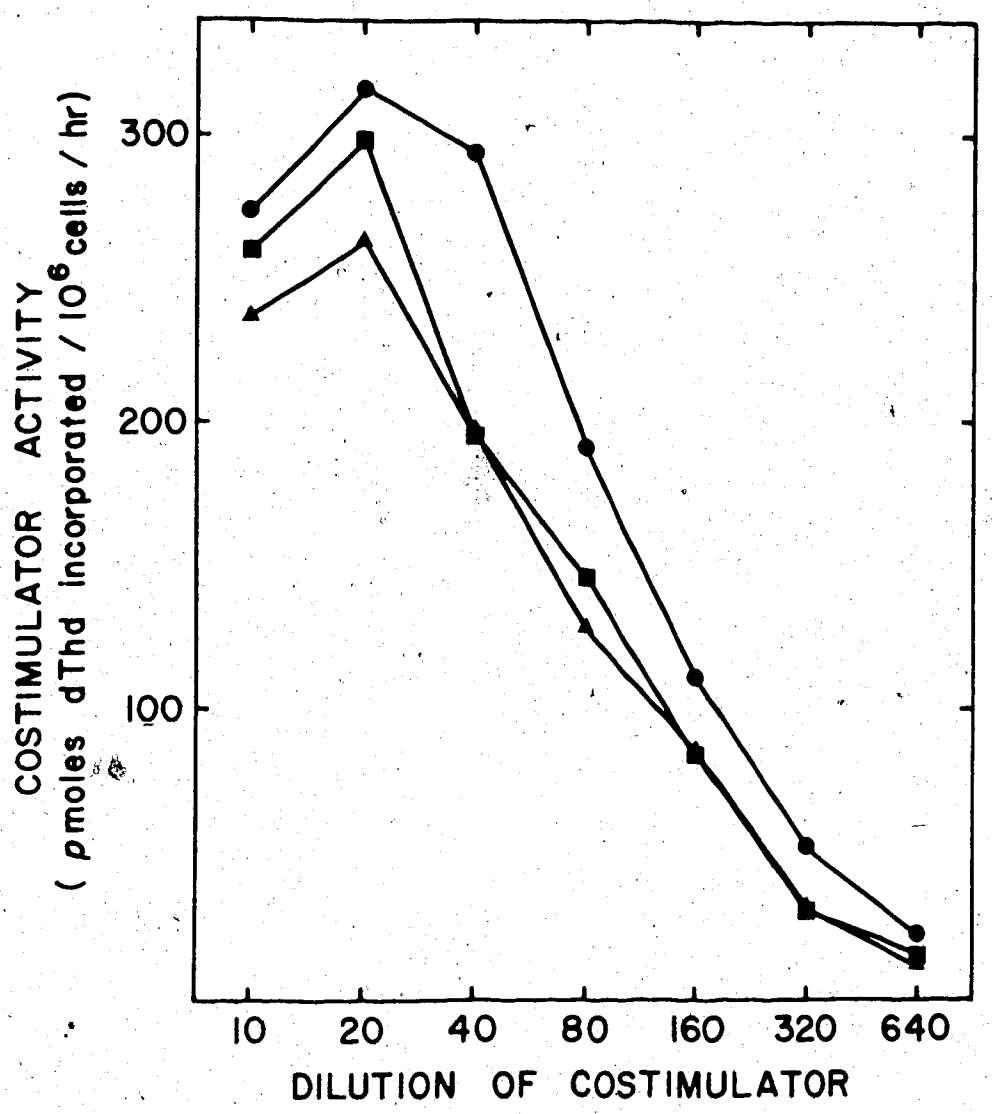


Figure 16. Assay of costimulator activity after passage over anti-Ia immunoadsorbant columns. Fraction 3 costimulator from CBA/CaJ (H-2k) mice was untreated (•), passed over anti-Ia (s)(■), or over anti-Ia(k) (▲) columns, and the eluted material tested at various dilutions in the thymocyte proliferation assay. (The anti-Ia(s) and anti-Ia(k) treated samples are aliquots of those used in Table 24, chapter V.) The passage of costimulator over these immunoadsorbant columns were performed by Dr. T.L. Delovitch at the University of Toronto.

contain[illegible]l mannose residues (Eshhar *et al.*, 1977). Costim[illegible]s not retarded by Concanavalin A - Sepharose, u[illegible]er co[illegible]ns where it bound serum glycoproteins (J. Shaw a[illegible]. P[illegible]au, unpublished observations). Unlike AEF (D[illegible]ovit[illegible] *et al.*, 1978) and many of the antigen specific help[illegible]ctors (Chapter I, section J; Table 1) costimulator does[illegible] have detectable Ia determinants.

[illegible]e molecular weight determined for costimulator, 30,50[illegible]s considerably lower than would be predicted for a globul[illegible] protein with a Stokes radius of 29.6. Ovalbumin, which h[illegible]s a Stokes radius of 27.5 has a molecular weight of 44,000. Thus on the basis of gel filtration data alone, a molecular weight of 45-50,000 would have been predicted. The behavio[illegible] costimulator upon gel exclusion chromatography may be explained by a high frictional coefficient (f/fo=1.45,Table 7). Molecular asymmetry could be responsible for the high frictional coefficient (Andrews, 1964).

Comparison of Costimulator's Physical Properties to Those of LAF (IL1)

Similar studies of hydrodynamic properties have been performed for murine LAF (Economou and Shin, 1978) and for guinea pig mitogenic factor (MF), which may be the equivalent of LAF for that species (Gately *et al.*, 1975). Their properties are compared in Table 10, along with those of guinea pig lymphotoxin (Gately and Mayer, 1974), a cytotoxic lymphokine which is released during the first 8-10

Table 10.

Comparison of molecular properties of costimulator, LAF, Mitogenic Factor, and Lymphotoxin[1]

	Factors			
Property	Costimulator	LAF[2]	MF[3]	LT[4]
Hydrodynamic (Stokes) radius a (cm, x 10^{8})	29.6	22	--	--
Diffusion coefficient, D (cm^2/sec) x 10^{7}	7.25	9.7	9.9	8.0
Sedimentation coefficient, S (10^{-13}sec)	2.63	2.0	2.4	4.2
Partial specific volume, $\bar{v}$ (cm^3/g)	.71	.72	.71	.72
pI values	3.8-4.8	4.8,5.3	--	--
Frictional coefficient f/fo	1.45	1.25	1.2	1.1
Molecular weight	30,500	18,000	20,000	45,000

[1]Adapted from Paetkau (1979).
[2]Lymphocyte activating factor (Economou and Shin, 1978).
[3]Mitogenic factor from guinea pig (Gately *et al.*, 1975). Molecular weight calculated using the Svedberg equation.
[4]Lymphotoxin from guinea pig (Gately and Mayer, 1974).

hrs of lymphocyte stimulation with mitogens such as Con A (Hiserodt *et al.*, 1979). Costimulator can readily be distinguished from LAF (MW 18,000) and MF (MW 20,000) on the basis of its higher molecular weight. The S20,w is significantly higher than for murine LAF. The pI of LAF appears to be slightly higher, but due to the heterogeneity observed in pI, the factors could not be distinguished from each other on that basis. LAF and MF have lower frictional coefficients than costimulator.

Comparison of Costimulator's Physical Properties to Those of Other IL2 Factors

Most of the IL2 factors (Table 2) have 'molecular weights' estimated to be 30-60,000 by gel filtration. Some have reported lower molecular weights than we find using gel filtration chromotography: 30-32,000 for TSF (Di Sabato *et al.*, 1975) and 35-38,000 for TMF (Simon *et al.*, 1979). The reason for this discrepancy is unclear. The Stokes radius of costimulator was determined in many experiments using calibrated G-100 columns. It should be pointed out that the Kd varied with the batch of Sephadex used, however. Every new column had to be calibrated with protein markers before use.

Direct comparison between TRF (Watson *et al.*, 1979b) and costimulator was carried out by exchanging factors at various stages of purification. TRF enables T cell depleted spleen cells to mount an AFC response against heterologous erthyrocytes *in vitro*. The two activities co-purified

through gel filtration, ion-exchange chromatography and IEF (Watson *et al.*, 1979a). Watson used a flat bed system for isoelectric focusing of TRF, in which the sample and ampholytes are mixed in a thin layer of Sephadex. In this system, TRF activity is resolved into three different peaks of pI 3.0-4.0, 4.3, and 4.9. Only the 2 peaks of higher pI had activity in the thymocyte proliferation assay (J. Shaw and J. Watson, unpublished observations).

A similar comparative study was carried out by Watson *et al.* (1979c) between TRF and T cell growth factor (TCGF), which allows CTL to continuously proliferate in tissue culture. Again, the two activities copurified, except that material of low pI which had TRF activity could not be detected in the TCGF assay. The TRF of pI 3.0-4.0 may be an antigen specific factor which acts directly on B cells, but has no effect on T cells (J. Watson, personal communication). Its activity is sensitive to treatment with 0.1 M 2-Me for 0.5 hr at room temperature, whereas costimulator activity is not affected by this treatment (data not shown).

IV. Cellular Origins of Costimulator and Conditions Required for Production

A. Introduction

Costimulator is generated when a heterogeneous population of leukocytes from spleen or lymph node is stimulated with Con A. One of the objectives of this project was to identify the functional and physical characteristics of the types of cells involved. Several earlier reports indicated that T cells were required for production of costimulator (Paetkau *et al.*, 1976), or similarly-induced factors (reviewed in Table 2). However it was not known whether T cells actually secrete the factor, or whether they merely participate in inducing it from another cell type, such as a macrophage. There was some evidence that adherent (A) cells are required for costimulator production (Mills *et al.*, 1976; Paetkau *et al.*, 1976), but it had not been established that the A cell is of the monocyte/macrophage lineage.

The importance of determining the types of cells responsible for costimulator secretion is two-fold. Identifying the cell producing the factor in functional terms may be helpful in determining its biological role. Secondly, knowing the physical characteristics of the producing cell would facilitate attempts to generate it on a large scale for further biochemical analysis. This could be approached either by screening tumor cell lines of

appropriate phenotype, as has been done for IL1 (Table 3), or by producing hybridomas between Con A - stimulated cells and tumor cells derived from the same cell type as the costimulator-secreting cell. The latter approach has been used to produce hybridomas secreting antigen-specific suppressor factors (Table 1).

In this chapter, results are presented pertaining to the type of T cell required for costimulator production. Antisera directed against several lymphocyte differentiation antigens (Chapter I) Ly 1,2,4,5,6 and 7, were used to characterize the types of cells involved. Since costimulator appears to replace the requirement for helper T cells in both AFC (Watson *et al*, 1979a) and CTL responses (chapter V) we considered the possibility that the T cell involved might have a phenotype characteristic of helper T cells. Cantor and Boyse (1976b) have determined the Ly phenotype of both CTL - and AFC - helper T cells to be Ly $1^+,2^-,3^-$. The phenotype of the CTL helper has been further characterized by Pilarski and colleagues (using the same antisera employed in experiments to be reported here) as Ly $1^+,2^-,4^-,5^+,6^-$, and 7^+ (Al-Adra *et al.*, 1980; Pilarski *et al.*, 1980).

Antisera against these lymphocyte differentiation antigens were used to delete specific subpopulations of lymphocytes from spleen cell suspensions. The costimulator-generating capacity of the remaining cells was then assessed. One of the difficulties in using these antisera is that they are often contaminated with antiviral

or auto-antibodies, and soluble immune complexes (McKenzie and Potter, 1979). These complications occur in spite of careful adsorption of antisera with appropriate mouse tissue. They can only be completely overcome by the use of monoclonal antibodies secreted by hybridomas (produced by fusing spleen cells from immunized mice with mouse myeloma cell lines). One such hybridoma, which secretes anti-Lyt 1.1, has been produced (Hogarth and McKenzie, personal communication) and was used in experiments reported here.

Results presented in this chapter also analyze the macrophage contribution to costimulator production. Adherent (A) cells, which can be removed by nylon wool columns appear to be a requirement. Cells which adhere to nylon wool can be of several types: activated T cells (Arala-Chaves *et al.*, 1978), B cells (Julius *et al.*, 1973) or macrophages (Schwartz and Paul, 1975). Attempts were made to replace the A cell with purified macrophages. Evidence is presented that the active A cell in the costimulator-producing cultures is a monocyte/macrophage.

The question as to whether a T cell or a macrophage secretes costimulator might be solved if one of the two types of cells could be replaced by a soluble product, which is itself inert in the costimulator assay. Synergy between LAF (IL1), a soluble macrophage product, and A-depleted, T-enriched leukocytes was observed. This indicates that costimulator is probably a T cell product.

Other experiments in this chapter characterize the

optimal cell culture conditions for costimulator generation. This involved experiments on the cell density, time course, and requirements for serum and 2-mercaptoethanol.

Most experiments employed Con A as the stimulant for the induction of the factor. However it appears that a factor which is similar, if not identical, to costimulator can be elicited in a 2-way mixed lymphocyte culture. The time course and G-100 profiles of the resultant factor are examined.

B. Results

The Cell Density Requirements for Costimulator Production

Results presented in Figure 17 indicate that a minimum cell density of 2.5×10^6/ml is required for the efficient production of costimulator from spleen cells. In this experiment, cells were cultured in 1 ml of medium at various cell densities with Con A to produce costimulator, and the crude supernatants were titrated in the thymocyte proliferation assay. The results are graphed so that the numbers on the horizontal axis refer to the number of generating cells represented by the costimulator present in a given assay well, and not the number of cells actually present in the generating cultures. Thus Figure 17 shows that the amount of factor produced per cell decreases as the cell density is decreased from 10×10^6/ml to 2.5×10^6/ml. Below 2.5×10^6/ml, the production of factor falls off sharply

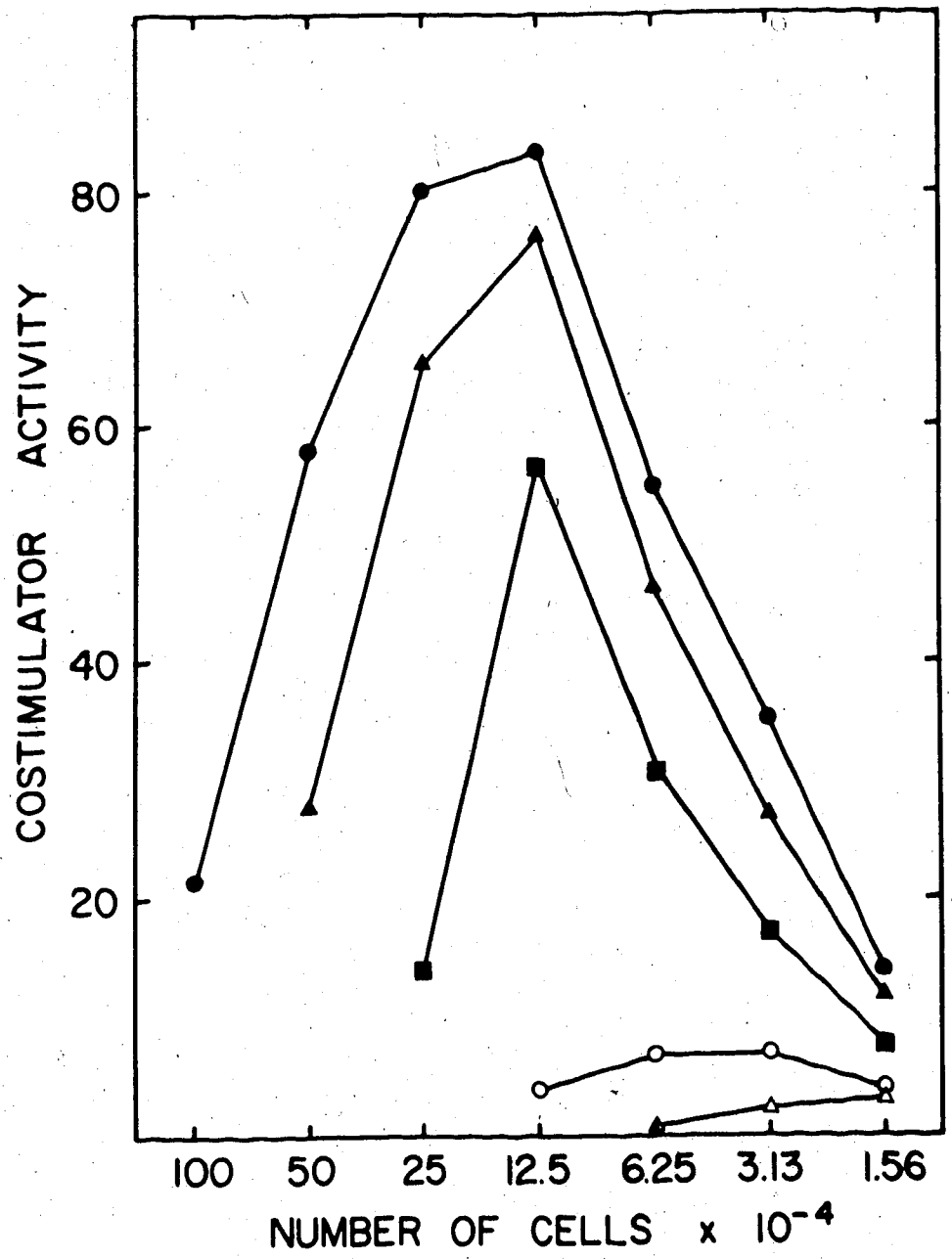


Figure 17. Effect of cell density on costimulator production. CBA/J spleen cells were cultured in 1.0 ml MHM with Con A at various cell densities to generate costimulator:
(•) 10×10^6/ml
(▲) 5×10^6/ml
(■) 2.5×10^6/ml
(o) 1.25×10^6/ml
(△) 0.625×10^6/ml
Crude supernatants were titrated in the thymocyte proliferation assay. Results are graphed so that the "number of cells" on the horizontal axis refers to the number of cells in a generating culture which are represented by the costimulator present in an assay well. Each point represents the mean of 6 replicate assay cultures. Thus, the generated activity for a given abscissa value is on a per-cell basis.

The cell density requirements were also examined for smaller cultures in microtitre wells, which were to be used for the analysis of the types of cells involved in costimulator production. Small cultures using minimal numbers of cells were necessary for these experiments because the antisera required were limiting in quantity. The cell density requirement in 0.05 ml cultures (Figure 18) were similar to those observed in 1.0 ml culture. A density of $20x10^6$/ml was too high. The smallest number of cells from which costimulator could be detected was $0.1x10^6$, or $2.0x10^6$/ml. In 0.1 ml cultures, which were used in most subsequent microculture experiments (because they gave more consistent results), the optimal cell concentration was $10x10^6$/ml (data not shown).

Time course of Con A - Induced Costimulator Production

The level of costimulator present in the supernatant of Con A-stimulated cells over a 96 hour period is shown in Figure 19. The factor is first detectable in the medium 4 hrs after culture with Con A, and reaches its maximal level by 21 hrs (Figures 19 and 20). In FBS-containing cultures the level of costimulator remains constant for the next three days. In the absence of FBS, the level declines, perhaps due to proteases (Figure 19).

The greatest apparent yield of costimulator occurs if the cells are resuspended in fresh medium at 8.5 hrs, and then cultured until 21 hrs (compare 8.5-21 hr and 0-21 hr in Figure 20). This may reflect the removal of an inhibitor of

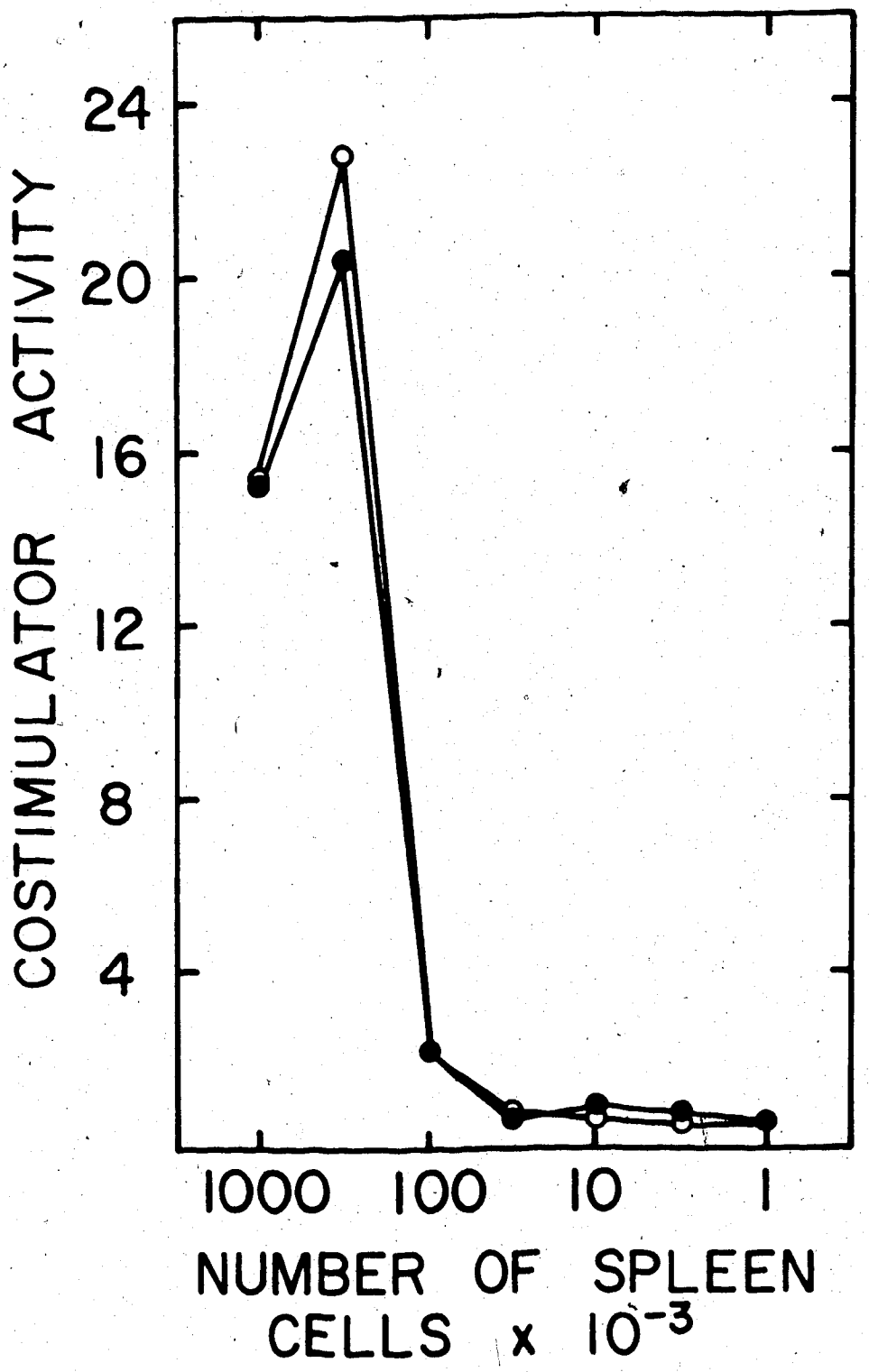


Figure 18. Effect of cell density on costimulator production in microcultures. Various numbers of CBA/J spleen cells (indicated on the horizontal axis) were incubated in 0.05 ml cultures in round-bottomed (•) or V-bottomed (o) microtitre wells with Con A to generate costimulator. The crude supernatants were tested for costimulator activity in the thymocyte proliferation assay. The background in the assay with no added costimulator was 0.9. The maximal stimulation was 48. Each point represents the mean of 6 replicate assay cultures.

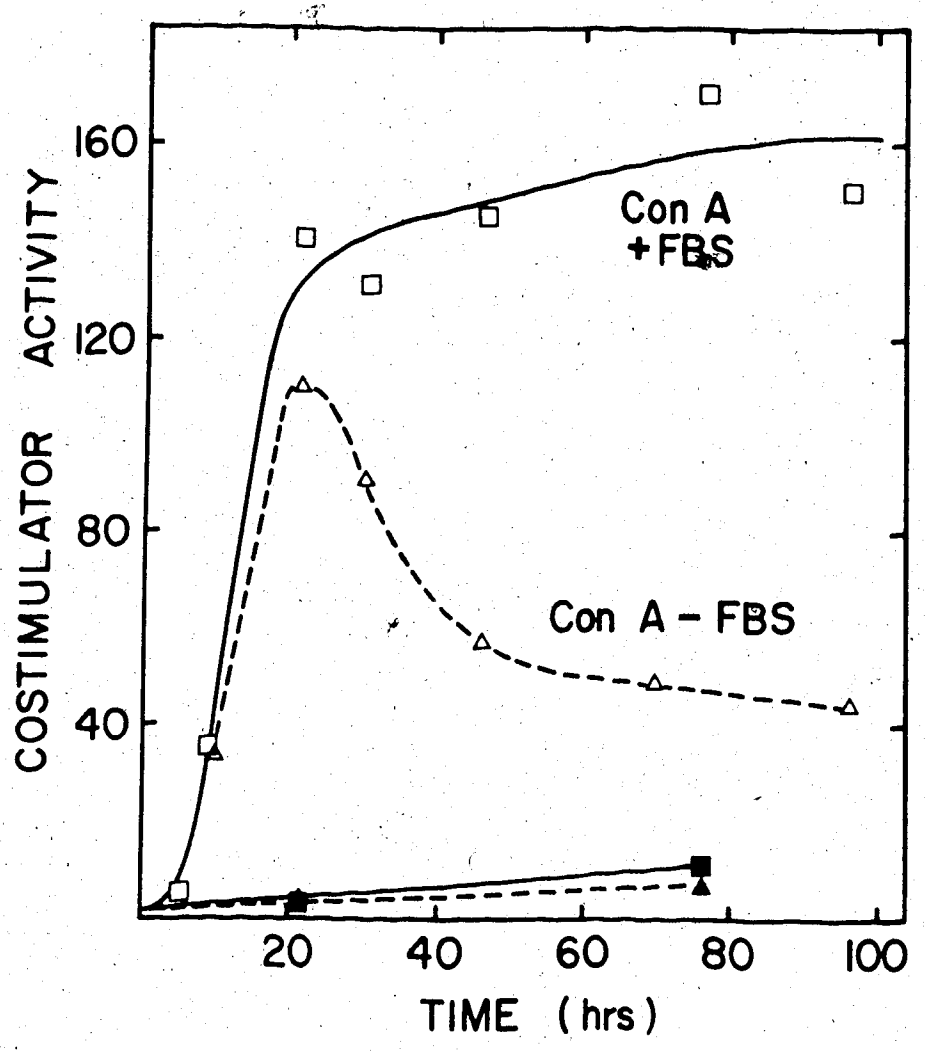
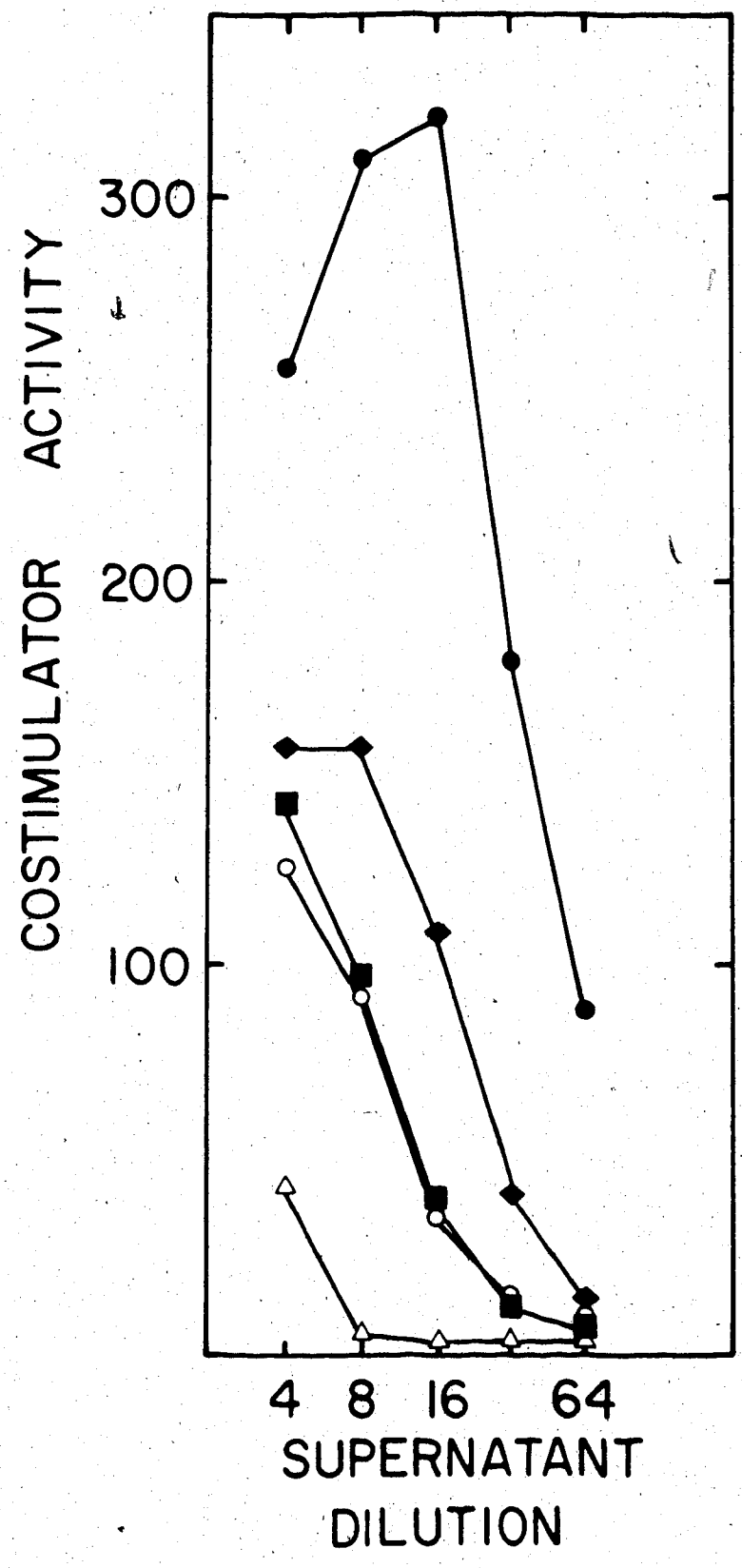


Figure 19. Time course of costimulator production by CBA/J spleen cells stimulated with Con A. Spleen cells were incubated in 1.0 ml cultures at $10x10^6$/ml, with or without serum (FBS). The Con A concentration was 1.5 microgram/ml without serum and 4 with serum. Supernatants were harvested at various times, and titrated in the thymocyte proliferation assay. The results shown represent a 1/8 dilution of the supernatants, which was on the linear portion of the dilution curve for all samples. Control (-Con A) costimulator generating cultures gave values of 2 (-FBS) and 2 (+FBS) at 21 hr, and 6 (-FBS) and 10 (+FBS) at 72 hr. (■) No Con A + FBS; (▲) No Con A - FBS.

Figure 20. Costimulator produced by CBA/J spleen cells during various time intervals of incubation with Con A. Cultures were set up as in Figure 19, without serum. After various lengths of time, supernatants were harvested. If cells were to be recultured, they were washed once in warm medium, and resuspended in the original volume of fresh Con A-containing medium. Supernatants were titrated in the thymocyte proliferation assay. The background value in the assay with no added costimulator was 5.
(△) 0-4 hr
(■) 0-8.5 hr
(◆) 0-21 hr
(●) 8.5-21 hr
(o) 21-46 hr


COSTIMULATOR ACTIVITY
300
200
100
4
8
16
64
SUPERNATANT
DILUTION

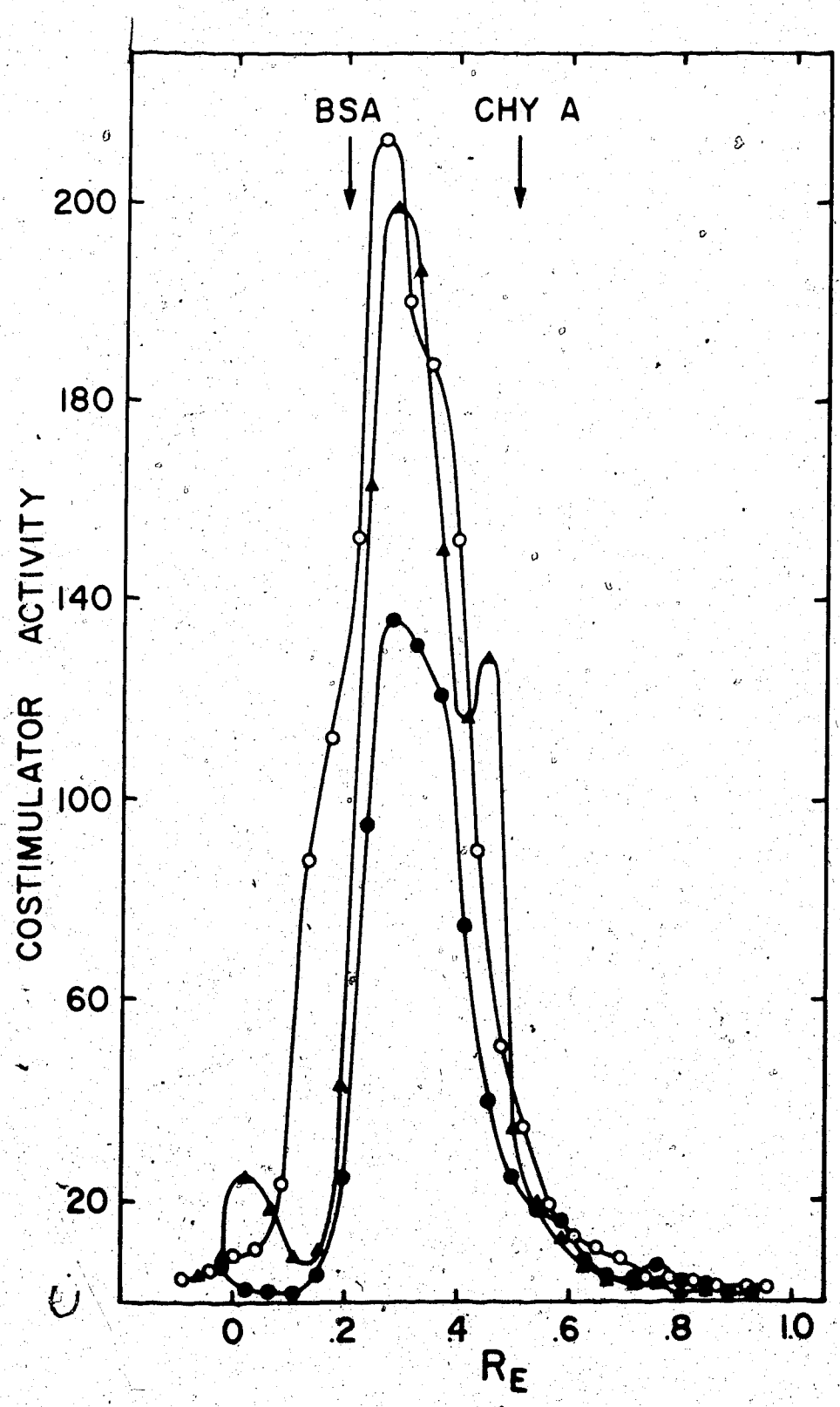
costimulator production from the medium. Alternatively, the 0-8.5 hr supernatant may contain an inhibitor of the thymocyte proliferation assay, or of the costimulator producing cell itself. The effect of changing the medium was not further investigated.

Costimulator was routinely produced in cultures of 18-24 hr duration. Serum was not used in bulk cultures, to simplify the purification procedure, but was sometimes used in microcultures containing A cells, which clump in the absence of serum. The molecular size of factors produced after 24 hr of culture with and without serum, and after 72 hr of culture with serum were compared by gel filtration chromatography on G-100 Sephadex (Figure 21). The 3 samples eluted in similar positions, indicating that the size is not grossly affected by culture with serum, or length of time in culture. Some of the TRF activity harvested from 30-38 hr cultures consists of smaller molecular weight material (about 15,000 daltons) than that from 24 hr cultures (30,000 daltons; Watson, 1979a). This does not appear to be the case with costimulator.

Other Conditions Necessary for Costimulator Production

The optimal concentration of Con A in the absence of serum was 1-1.5 microgram/ml (V. Monticone and V. Paetkau, personal communication). If serum was used, the level of Con A was increased to 4 microgram/ml. The continuous presence of Con A was not required . A one hr incubation with Con A, [illegible]hing and resuspending the cells in fresh

Figure 21. Chromatography of fraction 2 costimulator produced at 21 hr with no serum, and 21 and 68 hr with serum on G-100 Sephadex. Fifty ml of crude costimulator was generated for each condition. Samples were concentrated and run over G-25 Sephadex to remove salts and Con A before applying to the calibrated 1.5x60 cm G-100 column. The void volumes (Vo) and total volumes (Vt) were determined as in Figure 7. The position of protein markers is indicated. Fractions were assayed in the thymocyte proliferation assay at 1/20 dilution. The abscissa indicates the position of column fractions (Vo=0, Vt=1.0).
(•) 21 hr (+FBS)
(▲) 21 hr (-FBS)
(o) 68 hr (+FBS).
I acknowledge the assistance of Mr. Cliff Gibbs in performing this experiment.


BSA
CHY A
200
180
140
100
60
20
COSTIMULATOR ACTIVITY
0
.2
.4
.6
.8
1.0
R_E

medium, gave equivalent results (data not shown).

All of the culture systems for costimulator generation contained 2-ME ($1x10^{-5}$ or $5x10^{-5}$M). This was not an absolute requirement for production, but it greatly enhanced the yield (Figure 22).

Production of a Costimulator-like Factor in Mixed Leukocyte Culture (MLC)

Since costimulator is produced by strong T cell activation by mitogens, the possibility that it can also be produced during T cell stimulation with alloantigens was investigated. Earlier reports (Schimpl and Wecker, 1973; Dutton *et al.*, 1971) indicated that TRF activity is induced during MLCs. Spleen cells from CBA (H-2k) and BALB/c (H-2d) mice were cultured together for various lengths of time (with no Con A added), and the culture supernatants assayed for costimulator activity (Figure 23). Activity in MLC supernatants (first tested at 24 hrs) was comparable to levels induced by Con A, but was negligible in control supernatants (from CBA or BALB/c cells cultured alone and their supernatants pooled for the assay).

Costimulator generated in a 72 hr MLC was processed to the level of fraction 2, and then run over a calibrated G-100 column (Figure 24). Its elution position was identical to a sample consisting of Con A-generated costimulator mixed with the pooled supernatants of CBA and BALB/c cells cultured separately. Thus the Con A- and MLC- elicited factors have the same hydrodynamic radius. It remains to be

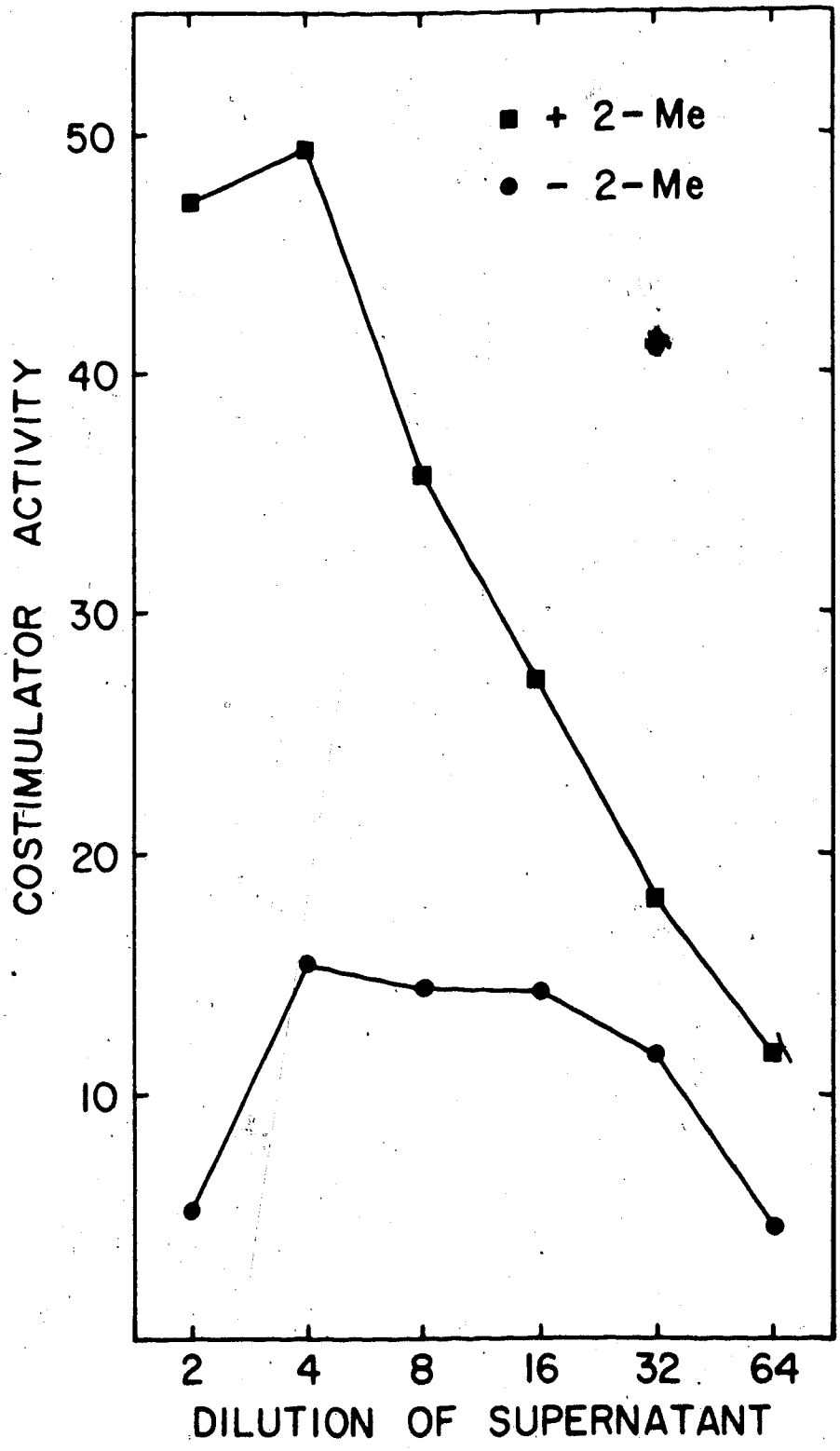


Figure 22. Enhancement of costimulator production by 2-Me. CBA/J spleen cells were cultured with Con A for 24 hr to produce costimulator. One ml cultures contained 10×10^6 cells; 2-Me was absent, or present at 5×10^{-5}M. The resultant supernatants were titrated in the thymocyte proliferation assay (0.2 ml cultures).

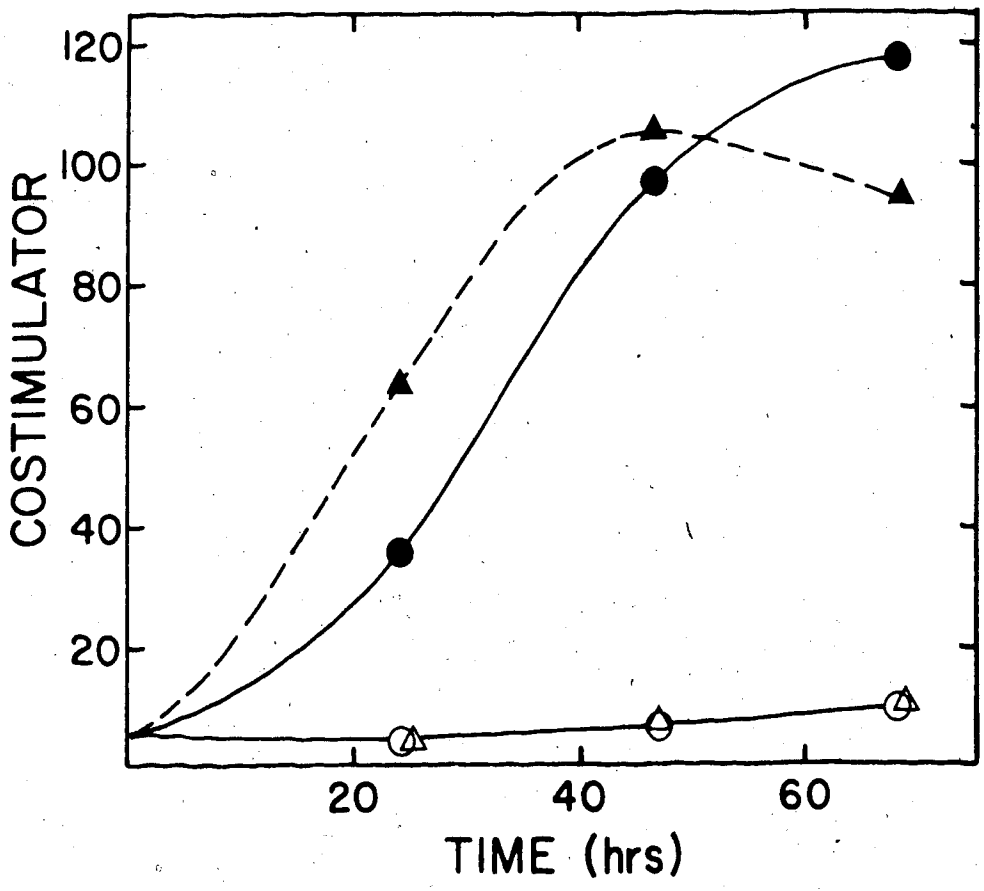


Figure 23. Time course of costimulator generation in mixed leukocyte cultures. Spleen cells from BALB/c (H-2d) and (CBA/J (H-2K) were cultured in 1.0 ml either together (MLC) or separately (controls). MLC cultures contained either 1.0×10^6 (•) or 2.5×10^6 (▲) cells/ml from each strain. Control cultures contained 2.0×10^6 (o) or 5.0×10^6 (△) of either type. The control cell supernatants were later mixed so that these samples represented a 1:1 mixture of medium from CBA and BALB/c cultures. Supernatants were harvested at various times. They were assayed at 1/5 dilution in the thymocyte proliferation assay.

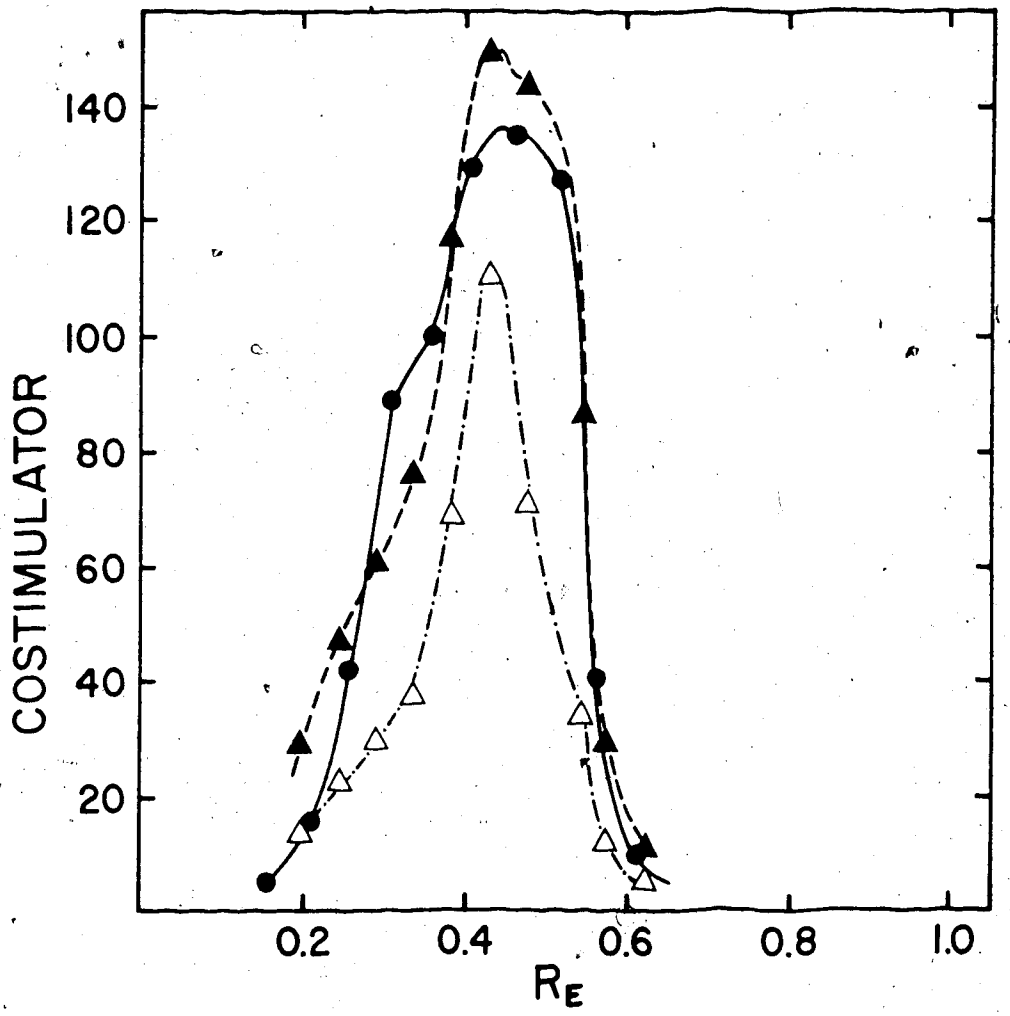


Figure 24. Chromatography of MLC- and Con A-generated costimulator on G-100 Sephadex. Spleen cells from CBA/J and BALB/c mice were cultured together in a 1:1 ratio at a total cell density of 2.0×10^6/ml in 400 ml of FBS-containing medium (MLC). Control cultures consisted of 2.0×10^6/ml (200 ml) of either cell type. The MLC and control cultures were incubated for 72 hr. Con A-generated costimulator (50 ml) was prepared by incubating CBA/J spleen cells at density of 16×10^6/ml with 1 microgram/ml Con A for 18 hr. The Con A-generated material was added to the supernatant from CBA/J and BALB/c control cultures. (This control was done to account for the effects of incubating for 72 hr in the presence of FBS.) The MLC and Con A + control samples were processed to the level of fraction 2, and chromatographed on a G-100 Sephadex column as described in Figure 7. Fractions were assayed for costimulator activity in the thymocyte proliferation assay. (△) Con A + control sample assayed at 1/100 dilution; (▲) Con A + control sample assayed at 1/50; (•) MLC sample assayed at 1/50. The RE gives the position of the fractions relative to the total volume (Vt) of the column, which was determined as described in Figure 7. I acknowledge the assistance of Ms. V. Monticone in performing this experiment.

determined whether they are the same molecular entities.

Effect of Ultraviolet (UV) and Gamma Irradiation on Costimulator Production by Spleen Cells

(CBA/J x DBA/2J)F1 spleen cells were exposed to either UV or gamma irradiation, and cultured with Con A for 24 hrs to generate costimulator. Gamma irradiation up to 1600 rad did not affect costimulator production by spleen cells; irradiation of 3200 rad reduced it by 2/3 (Table 11). The ability of spleen cells to generate costimulator was highly sensitive to UV irradiation. It was completely abolished by 0.1-0.25 min exposure at a distance of 11 cm from a 15 watt UV bulb (Table 11). The minimum distance from the UV source for complete abrogation of costimulator production in 10 sec irradiation was 10-15 cm (Table 12).

T Cells are Required for Costimulator Generation

Previous work in our laboratory showed that treatment of CBA spleen cells with anti-Thy 1.1 serum and complement abrogated their ability to secrete the factor (Paetkau *et al.*, 1976). The T cell requirement was further substantiated by the demonstration that spleen cells from athymic (BALB/c nu/nu) mice fail to produce costimulator when cultured under the same conditions as normal BALB/c cells, which do produce it (Table 13).

A Cells are Required for Costimulator Production

Removal of an adherent cell population (presumably macrophages) from thymus cells with carbonyl iron abrogated their ability to generate costimulator (Paetkau *et al.*,

Table 11.

Effect of ultraviolet and gamma irradiation on the ability of spleen cells to produce costimulator[1]

Treatment of spleen cells	Costimulator activity (supernatant dilution)			
	1/8	1/16	1/32	1/64
none (control)	319	231	134	38
UV 0.1 min	19	14	6	4
UV .25	4	4	4	7
UV .5	6	4	6	7
UV 1.0	6	7	3	6
none (control)	302	243	163	57
gamma 100 Rads	361	277	140	49
gamma 200	348	287	140	60
gamma 400	332	295	157	56
gamma 800	338	279	148	60
gamma 1600	338	308	144	67
gamma 3200	189	87	45	14
No costimulator	5.6			

[1] (CBA/J x DBA/2J)F1 spleen cells were subjected to gamma or ultraviolet (UV) irradiation as described in Materials and Methods. UV irradiation was carried out at a distance of 11 cm from a 15 Watt bulb for the times indicated. "Control" cells were kept in petri dishes or test tubes for the length of time it took to complete the irradiation procedures. Treated cells were cultured with Con A in 0.1 ml cultures to generate costimulator, and the crude supernatants were titrated in the thymocyte proliferation assay.

Table 12.

Effect of distance from lamp during UV exposure on the ability of cells to produce costimulator[1]

Distance of cells from 15 watt UV bulb (cm)	Costimulator activity generated (dilution of supernatant)			
	1/8	1/16	1/32	1/64
unirradiated (control)	198	173	96	27
5	7	8	6	8
10	8	6	6	9
15	19	13	11	8
20	101	32	23	8
25	83	52	23	12
30	87	44	24	17
40	64	33	20	12
50	119	97	51	27
60	142	107	57	28

No costimulator = 2

[1] (CBA/J x DBA/2J)F1 spleen cells were exposed to UV light for 10 sec as described in Materials and Methods. Costimulator was generated in 0.1 ml microtitre cultures containing 10^6 irradiated or control cells per culture. Crude supernatants were titrated in the thymocyte proliferation assay (0.1 ml cultures). Costimulator activity is expressed as pmoles dThd incorporated by the test thymocytes per hour, per 10^6 cells.

Table 13.

Spleen cells from athymic mice do not produce costimulator[1]

Source of costimulator	Costimulator activity (Supernatant dilution)	
	1/10	1/40
BALB/c(+/+)6x10^6/ml	56.6	51.0
BALB/c(+/+)3x10/ml	41.3	37.5
BALB/c(+/+1x10^6/ml	42.0	15.5
BALB/c(nu/nu)6x10^6/ml	1.3	1.8
BALB/c(nu/nu)3x10^6/ml	1.2	1.3
BALB/c(nu/nu)1x10^6/ml	1.2	1.7
CBA/J fraction 3 (1/200)	50.4	
no costimulator	1.8	

[1] Spleen cells from wild type (+/+) or athymic (nu/nu) BALB/cCr mice were incubated for 24 hrs in 1 ml cultures at the concentrations indicated to produce costimulator. Crude supernatants were tested for costimulator activity at 1/10 and 1/40 dilutions in the thymocyte proliferation assay.

1976). However, this method did not prevent secretion by spleen cells, which produce more factor than thymocytes do. To resolve the question of A cell dependence, costimulator production by lymph node cells depleted of adherent cells on nylon wool columns was examined. Filtration through glass wool, followed by incubation on nylon wool columns abolished costimulator production by CBA (Figure 25a) and DBA (Figure 25d) lymph node cells. Filtration through glass wool alone decreased costimulator production by 50% or more (data not shown). In agreement with previous results, anti-Thy 1.2 treatment of spleen cells from the same strains of mice resulted in decreased production of the factor (Figures 25b and e), the effect on CBA cells being greater than on DBA. When anti-Thy 1.2 treated cells were mixed with nylon wool treated cells, synergy between the "T-depleted" and "A-depleted" populations was observed, in both syngeneic and allogeneic combinations (Figures 25c and f).

Costimulator production in cultures of mixed allogeneic cells was totally Con A dependent (data not shown for simplicity). Although costimulator can be elicited in primary MLC (Figure 23), under the conditions and with the strain combination used here (24 hours of culture, no serum, CBA and DBA spleen cells), no detectable factor was produced in the absence of Con A. The synergy observed in allogeneic combination of "T cell" and "A cell" populations does not necessarily mean that their interaction is not normally H-2 unrestricted. Con A could stick cells together as it does in

cytotoxic cell assays (Bevan and and Cohn, 1975), bypassing any requirement for specific recognition.

The results presented in Figure 25 do not eliminate the possibility that the adherent cell is a B lymphocyte. It is clearly not a nylon wool adherent T cell, because the A cell requirement could be met by anti-Thy 1.2 treated spleen cells. However, the requirement for adherent cells is largely met by the addition of macrophages (cultured from bone marrow stem cells *in vitro*) to A cell-depleted lymph node cells (Table 14). The cultured cells were > 99% phagocytic and adherent; by morphological criteria they appeared to be macrophages. Furthermore, the medium used to culture the cells, mouse L cell conditioned medium, is known to primarily support the growth of macrophages; precursors of T and B lymphocytes do not develop (Lee, 1980). Thus the active A cells are probably macrophages and not B cells.

Macrophages are a heterogeneous population of cells. One basis upon which functional subsets of macrophages have been distinguished is that of size. Lee (1980) has fractionated macrophages from peritoneal cavities of mice using "Sta-put" gradients (linear gradients of FBS), and found that certain functions correlate with the size of the macrophages. For example, small macrophages present antigen to T cells, whereas large macrophages are poor antigen-presenters, but are effective in cytostasis of tumor cells *in vitro*. In collaboration with Dr. Lee, CBA/CaJ peritoneal macrophages were fractionated on Sta-put

Table 14.

Synergy between nylon wool-purified lymph node cells and macrophages derived from 8 day bone marrow cultures in costimulator production[1]

	Costimulator activity generated:		
	$+10^6$ nylon wool purified LN T cells +Con A	$+10^6$ nylon wool purified LN T cells -Con A	+Con A (no LN cells)
Number of macrophages per culture			
none	4	9	
$3x10^2$	7	5	3
$1x10^3$	10	4	5
$3x10^3$	18	5	3
$1x10^4$	22	10	5
$3x10^4$	41	12	17
$1x10^5$	71	ND	16
unfractionated LN cells +Con A =	154		
unfractionated LN cells -Con A =	12		

[1] Bone marrow macrophages were a gift from Dr. K.-C. Lee. They were cultured in L cell-conditioned medium for 8 days (Lee, 1980). Lymph node (LN) cells from CBA/J mice were depleted of adherent cells by passage over nylon wool. Each generating culture contained 10^6 LN cells (except controls), in a total volume of 0.1 ml. Cells were incubated for 24 hrs with or without Con A, and the supernatants assayed at 1/16 dilution for costimulator activity in the thymocyte proliferation assay. Costimulator activity is expressed as pmole dThd incorporated by the test thymocytes per hour, per 10^6 cells.

gradients to determine whether any particular size class was active in restoring the ability of A-depleted LNC to produce costimulator (Figure 26). Five pools, A-E, in increasing order of size are considered. The smallest cells (A and B) appear to be the most active. However A is predominantly lymphocytes, and B is contaminated with lymphocytes (see legend to Figure). The controls (Sta-put fractions alone) for both A and B show significant amounts of costimulator production. Therefore the active cells in fractions A and B are not necessarily macrophages. The important point established by this experiment, however, is that the larger macrophage fractions, C, D, and E, which do not produce costimulator without added T cells, do synergize with A cell depleted LNC. It can be concluded that the ability to cooperate with T cells to produce costimulator is a function of macrophages distributed over a wide size range.

The Adherent Cell Requirement can be Partially Met by a Soluble Macrophage Factor

To determine whether costimulator is a T cell product, with A cells playing an auxiliary role in its production, we attempted to replace the A cell requirement with soluble factors from an LPS-stimulated macrophage tumor cell line, P388-D1. Supernatants from stimulated P388-D1 cells were expected to contain LAF (IL1) (Mizel *et al.*, 1978b; Lachman, 1977a). Crude supernatants were concentrated, and run over G-100 columns to separate the activity from inhibitory material. The G-100 fractions were assayed for their ability

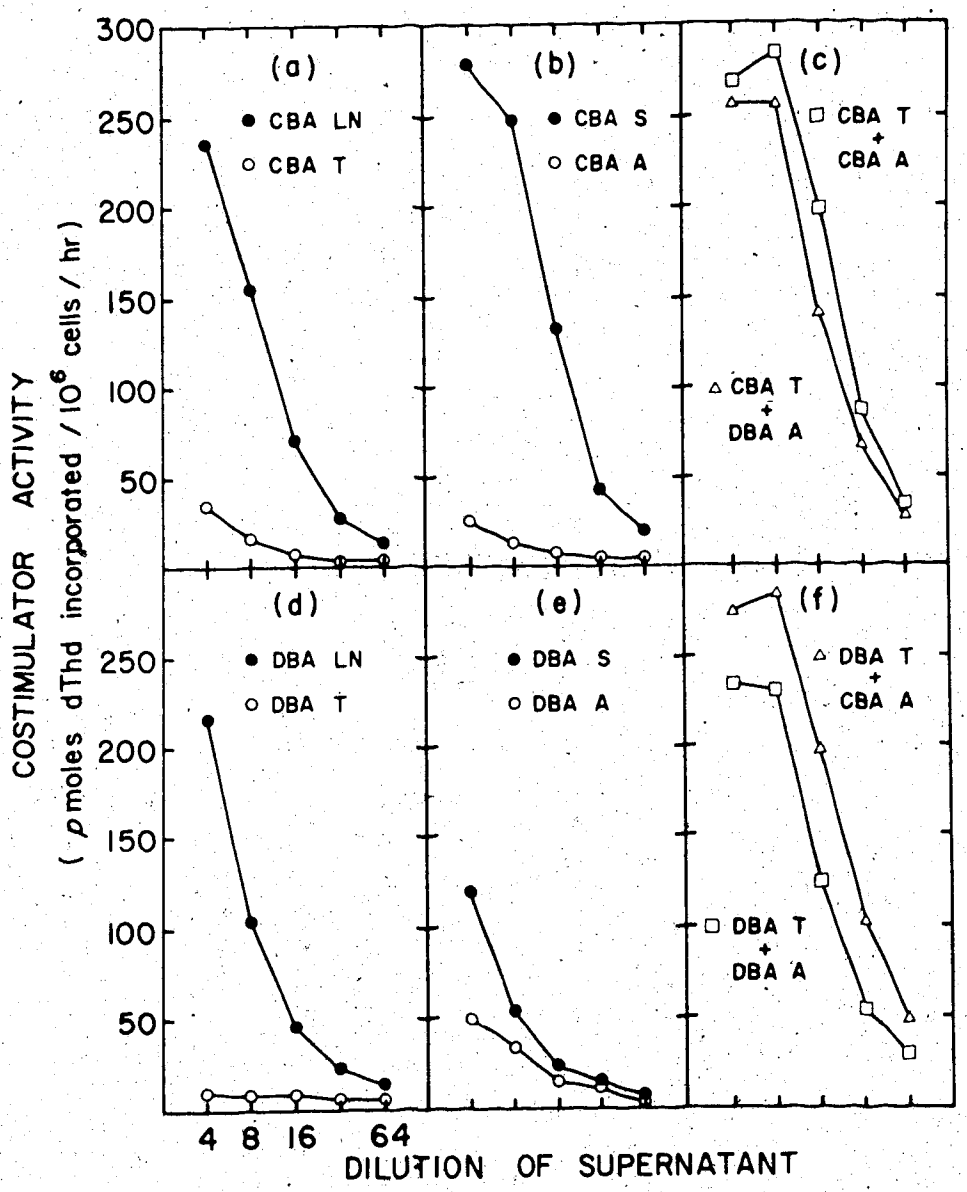


Figure 25. Synergy between nylon wool purified lymph node cells and anti-Thy 1 treated spleen cells in costimulator production. CBA and DBA lymph node cells were enriched for T cells on nylon wool columns. Spleens from the same mice were treated with anti-Thy 1.2 serum and complement (to obtain a T cell-free source of A cells) or heat inactivated normal rabbit serum (NRS) and complement as a control. The following denotations are used in the figure: LN (unfractionated lymph node cells), T (nylon wool purified lymph node cells); S (NRS and complement treated spleen cells); A (anti-Thy 1.2 and complement treated spleen cells). The cells were cultured in RHM with Con A for 24 hours to generate costimulator. Cultures contained a total of 1×10^6 viable cells in 0.1 ml. Cultures in which T and A cells were mixed (panels c and f) contained 0.5×10^6 of each cell population, previously determined to be the optimum ratio. Supernatants were assayed at various dilutions for costimulator activity in the thymocyte proliferation assay.

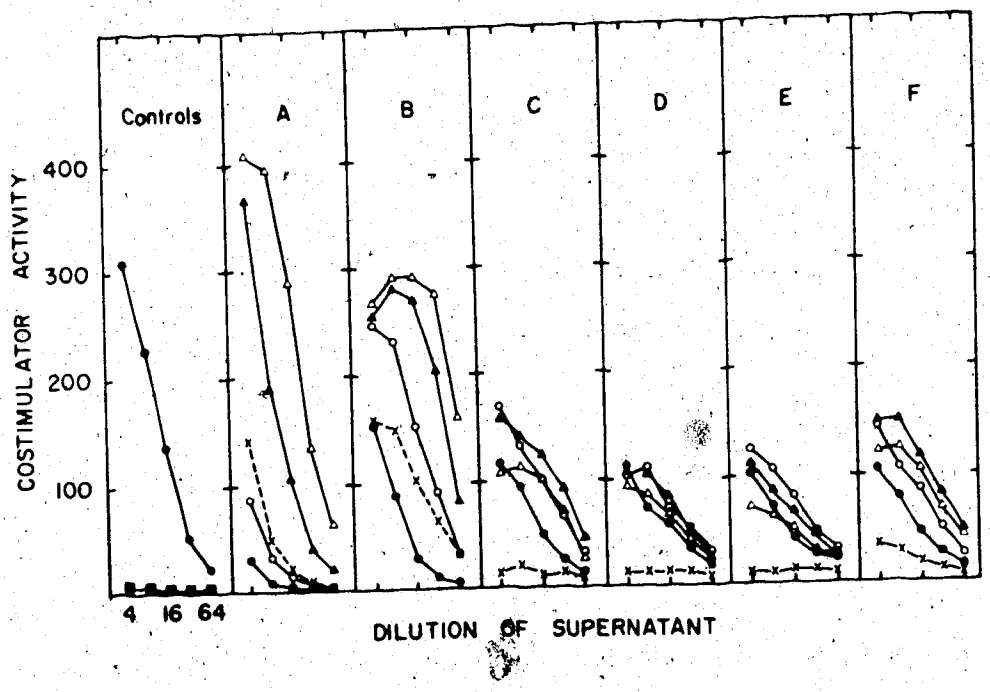


Figure 26. Ability of peritoneal macrophages of different sizes to synergize with A cell-depleted LNC in costimulator production. Normal CBA/CaJ peritoneal cells were fractionated by velocity sedimentation on a "Sta-put" gradient of FBS (details described in Lee and Berry, 1977). The fractionation was performed by Dr. K.-C. Lee. Fraction A consists of the smallest cells, and E the largest. The composition of the cells in various fractions after this procedure are
A: 98.5% lymphocytes, 1.2% macrophages;
B: 57.5% lymphocytes, 41.0% macrophages;
C: 7.1% lymphocytes, 92.6% macrophages;
D: 1.8% lymphocytes, 96.6% macrophages;
E: 0.8% lymphocytes, 71.5% macrophages, 27.5% mast cells.
(Any cells not accounted for were polymorphonuclear or mast cells.) Unfractionated (F) cells consisted of 57.4% lymphocytes and 40.4% macrophages. CBA/CaJ LNC were depleted of A cells on glass and nylon wool columns, and cultured at 10^6 cells/0.1ml with Con A to generate costimulator in RHFM with various numbers of fractionated PC added to some cultures. Control cultures contained peritoneal cells alone (no added LNC). Supernatants were titrated in the thymocyte proliferation assay. Controls: (•) untreated LNC; (▪) LNC depleted of A cells on nylon wool. A-E and F: (x) $3x10^5$ peritoneal cells (no LNC); A cell-depleted LNC plus the following numbers of peritoneal cells: (•) $0.1x10^5$; (o) $0.3x10^5$; (▲) $1x10^5$; (△) $3x10^5$.

to induce mitogenesis in mouse thymocytes (LAF activity). Active fractions were pooled. The ability of A cell-depleted LNC to produce costimulator was partially restored by the addition of pooled LAF fractions to generating cultures. Material from 2 areas of the G-100 column were active in inducing costimulator: excluded fractions (Figure 27d), and included fractions representing a molecular weight range of 12-20,000 (Figure 27b and c). These fractions themselves had slight activity in the thymocyte proliferation assay, but obviously are not the main source of the activity measured. It is likely that the low MW material is LAF (Economou and Shin, 1978), and that the high MW material is high MW LAF (Mizel and Rosenstreich, 1979; Lachman *et al.*, 1977a). This experiment strongly suggests that costimulator is secreted by T cells.

Ly and Ia Phenotype of Cells Required for Costimulator Production

Figure 25 shows that Thy 1$^+$ cells are required for costimulator production by Con A stimulated spleen cells. To further identify the cells involved, costimulator production was tested after treatment of cells with antisera directed against several lymphocyte differentiation antigens. CBA spleen cells, which carry the Ly alleles 1.1, 2.1, 4.1, 6.1, and 7.2, and express Ia antigens determined by the I(k) haplotype, were used. Two representative sets of data are given in Figures 28 and 29.

Figure 28 shows the effects of anti-Ly 1.1, 2.1, 6.1,

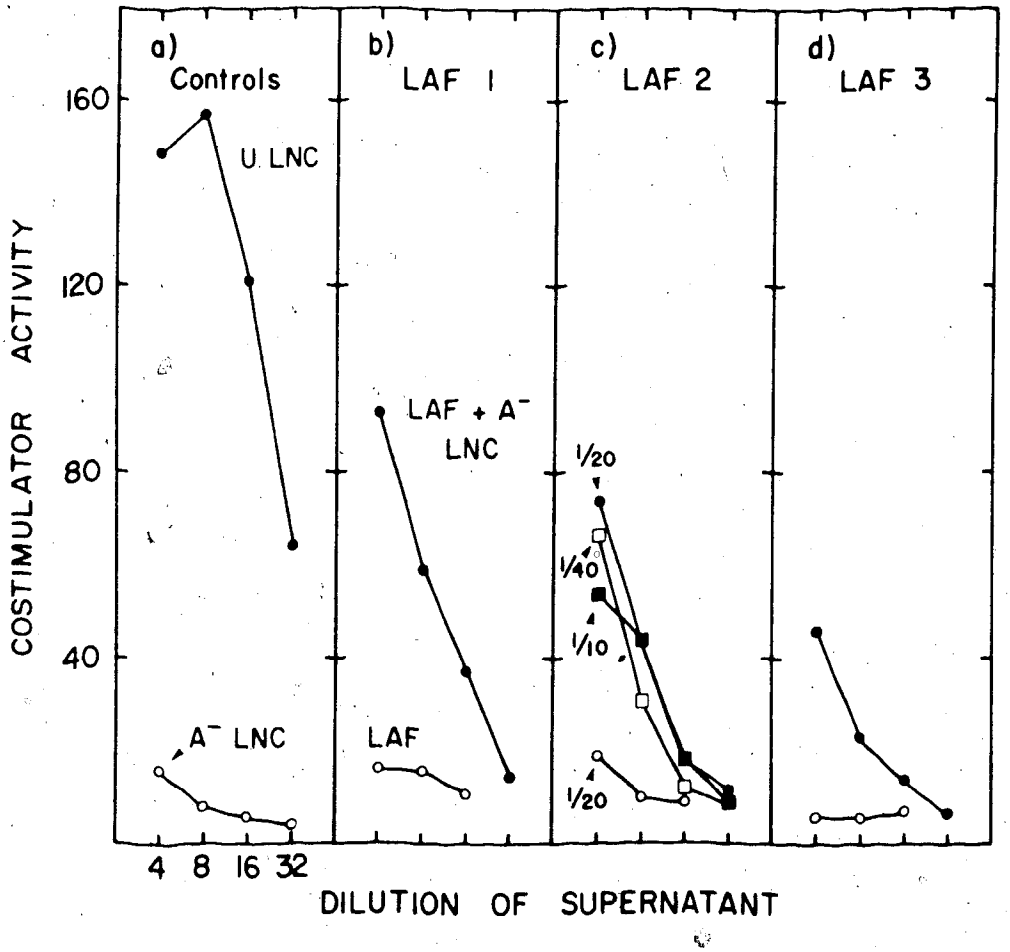


Figure 27. Synergy between A-cell-depleted LNC and a macrophage factor from P388-D1 cells in costimulator production. G-100 Sephadex purified LAF fractions were gifts from Dr. G. Mills, Ms. T. Lee and Mr. B. Caplan. P388-D1 cells were cultured for 72 hr with LPS (10-20 microgram/ml). The supernatants were harvested, concentrated and run over G-100 Sephadex. Fractions were assayed for their ability to induce mitogenesis in mouse thymocytes cultured at high cell density. Peak fractions were either pooled (panels c and d) or the peak fraction tested (panel b). CBA/J LNC were depleted of LNC on glass and nylon wool columns. They were cultured with Con A in 0.1 ml cultures (10^6 cells/culture) to generate costimulator. LAF was added at various dilutions to the cultures. Supernatants were harvested after 22 hr and titrated in the thymocyte proliferation assay. Controls (LAF alone) were set up so that the LAF was incubated with Con A for 22 hr, under the same conditions as the cultures containing LNC. (a) U: unfractionated; A⁻: A cell depleted on nylon wool; (b-d) (o): LAF alone at 1/20 dilution; no LNC; (b) LAF 1 = G-100 fraction with peak LAF activity, used at 1/20. (c) LAF 2 = small MW pool of LAF; (d) pool of excluded fractions with LAF activity, used at 1/20.

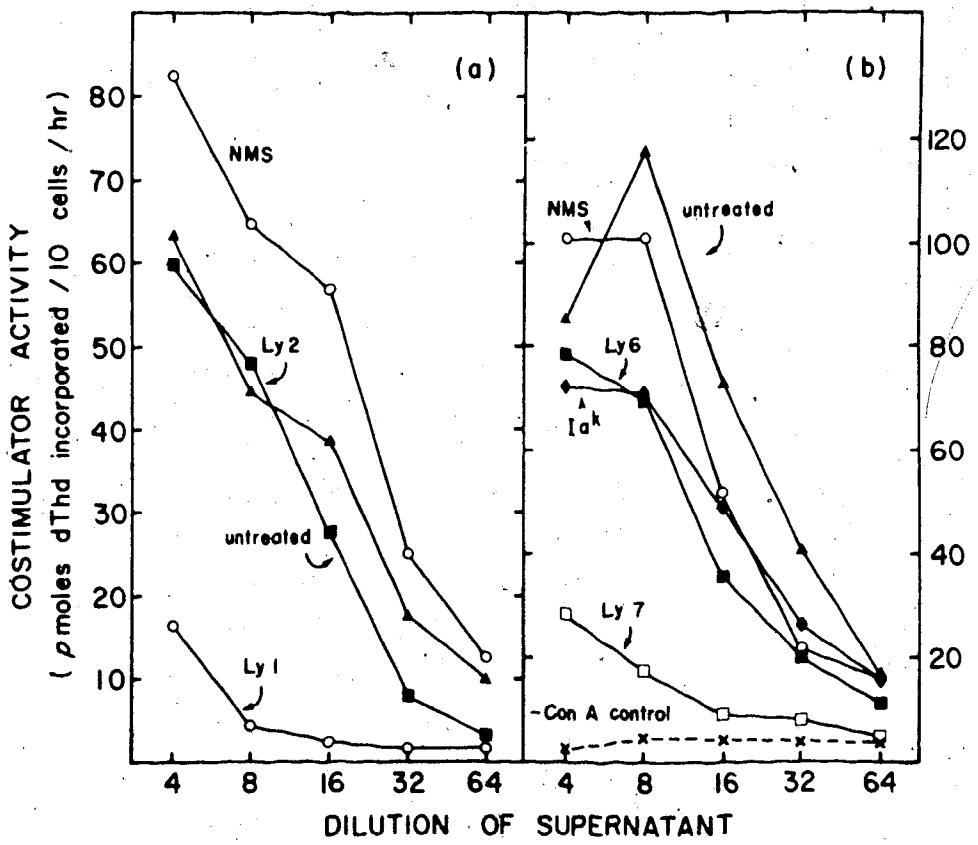


Figure 28. Ability of CBA/CaJ spleen cells to produce costimulator after treatment with anti-Ly 1.1, 2.1, 6.1, 7.2 and anti-Ia(k) sera. Data for (a) and (b) were obtained in separate experiments. Spleen cells were treated with the indicated antisera and complement, and cultured with Con A to produce costimulator in microcultures. Cultures contained 1 x 10^6 cells (untreated and NMS-treated) or the cells surviving treatment of 1 x 10^6 cells with the indicated antisera. The cells killed by the antisera are indicated in parenthesis. Supernatants were tested at various dilutions in the thymocyte proliferation assay. a: Antisera used at 1/10 dilution. NMS (0%), anti-Ly 1.1 (#466, 37%), anti-Ly 2.1 (41%). b: Anti-Ia(k) serum used at 1/16, and all others at 1/10 dilution. NMS (33%), anti-Ia(k) (67%), anti-Ly 6.1 (48%), anti-Ly 7.2 (68%). (X---X) supernatant generated from untreated cells in the absence of Con A.

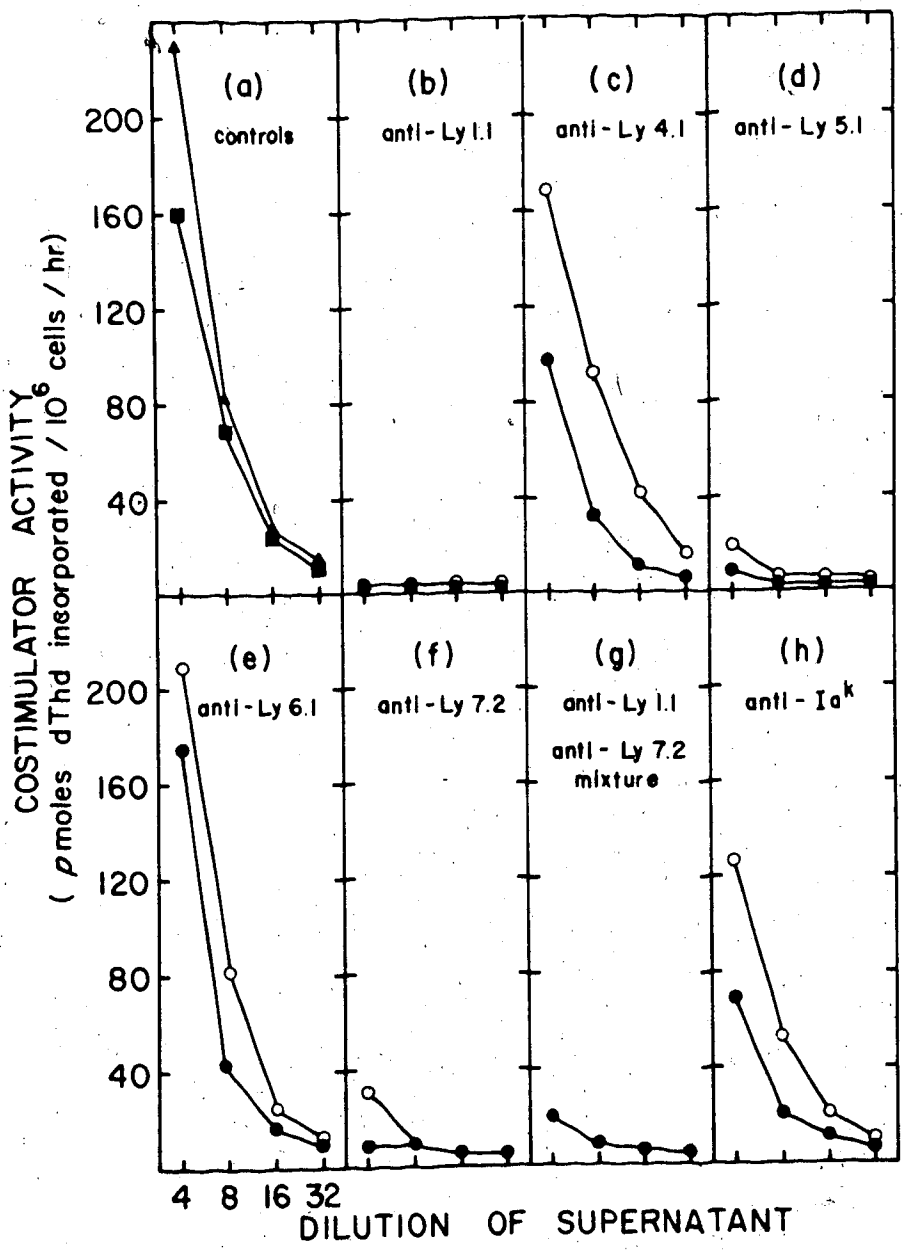


Figure 29. Costimulator production by CBA/J spleen cells treated with anti-Ly 1.1, 4.1, 5.1, 6.1 and 7.2 and anti-Ia(k) antisera. Spleen cells were treated with the antisera at 1/20 dilution and complement, and cultured with Con A to produce costimulator. The supernatants were assayed in the thymocyte proliferation assay. The cells killed by treatment with the antisera are given in parenthesis. a: 1 X 10^6 cells/culture. (■) untreated, (▲) NMS (5%). b-h: (●) cells surviving antiserum treatment of 1 X 10^6 cells in each culture. (o) treated cells, adjusted to 1 X 10^6 viable cells per culture. b: anti-Ly 1.1 (#458, 23%), c: anti-Ly 4.1 (45%), d: anti-Ly 5.1 (30%), e: anti-Ly 6.1 (14%), f: anti-Ly 7.2 (59%), g: mixture of cells treated with anti-Ly 1.1 and anti-Ly 7.2, both equivalent in number to 1 X 10^6 NMS treated, h: anti-Ia(K) (55%).

7.2 and Ia-(k) on costimulator production by CBA/CaJ spleen cells. CBA/J spleen cells were used in a similar experiment (Figure 29) which also shows the effects of anti-Ly 4.1 and 5.1 antisera. Costimulator production was Con A dependent in all of the experiments (cf Figure 28b). Antisera directed against Ly 1.1 (Figures 28a and 29b), Ly 5.1 (Figure 29d), and Ly 7.2 (Figures 28b and 29f) abolished costimulator production by spleen cells. These results were consistently observed using 4 different batches of anti-Ly 1.1 (6 experiments), 1 batch of anti-Ly 7.2 (4 experiments) and 1 batch of anti-Ly 5.1 (2 experiments). Treatment with anti-Ly 2.1 had no effect on costimulator production, a result confirmed in 2 additional experiments. This same preparation of anti-Ly 2.1 serum did eliminate cytotoxic and suppressor T cells, however (A. Al Adra, Immunogenetics, in press). Treatment with anti-Ly 4.1 decreased costimulator production insignificantly (Figure 29c). Anti-Ly 6.1 reduced costimulator yield slightly in one experiment (Figure 28b) but had no effect in another (Figure 29c), although an aliquot of these anti-Ly 6.1 treated cells showed reduced generation of helper and suppressor cells (L. Pilarski, personal communication) and of killer cells (Table 18, below). Treatment of cells with anti-Ia(k) serum led to some reduction in costimulator production, more in one experiment (Figure 29h) than another (Figure 28b). Figure 29g shows that the ability to produce costimulator is not restored with a mixture of anti-Ly 1.1 and anti 7.2 treated cells,

indicating that these two markers are on the same cells.

Since treatment with some of the antisera killed a high proportion of the spleen cells, the abrogation of costimulator production might have been due to a decreased cell density (cf Figures 17 and 18). Therefore, in Figure 29, treated cells were cultured both at concentrations which reflected the amount of killing (those surviving treatment of 10^6 cells with antiserum), and also at $1x10^6$ surviving viable cells per culture. The costimulator yield from anti-Ly 1.1, 5.1 and 7.2 treated cells was not improved at the higher cell number, eliminating the possibility that cell density effects alone were responsible for the results. Costimulator production by anti-Ly 4.1 and anti-Ia(K) treated cells was enhanced in the higher cell density culture, either as a result of adding nearly twice as many of the relevant cells, or as a consequence of correcting the cell density to control levels.

The abrogation of costimulator production after killing of Ly 1.1^+ cells was also observed using a monoclonal anti-Ly 1.1 (147A, Figure 30) from a hybridoma. The use of monoclonal antibodies eliminates the possibility that irrelevant markers on cell surfaces are responsible for the effects observed. Unfortunately, such antisera are not available for the other Ly specificities.

These results indicate that the phenotype of cells required for costimulator production is Ly $1^+,2^-,5^+,6^-,7^+$. Cells bearing Ly 4.1 and Ia(k) antigens may be involved, but

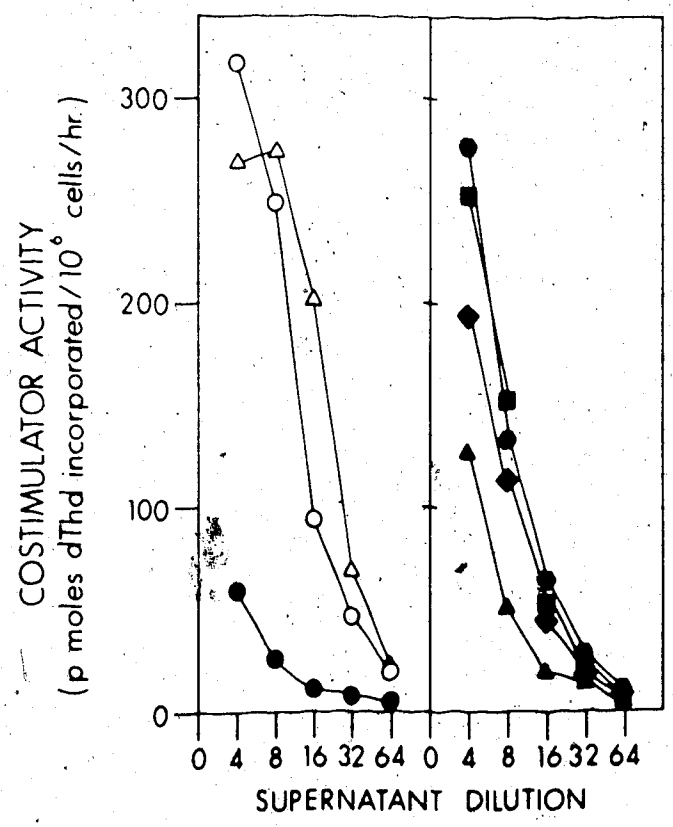


Figure 30. Effect of monoclonal anti-Ly 1.1 on the ability of spleen cells to produce costimulator. Costimulator generating cultures consisted of 1 X 10^6 CBA/CaJ splenocytes in 0.1ml RHF containing 1.5 microgram/ml Con A. Cells were treated with various antisera and complement before culturing. The monoclonal antiserum (147A) was tested at several concentrations. Cultures were terminated after 24 hr, and the supernatants titrated in the thymocyte proliferation assay. The number of cells killed by treatment with the antisera is given in parenthesis. Left: (△) untreated; (o) NMS + complement control (4%); (●) anti-Ly 1.1, #466, 1/20 (33%). Right: monoclonal anti-Ly 1.1, #147A, (▲) 1/25 (37%); (◆) 1/50 (42%); (■) 1/100 (28%); (●) 1/200 (24%).

do not appear to be an absolute requirement.

C. Discussion

This chapter describes experiments determining the optimal conditions for costimulator production, and defining the cells responsible for its secretion.

Conditions for Costimulator Production

Costimulator is secreted between 4 and 21 hr of stimulation of spleen or lymph node cells with Concanavalin A (Figures 19 and 20). The optimum cell density is 10×10^6/ml; below 2.5×10^6/ml, the efficiency of production on a per cell basis declines. The G-100 Sephadex profiles of the activity produced with and without serum are the same. The length of time in culture (+ serum), up to 72 hours, does not appear to alter the size of the active species (Figure 21).

A factor which runs similarly to costimulator on a Sephadex G-100 column is produced in a 2-way MLC between BALB/c and CBA spleen cells. Although allogeneic effect factor (AEF) (Delovitch and McDevitt, 1977; Delovitch *et al.*, 1978) is also produced in MLC, the factor we observe appears to be different. AEF activity is not obtained in a primary MLC; the cells used to produce it must be primed *in vivo* with alloantigen (T.L. Delovich, personal communication). AEF also elutes in a different position from Con A or MLC-derived costimulator on G-100 Sephadex, with a Kd slightly smaller than BSA, which has a molecular weight

of 67,000 (J. Shaw and T.L. Delovitch, unpublished results). These considerations indicate that MLC derived "costimulator" is not AEF.

Cells Required for the Production of Costimulator

Costimulator production requires cells that are Ly $1^+,2^-,5^+,6^-,7^+$ (Figures 28 and 29), a phenotype concordant with that of the T helper cells for CTL responses, characterized as Ly $1^+,2^-,5^+,Ia^-$ (Al-Adra *et al.*, 1980), and Ly $6^-,7^+$ (Pilarski *et al.*, 1980). The same antisera, and sometimes the same cells were used to characterize costimulator production and helper T cell function for CTL. Of the markers tested which did not affect costimulator production, Ly 2.1, 4.1, 6.1 and Ia, there are positive controls indicating that the antisera against Ly 2.1 and Ly 6.1 were active. (A. Al-Adra and L. Pilarski, personal communication). Although antisera against Ly 4.1 and Ia(k) killed a significant proportion of the cells (Figures 28 and 29) no functional tests which would serve as controls for postive effects of the sera were performed with them. Swain and Dutton (1980), who also used anti-Ia(k) antisera prepared by Dr. I.F.C. McKenzie, have reported that an Ia^+ non-T cell is required for the production of a Con A-induced helper T cell replacing factor. It is puzzling that such a cell was not detected in our experiments since we obtained the same levels of cytotoxicity on normal spleen cells with the antiserum and rabbit complement as they did (40-65%). Of the antigens not present on costimulator producing cells, Ly

2 is associated with alloreactive CTL, their precursors, and suppressor cells (Cantor and Boyse, 1976b), and Ly 6 with cytotoxic effector and suppressor (Pilarski *et al.*, 1980) T cells. The active cell in costimulator production is apparently different from the Ly 1^+2^+ amplifier cell described by Feldmann *et al.* (1977). The latter cell type may be similar to the thymic lymphocyte which synergizes with lymph node cells in the induction of GVHR (Cantor and Asofsky, 1970). The costimulator producing cell may be similar to the cell required for the MLC-stimulated production of TRF (Pickel *et al.*, 1976). The helper T cells which induce B cells to secrete antibody are also Ly 1^+, 2^- (Cantor and Boyse, 1976b).

The A cell required for costimulator production is clearly not an adherent T cell (Arala-Chaves *et al.*, 1978), since it is present in anti-Thy 1 treated spleen cells, which by themselves are unable to produce the factor (Figure 25). Several lines of evidence support the conclusion that the A cell is a macrophage. Essentially pure macrophages cultured from bone marrow are a source of the required A cell (Table 14). In addition, peritoneal macrophages, separated from small lymphocytes by velocity sedimentation, promoted costimulator production by A cell-depleted lymph node cells (Figure 26). The active cells were 96% macrophages, and contained less than 2% lymphocytes. The macrophage-rich populations did not produce the factor unless T cells were added. Finally, the A cell requirement

for costimulator production could be at least partially met by supernatants from the LPS-stimulated macrophage cell line P388-D1 (Figure 27). Preliminary analysis of the active mediator in the macrophage supernatants indicate that it is probably the same as the macrophage factor LAF (IL1) described by Lachman *et al.* (1979a), Economou and Shin (1978) and Mizel (1980) (B Caplan and G. Mills, personal communication).

In summary, these results are consistent with a model in which helper T lymphocytes secrete costimulator upon stimulation with Con A, and macrophages act as auxiliary cells. The role of the macrophage may be to provide a signal to the T cell in the form of a soluble factor, LAF. The Ly phenotype of the helper T cells for CTL responses, Ly $1^+,2^-,5^+,6^-,7^+$, is the same as that of the costimulator-secreting cells.

V. Effects of Costimulator on Immune Responses *in vitro*

A. Introduction

Costimulator activity was initially detected as a requirement for the Con A-induced proliferation of thymocytes cultured at low cell density (Paetkau *et al.*, 1976). The purpose of the experiments in this chapter was to explore the effects of the factor in other *in vitro* immune responses, in order to acquire information about its possible biological role. The systems employed include responses to mitogens other than Con A, antibody responses, and CTL responses.

The work of others suggested that crude preparations of factors produced under the same conditions as costimulator act to promote AFC responses *in vitro* under conditions where helper T cells are limiting (Watson, 1973; Hunter and Kettman, 1974; Dutton *et al.*, 1971; Schimpl and Wecker, 1973). Data are presented which indicate that costimulator, defined by its activity in the thymocyte proliferation assay, copurifies with the T cell replacing activity active in AFC responses.

The finding that costimulator is produced during a primary MLC reaction (Chapter IV, Figures 23 and 24) prompted an examination of its effects on the proliferative and cytotoxic phases of allogeneic responses. The identification of the costimulator producing cell as an Ly 1^+, 2^-, 7^+ cell (Chapter IV, Figure 28 and 29) suggested

that it may be a cell with helper T cell activity in CTL responses (Pilarski, personal communication). The demonstration that crude costimulator overcame the requirement for a metabolically active, lymphoid-derived stimulator cell in CTL responses (Talmage *et al.*, 1977) further supported the view that it replaces a second signal required for CTL generation. Helper T cells have been shown to promote an otherwise poor CTL response under three experimental conditions: (1) when thymocytes are used as a source of CTL precursors (Pilarski, 1977) (2) when splenic CTL precursors have been treated with anti-Ly 7.2 and complement (Pilarski *et al.*, 1980) and (3) when metabolically inactivated stimulator cells are used (Pilarski, 1979; Lafferty and Woolnough, 1977). The ability of costimulator to replace the requirement for helper T cells in these three situations will be described in this chapter. Again, the activity in CTL responses copurifies with the activity in the thymocyte proliferation assay.

Several other aspects of the role of costimulator in CTL responses were investigated. The CTL generated using costimulator were found to be antigen specific. As with mitogen responses, costimulator was not H-2 restricted in its CTL generating activity. The time course of addition and removal of factor from cultures shows that it is required during both the initial and late phases of a CTL response. Costimulator has activity not only in CTL responses against alloantigens, but against TNP-modified self antigens.

Since macrophages are necessary for costimulator production (Chapter IV, Figure 25 and Table 14), the question arises as to whether the macrophage requirement for CTL generation and other T cell responses *in vitro* might reflect a need for costimulator to be produced in the cultures. Experiments are described which indicate that costimulator can augment CTL and T cell proliferative responses to antigen if the responding lymphocytes have been depleted of macrophages.

The working hypothesis is that macrophages and helper T cells interact upon stimulation (probably by means of the macrophage product IL1), and helper cells secrete costimulator. The simplest model to explain the function of costimulator is that it acts on antigen-sensitive lymphocytes, in lieu of helper T cells, to promote their proliferation and differentiation.

B. Results

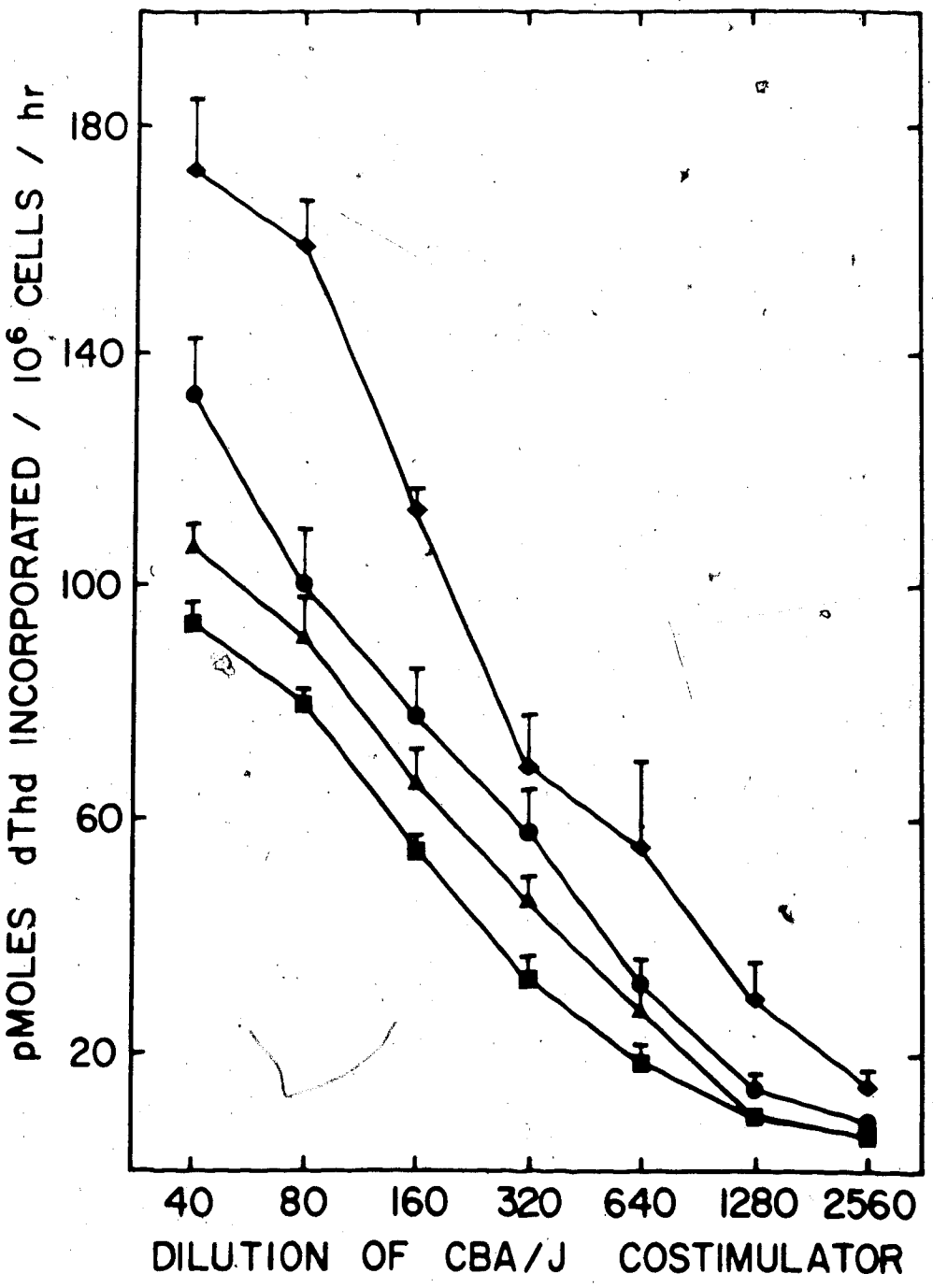
Effects of Costimulator on Proliferative Responses to the Mitogens Con A, LPS and PHA

The role of costimulator in the proliferation of CBA/J thymocytes in response to Con A has been extensively discussed in Chapter III. Figure 31 shows that costimulator is not H-2 restricted in this assay. Costimulator prepared from CBA/J (H-2k) spleen cells stimulated the Con A response of not only CBA/J, but also BALB/c (H-2d), DBA/2 (H-2d) and C57B1/10 (H-2b) thymocytes. Normal murine spleen cells,

Figure 31. Lack of H-2 restriction of costimulator activity in the thymocyte proliferation assay. The thymocyte proliferation assay was carried out as described in Materials and Methods, using CBA/CaJ costimulator fraction 3, and thymocytes from the following mouse strains:

(◆) BALB/cCr (H-2d)
(●) CBA/J (H-2k)
(▲) DBA/2J (H-2d)
(■) C57B1/10J (H-2b).

The background response with and without Con A, respectively, for the 4 thymocyte preparations was: BALB/c, 9.1, 0.7; CBA, 3.5, 0.8; DBA, 4.9, 1.2; C57B1/10, 3.0, 0.7.



unlike thymocytes, responded well to Con A. When costimulator was added to Con A-stimulated CBA/J or BALB/c spleen cell cultures, a 2-3 fold enhancement of the 72 hr proliferative response was observed (Table 15). The magnitude of the response of T-depleted (anti-Thy 1.2 treated or nu/nu) spleen cells was not enhanced significantly by costimulator. This finding is not concordant with that of Gillis and Smith (1979), who reported a good proliferative response of T-depleted spleen cells to Con A in the presence of TCGF. The discrepancy might be due to the use of crude factor by Gillis and Smith, and fraction 3 costimulator in Table 15. Costimulator synergized with the T cell mitogen PHA to allow CBA/J thymocytes to undergo a proliferative response comparable in magnitude to that obtained with Con A. The time course of the response using different concentration of CBA/J thymic responder cells is shown in Figure 32. The response of thymocytes to PHA in the absence of added costimulator was negligible.

In contrast to the enhancing effect of costimulator in T cell mitogen responses, there was no effect when saturating levels of the factor were added to cultures of spleen cells stimulated by the B cell mitogen LPS (Figure 33). This was true at several cell concentrations, and at both optimal and suboptimal concentrations of LPS. This lack of activity of costimulator in a B cell response may indicate that B cells are not a target for the factor.

Table 15

Response of normal, anti-theta-treated, and nude spleen cells to Con A and costimulator[1]

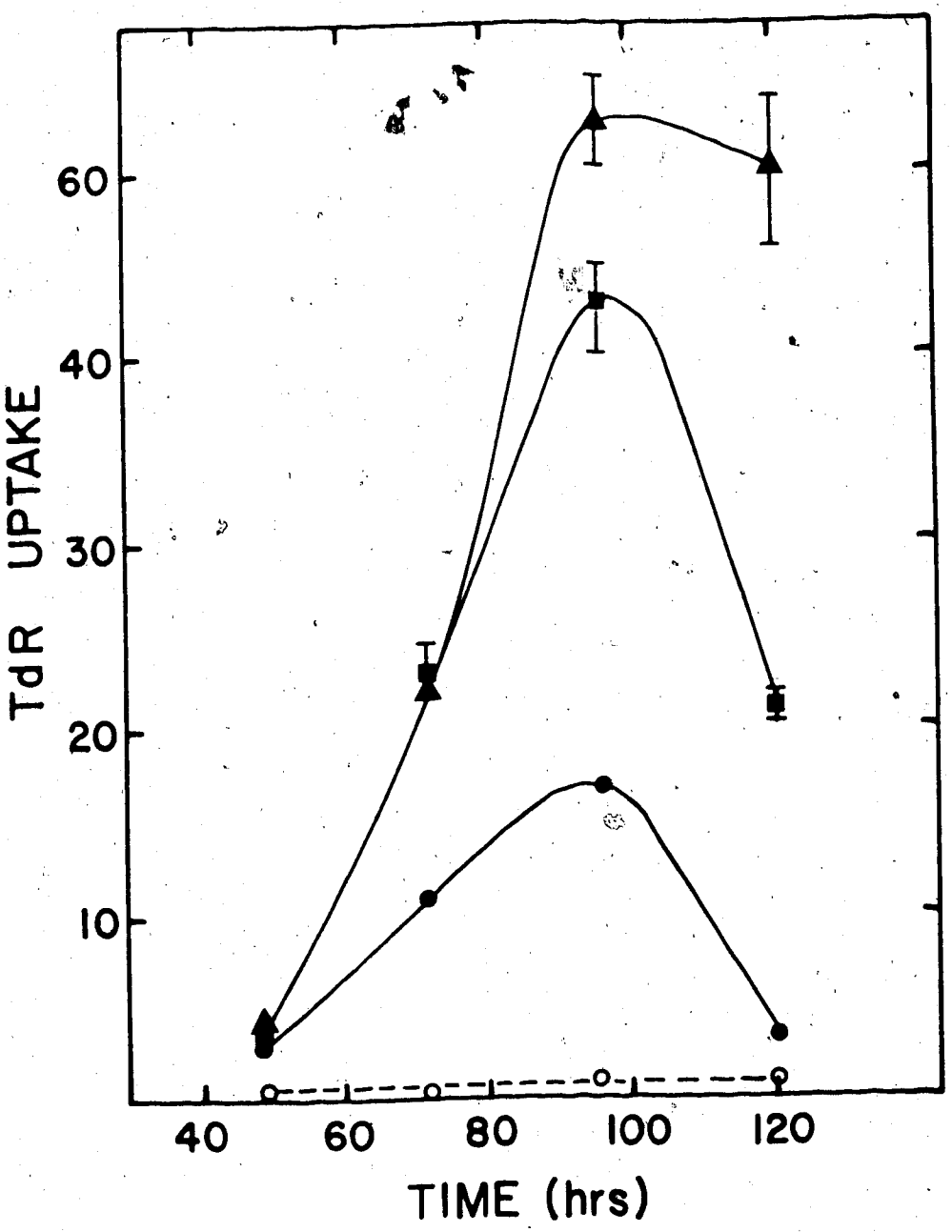
Spleen Cells	Con A	Costimulator -	Costimulator +
Complement-treated CBA/J	-	2	10
	+	115	434
Anti-Thy 1.2 + complement-treated CBA/J	-	3	12
	+	5	12
BALB/c	-	4	7
	+	141	292
BALB/c (nu/nu)	-	2	7
	+	2	5

[1] The spleen cells were cultured at 1.25×10^5/ml in the presence or absence of 3 microgram/ml of Con A and fraction 3 Con A-generated costimulator (CBA/J). The response was measured after 72 hr of culture (similar results were observed at 48 and 96 hr.)

Figure 32. Stimulation of thymocyte proliferation by PHA in the presence of costimulator. Thymocytes from 5 week old CBA/J mice were incubated at various concentrations with PHA (1/100), with or without fraction 3 costimulator. Uptake of ^{3}H-dThd was measured (pmoles/hr/10^{6} cells) at the times indicated on the abcissa. Cell concentrations and conditions:

(o) $4x10^{6}$/ml no costimulator
(•) $4x10^{6}$/ml plus costimulator
(▪) $2x10^{6}$/ml plus costimulator
(▲) $1x10^{6}$/ml plus costimulator.

Controls having 1 and $2x10^{6}$ cells/ml without costimulator gave values below 1 pmole/hr/10^{6} cells at all times (data not shown). The results using twice the concentration of PHA (1/200) gave qualitatively the same results, with the maximum rates of thymidine uptake shifted to 120 hr (data not shown). Ms. V. Monticone provided technical assistance in this experiment.



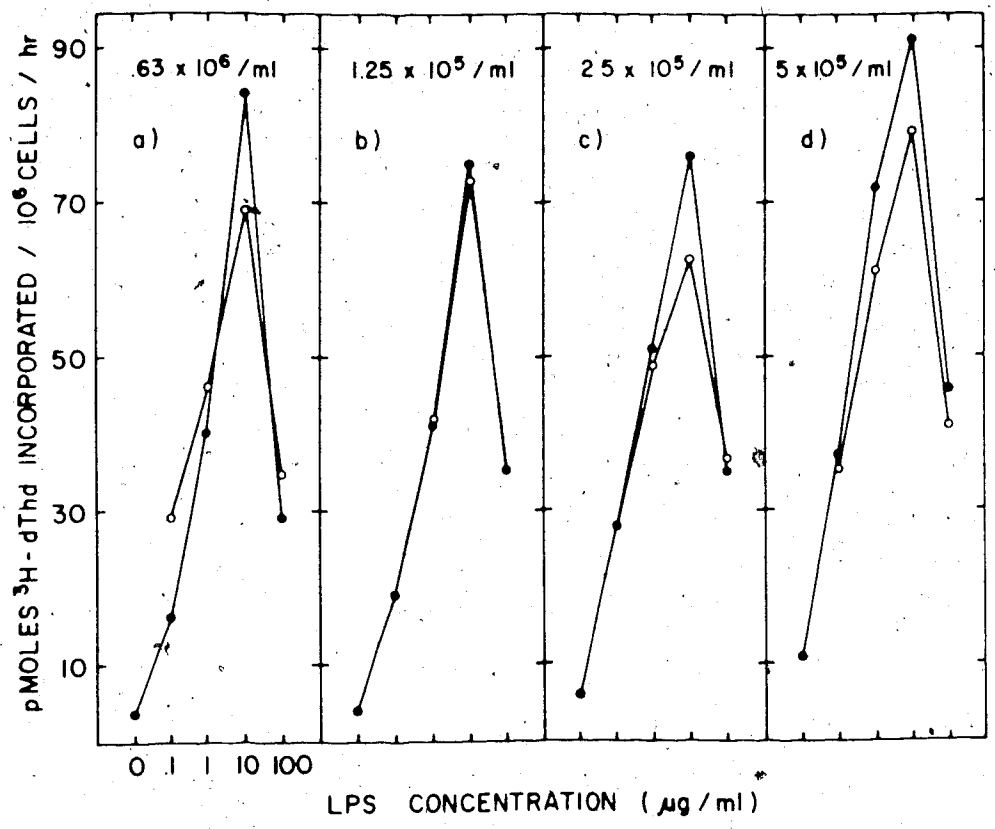


Figure 33. Failure of costimulator to enhance the proliferative response of CBA spleen cells to the B cell mitogen LPS. CBA spleen cells were incubated at the concentrations shown with or without LPS at various concentrations in flat bottom microtitre wells for 72 hr. CBA costimulator (fraction 3) was added to half the cultures at the level previously determined to be optimal in the thymocyte proliferation assay. The rate of DNA synthesis was determined as for the thymocyte assay. (•) no costimulator; (o) plus costimulator.

Effect of Costimulator on the Proliferative Response in an Allogeneic MLR

The ability of Con A-generated fraction 3 costimulator to enhance the level of DNA synthesis in an allogeneic MLC reaction is shown in Table 16. The degree of enhancement was most marked at dilute cell concentrations, perhaps because the level of endogenous costimulator production (by allogeneic stimulation, as in Chapter IV, Figure 23) was limiting. However, the factor by which the proliferative response was enhanced is considerably less than that observed for the cytotoxic activity in MLC (below). This might reflect the use of normal splenic responder and stimulator cells (rather than thymocytes or metabolically-inactivated cells) for the proliferative response, or an actual difference in the sensitivity of the cells proliferating in MLC and cytotoxic cells as targets for the factor.

Costimulator Allows the Generation of CTL from Thymocyte Precursors, and Enhances their Generation from Splenic Precursors

It was pointed out in the introduction to this chapter that thymocytes are normally poor responders in a CTL response, because they contain few mature helper T cells (Cantor and Boyse, 1977; Pilarski, 1977). We examined the ability of costimulator to replace the requirement for helper T cells in such a response. The CTL data to follow are mainly expressed in terms of a parameter, KA, which is

Table 16

Effect of costimulator on DNA synthesis in a one-way MLC[1]

BALB/c responder cells(H-2d) (X 10^{-6})	Irradiated stimulating cells	DNA Synthesis at 66 hr (±S.D.) Costimulator -	Costimulator +
0.63	CBA/J(H-2k)	8.1 ± 0.9	20.3 ± 2.6
0.31	CBA/J(H-2k)	2.2 ± 1.0	11.1 ± 0.7
0.15	CBA/J(H-2k)	1.2 ± 0.6	8.3 ± 2.3
0.63	BALB/c	0.4 ± 0.1	3.0 ± 0.1
0.31	BALB/c	0.4 ± 0.1	2.6 ± 0.2
0.15	BALB/c	0.7 ± 0.3	2.5 ± 0.8

[1] Spleen cells from BALB/c mice were cultured with either irradiated allogeneic spleen cells (CBA/J) or syngeneic spleen cells as stimulators. The stimulating cells were equal in number to the responding cells. The culture volume was 0.5 ml. DNA synthesis was measured as pmoles of ^{3}H-dThd/hr/10^{6} cells. Human serum, rather than FBS was used in the culture medium.

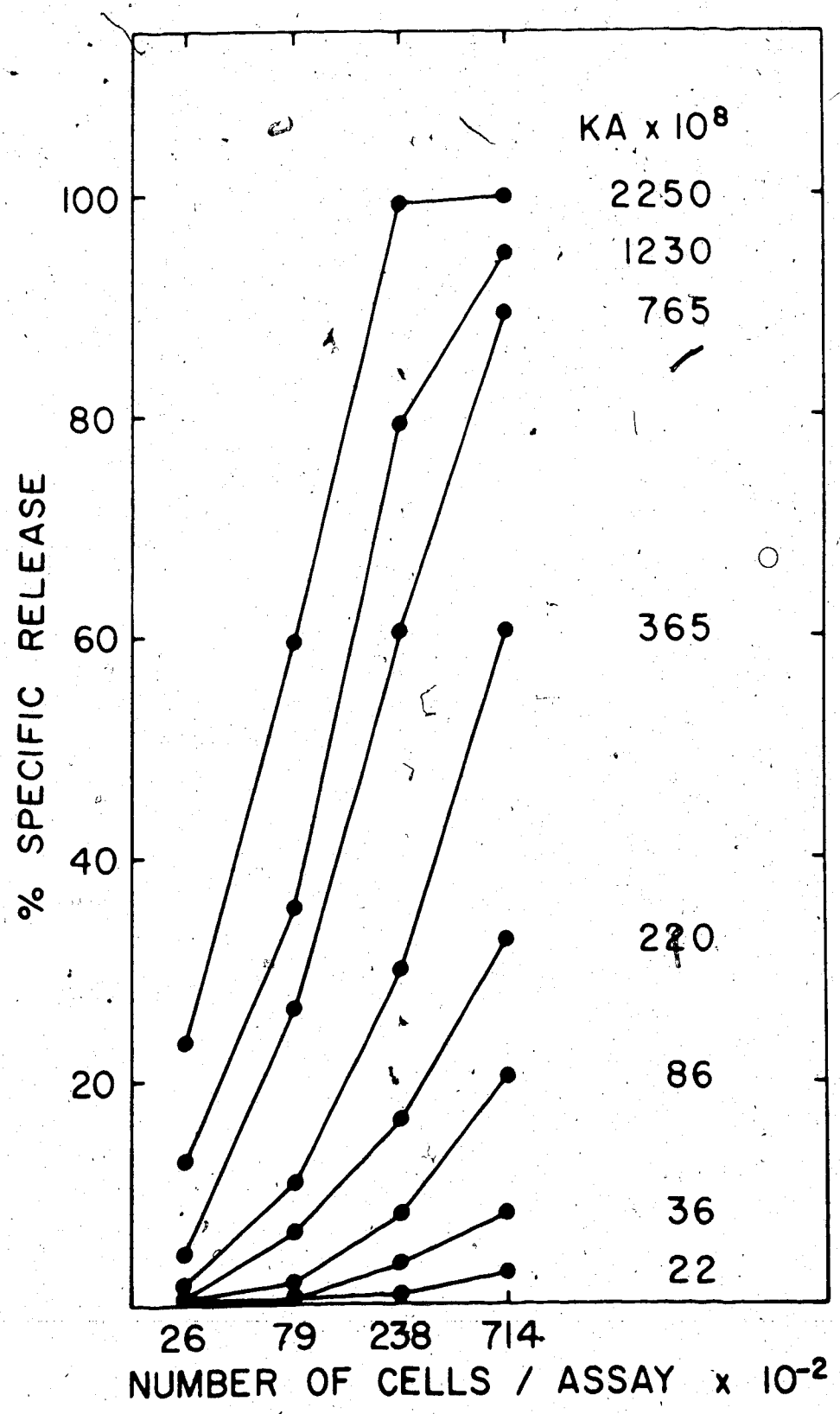
proportional to the number of cytotoxic cells in a culture (Materials and Methods). Figure 34 relates KA to the specific ^{51}Cr release observed in a typical experiment.

The CTL generating-activity found in crude costimulator preparations copurified with the activity in the thymocyte proliferation assay. CBA/J (H-2k) spleen cells responded to the allogeneic tumor cell P815 (H-2d) much more strongly if either fraction 3 (G-100 Sephadex-purified) or fraction 5 (IEF-purified costimulator) was added to the cultures (Table 17). The units of activity of a given costimulator preparation in the thymocyte proliferation assay correlated with its ability to stimulate CTL generation, suggesting that the two activities copurify. Table 17, Experiment 2, also shows that MLC-generated costimulator (fraction 2) is active in promoting the CTL response. Copurification of the activity detected in the thymocyte proliferation assay, and the ability to allow the generation of CTL from thymocyte precursors is shown in Figure 35. Fractions from a DEAE Sephacel column to which fraction 3 costimulator had been applied were tested in the thymocyte proliferation and CTL assays. Both activities were eluted from the resin at about 0.13 M NaCl, free of over 90% of the input protein. No further activity in either assay was recovered by elution with higher salt concentration (not shown).

Figure 34. Correlation between values calculated for KA x 10^8 ("Killing activity") and the fraction of ^{51}Cr released from target cells in a CTL assay. The curves represent experimental data obtained from cultures in which CBA spleen cells were cultured with (CBA x DBA) F1 splenic stimulator cells. Four 3-fold dilutions of each culture were assayed; cultures were done in quadriplicate. The number of starting cells represented in an assay well is shown on the abcissa. There were 30,000 P815 target cells per assay well. The parameter KA, used to express CTL activity, is directly analogous to the alpha parameter defined by Miller and Dunkley (1974):

$$F = 1-\exp(-N \bullet KA \bullet t)$$

where F is the fraction of specific lysis of ^{51}Cr-labelled target cells, N is the number of starting responder cells represented by the CTL and t is the assay time, in hours. The maximum ^{51}Cr release (detergent lysis) was 3802 cpm. The background, or spontaneous release was 355 cpm.



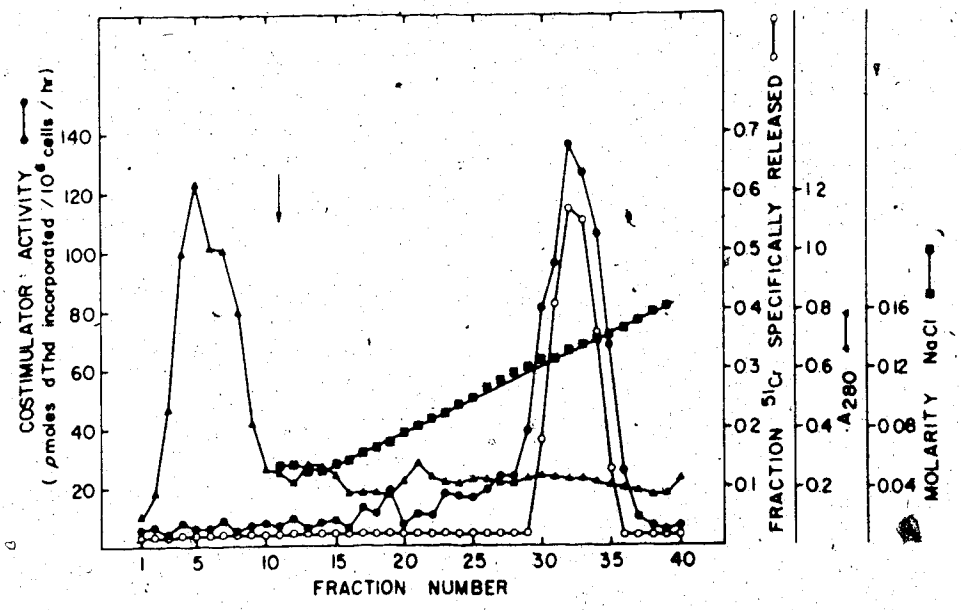


Figure 35. Copurification on DEAE Sephacel of costimulator activities which promote the Con A and CTL responses of thymocytes. Fraction 3 costimulator prepared from spleens of Swiss mice was passed through a DEAE column and eluted with a linear gradient of NaCl. The point at which the gradient (0.05 - 0.35 M NaCl) was applied is indicated with an arrow. The fractions (4.4ml) were analyzed for costimulator activity at 1/50 dilution in the thymocyte proliferation assay, and for activity in generating CTL at 1/40 dilution. The CTL generating cultures contained $3x10^5$ CBA thymocyte responders per well, and $3x10^3$ irradiated EL4 tumor cells as stimulators. The assay was performed on only one dilution of cultured cells. Data are expressed as the fraction of ^{51}Cr specifically release. Fractions were also assayed for CTL generating activity at 1/20 and 1/80 dilution, which resulted in the same peak of activity (data not shown). The DEAE chromatography and the thymocyte proliferation assay were done by Cliff Gibbs.

Table 17

Correlation between stimulation of thymocyte proliferation and stimulation of CTL responses by costimulator at various stages of purification[1]

Costimulator preparation (fraction and units/ml)	System for generating	KAx10^8 -Ag	KAx10^8 +Ag (P815)
Experiment 1:			
None		<4	13
3, 0.8 u/ml	Con A	<4	12
3, 3.1 u/ml	Con A	5	190
5, 0.5 u/ml	Con A	<4	22
5, 1.0 u/ml	Con A	<4	97
5, 2.0 u/ml	Con A	7	120
Experiment 2:			
None		<2	[illegible]
3, 0.39 u/ml	Con A	<2	25
3, 0.78 u/ml	Con A	5	65
3, 1.6 u/ml	Con A	8	250
3, 3.1 u/ml	Con A	55	645
5, 0.5 u/ml	Con A	5	235
5, 1.0 u/ml	Con A	5	275
2, 1/1280	MLC	<2	47
2, 1/320	MLC	<2	87
2, 1/80	MLC	6	410
2, 1/40	MLC	27	735

[1] CTL-generating cultures contained 1.5 x 10^5 CBA/J (H-2k) splenic responder cells, and 1 x 10^4 irradiated P815 (H-2d) cells as antigen, in 0.2 ml of MHFM medium. Con A- or MLC- (CBA/J versus BALB/c, see Chapter IV, Figures 23 and 24) induced costimulator was added to some of the cultures after various steps of purification. Fraction 2 has been chromatographed on Sephadex G-25, fraction 3 on Sephadex G-100. Fraction 5 has been subjected to isoelectric focusing and then rechromatographed on G-100. The units of activity/ml in the left hand column refer to the activity of the preparation in the thymocyte proliferation assay. Cultures were assayed using 3 x 10^4 ^{51}Cr-labelled P815 cells as targets.

Costimulator Replaces the Ly 7+ Cell in CTL Responses

The results in Chapter IV, Figures 28 and 29, show that the T cell involved in costimulator production is Ly 1+, 7+, which is the phenotype of a helper effector cell (Pilarski, 1980). It was therefore determined whether the factor could meet the requirement for helper T cells in CTL generation. In the system studied (CBA mice) we could separate helper T cells from CTL precursors by treatment with antiserum against Ly 7.2. CBA spleen cells were treated with this antiserum, and other antisera to serve as specificity controls, and cultured for 5 days with semi-allogeneic splenic stimulator cells with or without costimulator (Table 18). Treatment of precursors with anti-Ly 1.1, 5.1 and 7.2 reduced the CTL response to undetectable levels. In both experiments shown in the table, the response to anti-Ly 7.2 treated cells was restored by the addition of costimulator. The response of Ly 1.1 treated cells was not improved, as would be expected if the precursors had been killed by the antiserum. The ability of the factor to restore the CTL activity of anti-Ly 5.1 treated cells in experiment 2, and the failure to do so in experiment 1, is probably a reflection of the level of cytotoxicity obtained with the antiserum in the two experiments (30% and 87%, respectively, in experiments 2 and 1, using the antiserum at 1/20 and 1/7.5). Residual CTL precursors may have been present in experiment 2. Less drastic reductions in killer cell activity were observed with anti-Ly 4.1 and 6.1 treated

Table 18

Effect of costimulator on the CTL response of CBA spleen cells treated with Ly antisera[1]

Antiserum used to treat responder cells	% Cells killed by treatment	KA X 10^8 -Costimulator	KA X 10^8 +Costimulator
Experiment 1			
NMS	0	1050	1900
anti-Ly 4.1	52	934	1860
anti-Ly 5.1	87	10	10
anti-Ly 6.1	68	544	716
anti-Ly 7.2	88	10	688
Experiment 2			
NMS	7	202	690
anti-Ly 1.1(#466)	23	4	4
anti-Ly 4.1	45	136	597
anti-Ly 5.1	30	4	420
anti-Ly 6.1	14	85	460
anti-Ly 7.2	59	4	317

[1]Spleen responder cells were treated with the antisera indicated plus complement and cultured with irradiated stimulator cells in the presence or absence of costimulator generated from CBA/CaJ mice. The antisera were used at 1/7.5 dilution in Experiment 1 and at 1/20 dilution in Experiment 2. *Experiment 1*: Responders were 1.5 X 10^5 CBA/CaJ (H-2k) spleen cells, or the equivalent number of treated cells. Stimulators were 1.5 X 10^5 (C3H/HeJ X DBA/2J)F1 (H-2k/d) spleen cells. *Experiment 2*: Responders were 2.0 X 10^5 CBA/J (H-2k) spleen cells or equivalents; stimulators were 2.0 X 10^5 (CBA/J X DBA/2J)F1 (H-2k/d) spleen cells. ^{51}Cr-labeled P815 cells (H-2d) were used as targets in both assays. Fraction 3 and 4 costimulator were used in experiments 1 and 2, respectively. The cytotoxic activity in cultures, assayed on day 5, is expressed in terms of the parameter KA (see Materials and Methods), which is proportional to the number of killer cells. Cultures in which responder cells were incubated alone gave values of KA ranging from <10 to 33 X 10^{-8} (Experiment 1), and <4 X 10^{-8} (Experiment 2). Responder cells cultured with costimulator, but no antigen, gave KA from <10 to 84 X 10^{-8} (Experiment 1) and from <4 to 14 10^{-8} (Experiment 2).

precursors. In summary, the requirement for Ly 7^+ helper cells for CTL responses appears to be met by costimulator.

Costimulator Restores CTL Responses Against Stimulator Cells Inactivated by Glutaraldehyde Fixation or UV Irradiation

Treatment of allogeneic cells used as stimulators in CTL cultures with either glutaraldehyde or UV light renders them non-immunogenic (Lafferty and Woolnough, 1977). Addition of antigen-specific helper cells to cultures containing glutaraldehyde fixed stimulator cells restores the CTL response (Pilarski, 1979), and addition of crude supernatants from Con A stimulated spleen cells restores the immunogenicity of UV irradiated stimulators (Talmage *et al.*, 1977). Thus the alloantigens on stimulator cells appear to be effective after these two types of treatment, but their capacity to serve as stimulators has been lost. We examined the effect of costimulator fractions 3 and 4 on the immunogenicity of glutaraldehyde fixed and UV irradiated (CBA x DBA)F1 spleen cells in a CTL response mounted by CBA spleen cells. Figure 36 shows that costimulator restored the response against glutaraldehyde treated stimulator cells, if the concentration of glutaraldehyde used for the 3 minute treatment was less than 0.1% (v/v). At glutaraldehyde concentrations over 0.1%, the antigens on the cell surface may have been altered sufficiently not to resemble antigen expressed on a live target cell.

Exposure of stimulator cells to a 15 Watt UV lamp for 0.1 min at a distance of 11 cm was sufficient to competely

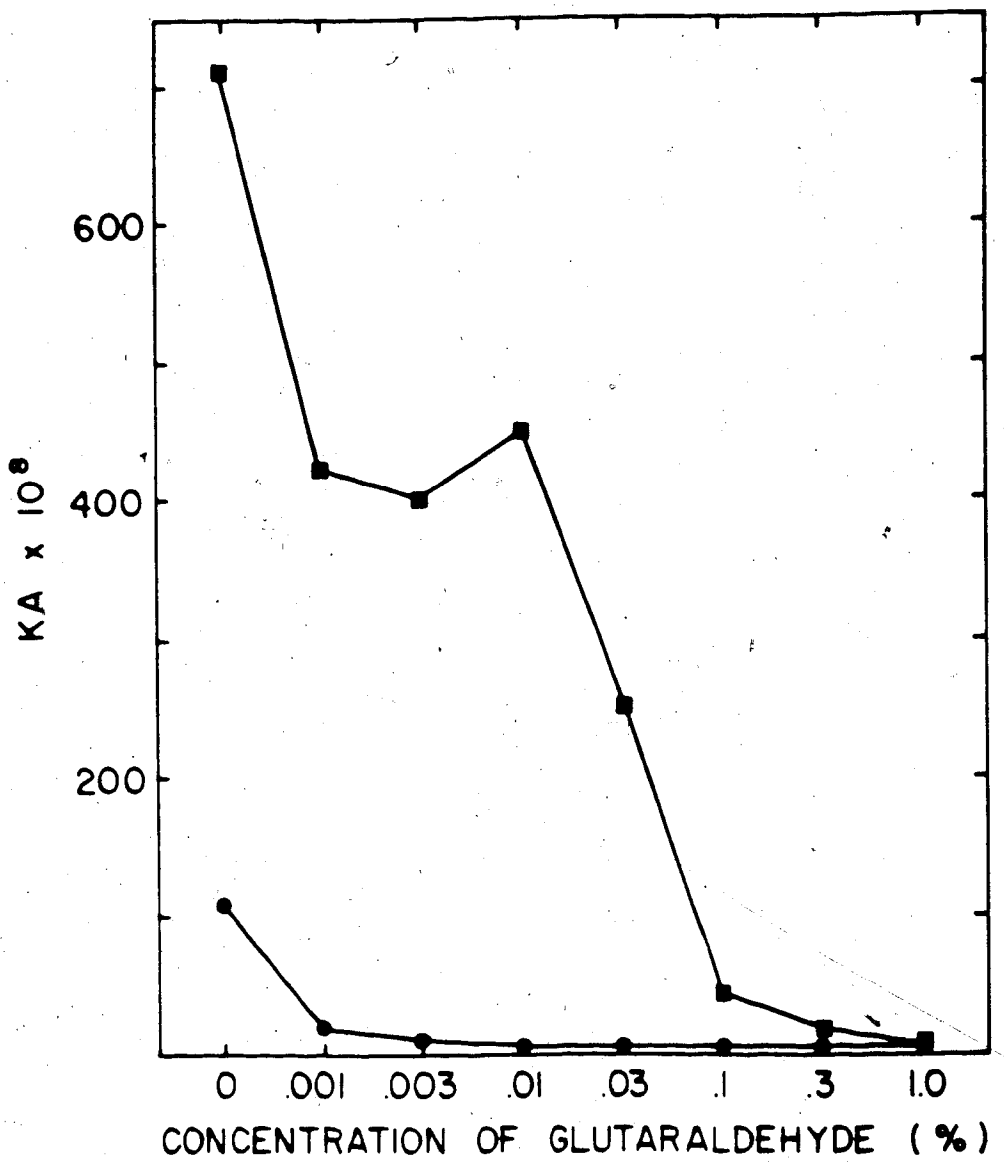


Figure 36. Costimulator restores the CTL response against glutaraldehyde fixed stimulator cells. (C3H/HeJ X DBA/2J)F1 spleen cells to be used as stimulators were exposed to the concentrations of glutaraldehyde indicated (v/v) as in Materials and Methods, section R. CTL generating cultures contained 2.0 x 10^5 CBA/J splenic responder cells, and an equal number of irradiated stimulator cells in a 0.2 ml volume. Costimulator fraction 3 from CBA/CaJ mice was used at 4 units/ml in half the cultures:
(•) with costimulator
(▪) without costimulator.
Cultures were assayed on 3 x 10^4 ^{51}Cr-labelled P815 target cells.

abrogate the generation of CTL by responding CBA cells (Table 19, Experiment 1). Costimulator restored the immunogenicity of the stimulator cells, even when the irradiation period was increased to 16 minutes. Experiment 2 in Table 19 shows that the stimulator cells must be held at a minimum distance of 20 cm from the UV source to be completely inactivated.

The enhancement by costimulator of the normal CTL response of spleen cells seen in Figure 36 and Table 19 may reflect the use of somewhat suboptimal conditions for the cultures. These might include limiting numbers of helper cells in the responder spleen cell population, limiting numbers of macrophages, or a non-optimal ratio of stimulating to responding cells. (This latter factor was discovered subsequently; the optimal ratio was not consistent throughout the series of experiments, as had been assumed.) Another possibility is that lower affinity clones are triggered in the presence of factor but not in its absence. At any rate, the augmentation of a low normal response by costimulator in no way invalidates the qualitative conclusions indicated by the experiments.

Costimulator Promotes a CTL Response in the Presence of Limiting numbers of Stimulator Cells

One of the explanations for the lack of immunogenicity of metabolically inactivated stimulator cells is that they express a lower density of the relevant antigens. Thus if stimulators were inactivated using actinomycin D, they still

Table 19

Generation of CTL against UV irradiated stimulator cells in the presence of costimulator: effects of time of UV exposure and distance from source[1]

Minutes stimulator cells exposed to UV light[2]	KA x 10^8 -costimulator	KA x 10^8 +costimulator
no stimulators	<4	17
0	92	688
0.1	<4	711
0.25	<4	638
0.5	<4	708
1.0	<4	660
2.0	<4	557
4.0	<4	429
8.0	<4	361
16.0	<4	404
Experiment 2:		
Distance of stimulator cells from UV light(cm)[3]		
no stimulators	<10	<10
no UV exposure	190	1560
5	<10	610
10	<10	820
20	<10	980
30	72	980
40	87	1141
50	104	1060
60	137	1249

[1] CTL-generating cultures contained 1.5 x 10^5 CBA/J spleen responder cells and equal number of (CBA/J x DBA/2J)F1 stimulator cells per 0.21 ml. Costimulator fraction 4 (Swiss mouse-derived) was added to some cultures. All stimulator cells were gamma irradiated, and some also received ultraviolet (UV) irradiation as indicated. Cultures were harvested after 5 days and assayed on P815 targets. Killing activity in cultures is expressed as KA x 10^8.

[2] Cells exposed to UV light at a distance of 11 cm.

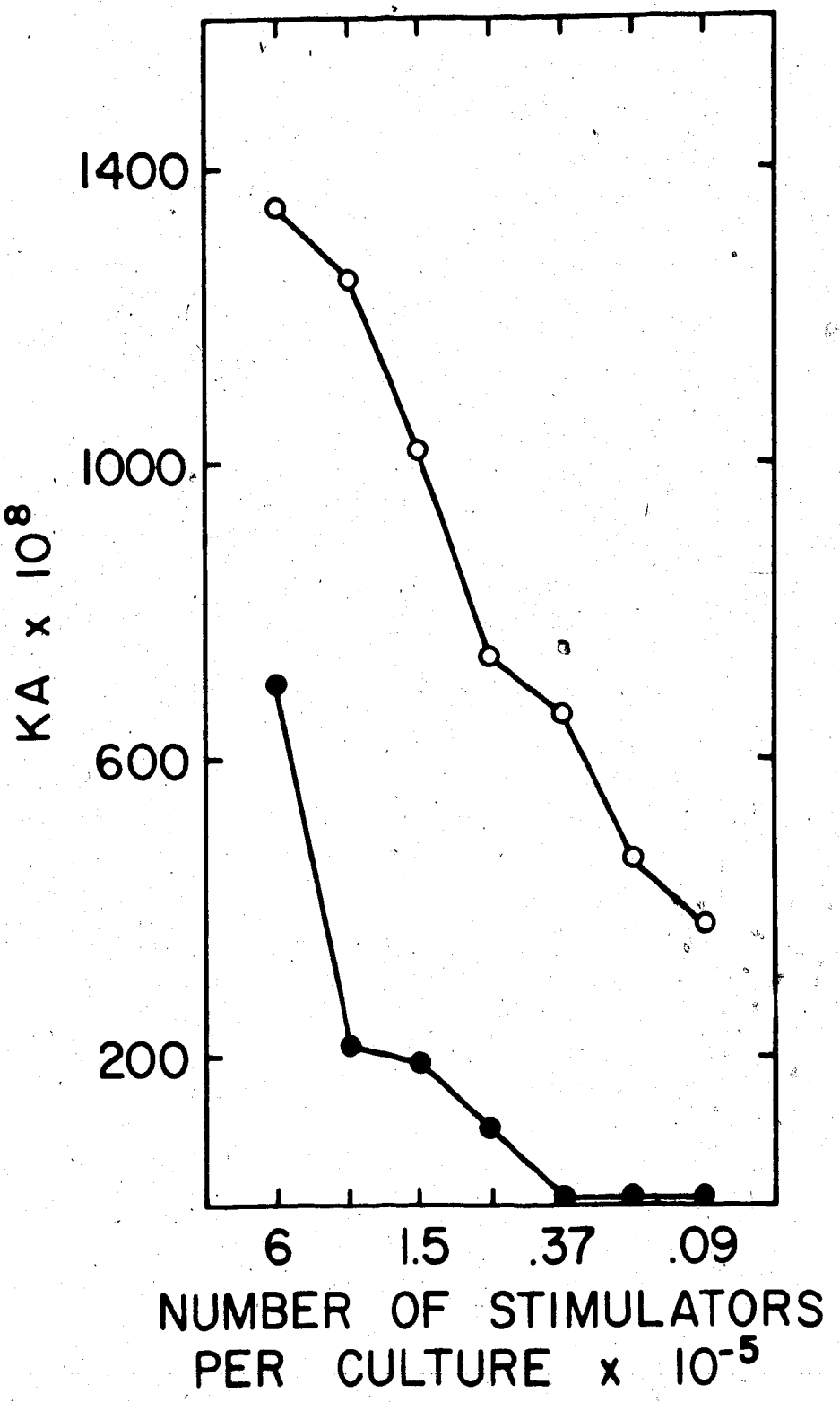
[3] Cells exposed to UV light for 10 seconds.

provoked a response if they were added to cultures in higher numbers (Wagner, 1973). The experiment in Figure 37 was designed to determine whether costimulator would help to generate a CTL response in the presence of limiting numbers of stimulator cells. The response of 1.5×10^5 CBA spleen cells to 6×10^5 (CBA x BALB/c)F1 stimulator cells was good, but as the number of stimulator cells decreased to 0.37×10^5, the response dropped to undetectable levels. Costimulator increased the response significantly at low antigen dose, although the responses at high antigen doses were even better if factor was added. The reason that the factor replaced the requirement for an optimal number of stimulator cells is unclear. One possibility is that the responder + stimulator interaction produces more endogenous costimulator at the optimum ratio. Another is that the responder's antigen-specific helper T cells are induced more efficiently at high doses of antigen. Costimulator might act to overcome such a requirement for helper T cells, as has been discussed above.

Generation of CTL Against TNP-Modified Self from Thymocytes in the Presence of Costimulator

For costimulator to have biological relevance in CTL generation *in vivo*, and for *in vitro* applications in cancer immunotherapy, the factor must be effective in promoting CTL against *autologous* cells which have become modified. One model system of CTL responses against "modified self" is the response against syngeneic lymphocytes which have been

Figure 37. Costimulator promotes a CTL response when the number of stimulator cells is limiting. Cultures contained 1.5×10^5 CBA/J spleen responder cells and the indicated number of irradiated (CBA/J x DBA/2J)F1 spleen cells as stimulators in 0.21 ml volume. Quadruplicate cultures were assayed on 3×10^4 ^{51}Cr-labelled P815 target cells.
(•) Cultured without costimulator.
(o) Cultures contained 4 units/ml of fraction 4 costimulator from Swiss spleen cells.



trinitrophenylated (Shearer, 1975). (DBA/2 x BALB/c)F1 or DBA spleen or thymus cells were cultured with TNP-modified spleen cells as stimulators with and without costimulator (Table 20). The response of thymus cells was negligible without added costimulator (KA x 10^8 <1), but a good response was observed if cultures contained factor (KA x 10^8=68). The response of spleen cells to TNP-modified self was enhanced in one experiment (1), but not in the other (2). The CTL from such cultures did not lyse P815 targets which had not been trinitrophenylated (experiment 2). The TNP-modified splenic stimulator cells were an absolute requirement for the response - unmodified gamma irradiated spleen did not stimulate (experiment 2). Thus costimulator is active in generation of anti-modified self, as well as anti-allogeneic CTL responses.

CTL Generated Using Costimulator are Antigen Specific, and Carry the Ly 1.1 Antigen

To ensure that the CTL generated from splenic and thymic precursors in the presence of costimulator are specific for the target cell which was used as antigen in the cultures, CBA/J (H-2K) cells were cultured with either P815 (H-2d) or EL4 (H-2b) tumor cells as stimulators, and then assayed on both targets. CTL generated in the presence of P815 and costimulator lysed mainly P815 targets, but showed some increase in their ability to lyse EL4 over that of cells which had been cultured either alone, or only with costimulator (Table 21). The lysis of EL4 was greater for

Table 20

Costimulator promotes a CTL response by thymocytes against TNP-modified syngeneic spleen cells[1]

Responder cells	Stimulator cells	Cos	KA x 10^8 (target cell)	
			TNP-P815	P815
Experiment 1:				
thymus	none	-	<1	ND
thymus	none	+	2	ND
thymus	TNP-spleen	-	<1	ND
thymus	TNP-spleen	+	68	ND
spleen	none	-	4	ND
spleen	none	+	8	ND
spleen	TNP-spleen	-	56	ND
spleen	TNP-spleen	+	126	ND
Experiment 2:				
spleen	none	-	3	2
spleen	none	+	3	2
spleen	normal spleen	-	<1	<1
spleen	normal spleen	+	2	<1
spleen	TNP-spleen	-	74	<1
spleen	TNP-spleen	+	50	2

[1] CTL generating cultures contained 1 x 10^6 responder cells and an equal number of gamma irradiated, syngeneic splenic stimulator cells (which had been TNP-modified or not, as indicated) in a final volume of 0.2 ml. After 5 days, cultures were assayed on 3 x 10^4 P815, or TNP-modified P815 target cells (Materials and Methods). Killing activity in cultures is expressed as KA x 10^8. Experiment 1: DBA/2J responder and stimulator cells. Experiment 2: (DBA/2J x BALB/cCr)F1 responder and stimulator cells. Costimulator (Cos) fraction 3 was used in both experiments.

Table 21

Antigen specificity of CTL generated from thymic and splenic lymphocytes in the presence of costimulator[1]

Responding cells	Antigen	KA x 10^8 (Target) P815 Costimulator -	P815 Costimulator +	EL4 Costimulator -	EL4 Costimulator +
spleen	none	<3	20	<3	10
$3.3x10^5$/ml	P815	120	640	4	20
	EL4	<3	110	15	610
thymus	none	<3	<3	<3	3
$10x10^5$/ml	P815	<3	65	<3	5
	EL4	<3	10	<3	85
thymus	P815	ND	140	ND	5
$3.3x10^5$/ml	EL4	ND	10	ND	70

[1] CBA/J (H-2k) responder cells were cultured with 1 x 10^4 irradiated P815 (H-2d) or EL4 (H-2b) tumor cells as antigen, in the presence and absence of CBA/J fraction 3 costimulator at 4 units/ml. The culture volume was 1.0 ml. After 5 days, the cultures were divided into two portions, and assayed on both EL4 and P815 targets. Killing activity in cultures is expressed as KA x 10^8. ND, not determined.

spleen than for thymus-derived CTL, and probably represents the amplification of CTL clones which crossreact with antigens present on EL4. Thus costimulator generates CTL which are antigen specific. The CTL harvested from cultures of CBA spleen cells stimulated by (CBA X BALB)F1 spleen cells in the presence of costimulator are sensitive to treatment with anti-Ly 1.1 antiserum and complement (Figure 38), or Thy 1.2 serum and complement (data not shown). In summary, the characteristics of CTL which develop in cultures containing costimulator are similar to those of CTL stimulated by antigen alone in terms of specificity and surface markers.

Costimulator is Required Throughout a CTL Response

In the CTL experiments presented so far, costimulator was added, along with antigen, at the initiation of cultures. In the experiment shown in Table 22, CBA thymocytes were cultured with P815 stimulator cells and costimulator was added or removed at various times. Addition of costimulator at the initiation of culture gave a good response, but later addition, at 24 or 48 hrs, was ineffective. Removing the factor after 24, 48 or 72 hrs also abrogated its efffect. Thus costimulator appears to be continuously required for at least the first 3 days of a 5 day response. A requirement for the factor through day 5 is shown in Table 23. CBA (H-2k) thymocytes were cultured with EL4 (H-2b) tumor cells as antigen for 5-7 days before assay. Costimulator (fraction 4) was removed from a group of

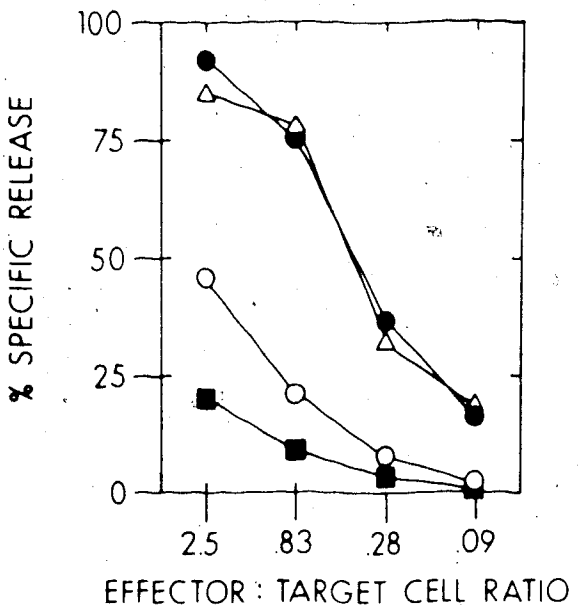


Figure 38. CTL generated from CBA spleen cells in the presence of costimulator bear the Ly 1.1 antigen. Cultures contained 1.5 x 10^5 CBA/CaJ responder spleen cells, 1.5 x 10^5 (CBA/CaJ x BALB/cCr)F1 irradiated spleen cells as stimulators, and costimulator fraction 4 from CBA/CaJ mice at 4 units/ml, in a total volume of 0.21 ml. After 5 days of culture, cells were harvested, treated with NMS or monoclonal anti-Ly 1.1 (antiserum 147A), and assayed for killer cell activity on 3 x 10^4 ^{51}Cr-labelled P815 target cells. Treatment with the sera (used at 1/20 dilution) was done at a cell concentration of 4.8 x 10^6 viable cells per ml. Seventy percent of NMS and 19% of anti Ly 1.1 treated cells survived. Effector to target cell ratios are based on the numbers of cells treated with antisera.
(●) control; no treatment prior to assay
(△) NMS + complement
(o) anti-Ly 1.1 (147A) + complement
(▪) control; cultured without antigen (no treatment).

Table 22

Effect of addition and removal of costimulator on the CTL response[1]

Time costimulator added (hrs of culture)	Time costimulator removed (hrs of culture)	Costimulator replaced	KA X 10^8
none added	-	-	<2
0	-	-	211
24	-	-	5
48	-	-	4
0	24	no	<2
0	24	yes	103
0	48	no	<2
0	48	yes	123
0	72	no	<2
0	72	yes	182

[1] One million CBA/J thymocytes were cultured with 1 X 10^4 irradiated P815 stimulator cells in 12 X 75 mm sterile plastic tubes. The culture volume was 1.0 ml, and the medium was MHFM. Costimulator (fraction 3 from CBA/CaJ mice, used at approximately 5 units/ml) was added or removed at various times. Costimulator was removed by aspirating the supernatant away from the cell pellet, washing the cells in 4 ml of medium and resuspending them in 1 ml. To control for the effects of manipulating the cells, fresh costimulator was added back to some cultures (marked "yes" in column 3). After 115 hours of incubation, triplicate cultures were assayed on ^{51}Cr-labeled P815 target cells.

Table 23

Effect of removing costimulator from CTL-generating cultures during the first 5 days[1]

Day costimulator removed	Fresh costimulator added	KA X 10^8 (Day of assay)[3]		
		5	6	7
1	no	<4	<4	<4
1	yes	<4	32	42
2	no	<4	<4	<4
2	yes	18	33	93
3	no	<4	<4	<4
3	yes	17	74	116
4	no	<4	<4	<4
4	yes	17	53	107
5	no		<4	<4
5	yes		37	93
7[2]	no			117
7	yes			139

[1] Cultures (0.21ml) contained 3 x 10^5 CBA/J thymocytes and 3 x 10^3 irradiated EL4 tumor cells as antigen. Fraction 4 costimulator was added at 4 units/ml to all cultures on day 0. Cultures were assayed on days 5, 6 or 7, using 2 x 10^4 ^{51}Cr-labelled target cells. Killing activity in cultures is expressed as KA x 10^8. Costimulator was removed from cultures and added back as described in Table 22, after transferring the cells from microtitre wells to plastic tubes.

[2] Cells were washed and resuspended in fresh medium 0.5 hr before addition of target cells.

[3] The values of KA x 10^8 for control cultures containing responder cells only, and responder cells plus costimulator were <4 on all days assayed.

cultures daily over the period of 1-5 days. There was no detectable CTL activity on days 5, 6 or 7 if costimulator was removed as late as day 4. Control cultures showed CTL activity on day 5. When costimulator was removed from parallel cultures on day 5, however, there was no killer activity in them 24 hr later. Costimulator is clearly not required for the killing process itself, since the level of killing in CTL cultures washed free of factor immediately prior to assay (on day 7) was not significantly diminished. The conclusion from Tables 18 and 19 is that costimulator is required at the beginning of the culture period, and is also required to maintain killing activity in the cultures. It appears as though the CTL depend upon costimulator either for proliferation, or to maintain viability or function.

Costimulator is not H-2 Restricted, Does not Bear Ia Determinants and is Probably Antigen-Nonspecific

Aliquots of the CBA/J (H-2k) costimulator which had been passed over anti-Ia(k) and anti-Ia(S) columns and assayed for activity in the thymocyte proliferation assay (Chapter III, Figure 16) were tested for their ability to stimulate the development of CTL from CBA thymocyte precursors. The material from the anti-Ia(k) column was as effective as that which had been passed over the control anti-Ia(S) column, indicating that Ia determinants are not present on the molecules responsible for CTL generation (Table 24). The lack of H-2 restriction of the factor is shown in Table 25. CBA/J (H-2k) thymocytes gave a good CTL

Table 24

CTL-stimulating activity of CBA/J costimulator after passage over anti-Ia immunoadsorbant columns[1]

Dilution of costimulator	Antigen	KA X 10^8 Adsorbed: Anti Ia(s)	Anti Ia(k)
1/10	+	82	50
1/20	+	94	82
1/40	+	38	61
1/80	+	31	49
1/160	+	15	8
1/20	-	3	4

[1] Fraction 3 costimulator generated from CBA/J (H-2k) spleen cells was passed over anti-Ia(S) or anti-Ia(k) columns. The unbound fractions were tested for CTL stimulating activity at various dilutions. CTL generating cultures contained 3 x 10^5 CBA/J (H-2k) thymic responder cells, and 3 x 10^3 irradiated EL4 (H-2b) stimulator cells. Cytotoxic activity, expressed in terms of KA, was measured on day 5 using ^{51}Cr-labeled EL4 cells as targets. The value for KA of cultures containing CBA/J thymocytes with no antigen or costimulator was <3 x 10^{-8}. The preparations of costimulator tested are the same as those used in Figure 16.

response to both P815 (H-2d) and EL4 (H-2b) tumor cells in the presence of either BALB/c (H-2d) or CBA/J - derived costimulator. If the factor were H-2 restricted, the CBA, but not the BALB material would be active on CBA precursors.

This experiment (Table 25) also suggests that the factor is nonspecific, rather than a polyclonal mixture of antigen specific factors. The argument assumes that the CTL response of CBA precursors against allogeneic tumor cells is mainly directed against major H antigens, and that an antigen specific factor against a self antigen would not be produced in as great a quantity as a factor specific for an alloantigen. If costimulator were specific it would be predicted that factor produced by BALB/c (H-2d) spleen cells would be less active against the near-self antigen P815 (H-2d) than against the alloantigen EL4 (H-2b). Costimulator produced by CBA spleen cells would be expected to have full activity against both tumors, since both are allogeneic. The results clearly show BALB/c costimulator to be as active in an anti-H-2(d) as in an anti H-2(b) response. These data suggest, but do not prove, non-specificity of the factor.

Ability of Costimulator to Bypass the Requirement for A Cells in CTL Responses and in the Secondary T Cell Proliferative Response to PPD

The possibility that the A cell requirement for CTL generation might reflect the role of macrophages in costimulator production was investigated. If this were so, the normal requirement for A cells in a CTL response might

Table 25

Costimulator does not show self tolerance for MHC antigens, and is not MHC restricted[1]

Antigen	Source of costimulator	KA X 10^8 Target: P815(H-2d)	EL4(H-2b)
none	none	<1	<1
none	CBA	2	<1
none	BALB/c	2	3
P815	none	<1	<1
P815	CBA	73	5
P815	BALB/c	66	3
EL4	none	<1	<1
EL4	CBA	6	107
EL4	BALB/c	4	54

[1] CBA/J (H-2k) thymocytes (1 x 10^6) were cultured with 1 x 10^4 P815 or EL4 tumor cells in 1.0 ml. Costimulator from CBA (fraction 3) or BALB/c (fraction 2) spleen cells was added at about 4 units/ml where indicated. After 5 days, cultures were divided into 2 portions and assayed on P815 and EL4 target cells.

be overcome by the addition of costimulator to the culture medium. Passage over nylon wool reduced the number of killer cells generated by BALB/c or (BALB/c x DBA/2)F1 lymph node cells co-cultured with EL4 stimulator cells (Table 26). CTL activity was restored to control levels by the addition of costimulator. In Experiment 2, fractions 3 and 4 were titrated. When added at the same levels, as defined by units of activity in the thymocyte proliferation assay, they gave a similar degree of stimulation in the CTL response. The addition of irradiated peritoneal cells (containing a high proportion of macrophages) at the optimal concentration was less effective than costimulator in restoring the response (Experiment 1).

Another system in which macrophages have been found to be an absolute requirement is in the secondary proliferative response of lymph node T cells to the antigen PPD (Lee, 1980). Dr. K.-C. Lee kindly performed an assay to determine whether costimulator can replace macrophages in this type of T cell response. Antigen-primed lymph node T cells depleted of macrophages on nylon wool do not proliferate in response to PPD (Table 27). The addition of costimulator fraction 4 or peritoneal macrophages stimulated the response. However, the macrophages were more efficient than costimulator in restoring the response, the latter causing only half the level of DNA synthesis seen in control cultures containing unfractionated lymph node cells. The greater efficacy of macrophages was more pronounced if the lymph node cells were

Table 26

Effect of costimulator on the CTL response of nylon wool-purified T cells[1]

Number EL4 stimulator cells per culture ($\times 10^{-4}$)	Number irrad. PC per culture ($\times 10^{-5}$)	Costim. fraction and units/ml	KA $\times 10^{8}$ Unfractionated LN responder cells	Nylon wool purified LN responder cells
Experiment 1				
0	0	none	8	<4
0	0	3, 4 U/ml	11	8
1	0	none	242	32
1	0	3, 4 U/ml	479	550
3	0	none	198	12
3	0	3, 4 U/ml	802	549
3	10	none	ND	<5
3	3	none	ND	<5
3	1	none	ND	248
3	0.3	none	ND	137
Experiment 2				
0	0	none	6	<6
0	0	3, 6 U/ml	6	<6
0	0	4, 12 U/ml	12	17
0	0	4, 6 U/ml	6	7
0.1	0	none	179	82
0.3	0	none	332	65
1.0	0	none	237	7
0.3	0	3, 12 U/ml	508	923
0.3	0	3, 6 U/ml	960	480
0.3	0	3, 3 U/ml	626	320
0.3	0	3, 1.5 U/ml	441	136
0.3	0	3, .75 U/ml	321	60
0.3	0	4, 12 U/ml	622	769
0.3	0	4, 6 U/ml	951	653
0.3	0	4, 3 U/ml	627	333
0.3	0	4, 1.5 U/ml	538	627

[1] Normal or nylon wool purified mesenteric lymph node cells were incubated with irradiated EL4 (H-2b) cells as stimulators. Irradiated peritoneal cells (PC) or costimulator were added to cultures at the concentrations indicated. The responder cells were 2×10^5 (BALB/cCr x DBA/2J)F1 (H-2d) lymph node cells in Experiment 1, and 2×10^5 BALB/cCr lymph node cells in Experiment 2. Cultures were assayed on ^{51}Cr-labelled EL4 targets. ND, not determined; LN, lymph node.

Table 27

Costimulator fraction 4 partially replaces the requirement for macrophages in a secondary proliferative response[1]

Responding cells	Additions	CPM NO PPD	CPM +PPD
Unfractionated	none	958	72,811
Nylon wool (once) purified	none	81	1810
	costimulator		
	3U/ml	6825	33,820
	1.5U/ml	2252	21,829
	.75U/ml	1781	12,282
	.38U/ml	353	7294
	.19U/ml	130	3989
	macrophages		
	10^3	66	17,730
	3×10^3	112	54,502
	10^4	430	111,829
	3×10^4	1679	131,664
	10^5	6804	30,061
Nylon wool (twice) purified	none	465	1891
	costimulator		
	3U/ml	3862	14,837
	1.5U/ml	2226	14,210
	.75U/ml	898	4843
	.38U/ml	312	3147
	.19U/ml	151	1600
	macrophages		
	10^3	272	8874
	3×10^3	468	22,331
	10^4	207	69,567
	3×10^4	791	109,479
	10^5	7538	55,015

[1] This assay was performed by Dr. K.-C. Lee; and details of the methods employed are given in Lee (1980). The cultured lymph node cells were from CBA/CaJ mice which had been primed with PPD (purified protein derivative of tuberculin). They were enriched for T cells by passage over nylon wool once or twice. The response was measured as the amount of 3H-dThd incorporated into DNA in the 24 hr prior to harvesting the cells at 96 hr. The results are expressed as total cpm incorporated.
Costimulator fraction 4 (Swiss) or peritoneal

macrophages were added to cultures in the presence or absence of PPD (100 micrograms/ml). Cultures contained 2 x 10^5 lymph node cells.

acutely depleted of macrophages by two passages over nylon wool (lower set of data in Table 27). Thus it is not clear whether costimulator can fully replace the requirement for macrophages in this system, or whether it can only act in the presence of residual macrophages. In evaluating the role of the factor, it must be taken into consideration that with a soluble antigen such as PPD, macrophages might be necessary for antigen presentation, as well as for the elaboration of soluble factors.

The Role of Costimulator in the Antibody Response to Heterologous Erythrocytes in vitro

Con A-induced factors have been reported to replace helper T cells in AFC responses against heterologous erythrocytes *in vitro* (Watson, 1973; Schimpl and Wecker, 1972). Experiments were carried out to determine whether the T cell replacing activity in crude supernatants from Con A-stimulated spleen cells copurified with costimulator, as defined by its activity in the thymocyte proliferation assay. Although fraction 3 costimulator enhanced the AFC response of CBA/J and BALB/c spleen cells to SRBC (Table 28), it failed to promote a response by T-cell deprived (nu/nu) spleen cells (data not shown). The enhancement of the 96 hr response of normal spleen cells occurred only when the factor was added at the time the cultures were initiated, or within the next day. Addition 2 or 3 days later proved ineffective.

Although nude mice on a BALB/c background failed to

Table 28

Costimulator enhances the AFC response of spleen cells to sheep erythrocytes[1]

Spleen cells	SRBC	Day of addition of costimulator: None	0	1	2	3
		PFC/10^6 cells ± S.D.				
Experiment 1						
CBA/J, 9×10^6	-	12±3	17±11	16±7	7±3	
" "	+	74±19	135±14	220±27	47+20	
Experiment 2						
CBA/J, 10×10^6	-	21±17		33±8		
" "	+	231±44		694±55		
Experiment 3						
BALB/c, 3.5×10^6	-	3±3		29±8		4±1
" "	+	5±4		244±33		8±8
Experiment 4						
BALB/c, 10×10^6	-	1±1		25±13		
" "	+	38±16		263±36		
" 3×10^6	-	7±8		26±2		
" "	+	44±11		506±68		
CBA/J, 10×10^6	-	11±4		20±9		
" "	+	46±9		132±13		
" 3×10^6	-	24±10		125±23		
" "	+	117±14		551±99		

[1] Spleen cells were cultured with SRBC in Mischell-Dutton cultures and assayed as described in Materials and Methods, section U. Costimulator fraction 3 (CBA), was added to the 1.0 ml cultures at approximately 3 units/ml at various times. Results are expressed as the number of PFC (plaque forming cells) per million starting cells in the cultures.

respond to SRBC in our experiments, the same strain of mice, used by Dr. J. Watson, were responsive to SRBC in the presence of costimulator. The reason for this difference is not known. However, in collaboration with Dr. Watson, using his BALB/c nude mice, we were able to confirm that costimulator activity in the thymocyte proliferation and CTL assays copurified with the T cell replacing activity in Con A supernatants. A preparation of fraction 5 costimulator from our laboratory was active in the AFC response of nu/nu spleen cells against SRBC (J. Watson, personal communication). Furthermore, T cell replacing factor (TRF) prepared and purified through isoelectric focusing in Dr. Watson's laboratory was active in the thymocyte proliferation and CTL assays in our laboratory (Figure 39). Upon isoelectric focusing, TRF activity can be resolved into three peaks, of pI 3.0 - 4.2, 4.3, and 4.5 (Watson *et al.* 1979b). The material from the pI 4.3 and 4.5 peaks had activity in the thymocyte proliferation and CTL assays (Figure 39 A and B). The CTL assay measured the response of CBA/J thymocytes against the allogeneic tumor cells P815. The pI 4.5 material had slightly more activity than the pI 4.3 pool in the CTL assay. The amount of factor required to give 1 unit of activity in the CTL response was about double that required for one unit of activity in the thymocyte proliferation assay. The amount of factor needed for a unit of TRF activity in the nude spleen cell cultures (also defined as 1/3 of the maximal response) is the same as for

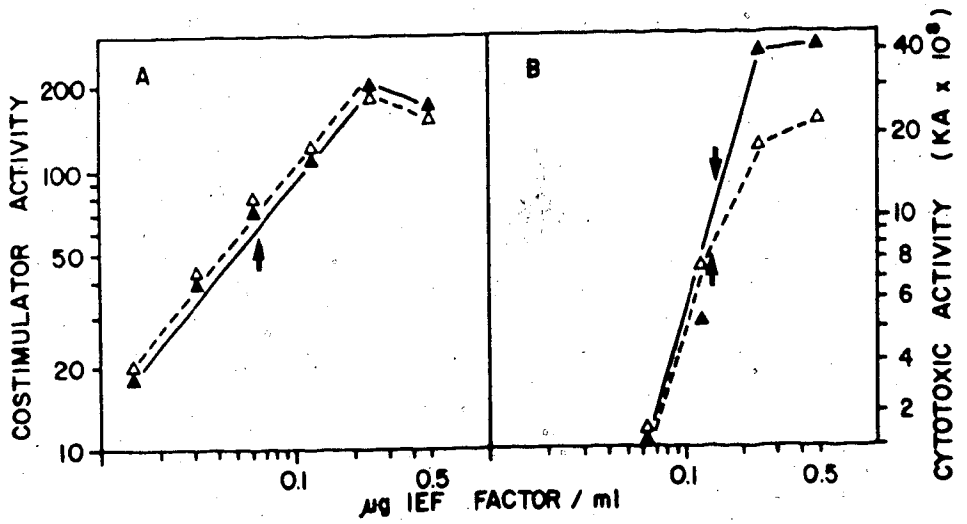


Figure 39. Costimulator activity in TRF preparations resolved by isoelectric focusing. Samples of TRF (T cell replacing factor) were provided by Dr. Jim Watson. They consisted of TRF which had been chromatographed on Sephadex G-100 and DEAE-cellulose, and then subjected to isolectric focusing. Two pools of TRF activity were made, representing material of pI 4.0-4.3 (△) and pI 4.3 to 4.5 (▲). Both pools had activity in the TRF assay, the ability to promote an antibody response against SRBC by nude spleen cells. A: activity in the thymocyte proliferation assay. B: Activity in the CTL assay. CTL generating cultures contained 1 x 10^6 CBA/J thymocytes and 3 x 10^4 irradiated P815 cells as antigen in 0.22 ml medium. Cultures were assayed on day 5 using 3 x 10^4 ^{51}Cr-labelled P815 cells as targets. The arrows mark a unit of activity - the amount of factor that produces 1/3 of the maximal response.

the CTL assay (Watson *et al.*, 1979c). The mitogeneic response might be more sensitive to factor than the CTL and AFC response. Alternatively, the requirement for more factor in the CTL and AFC assays may be a result of the much higher cell concentrations used in these assays (5×10^6/ml and 10×10^6/ml, respectively) compared to the concentration used in the thymocyte proliferation assay (0.5×10^6/ml). In view of the finding that IL2 factors are absorbed out by activated lymphocytes (Coutinho *et al.*, 1979; Smith *et al.*, 1979c) the latter explanation seems more likely.

The low pI (3.0 - 4.2) material with TRF activity (Watson *et al.*, 1979c) had no activity in the thymocyte proliferation and CTL assays, nor did it have any inhibitory activity which would mask the effect of costimulator (J. Shaw, J. Watson and V. Paetkau, unpublished results). This low pI TRF activity is thought to represent another molecular species with antigen-specificity (J. Watson, personal communication). Unlike IL2 factors, it is active if added to cultures during the final 1-2 days, and has been termed "late acting factor."

In summary, through collaborative studies with Dr. Watson, we have determined that costimulator activity, originally defined as the ability to promote the proliferative response to T cell mitogens of mouse thymocytes cultured at low cell density, and later found to co-purify with CTL generating activity, also co-purifies with "T cell-replacing activity" in AFC responses.

C. Discussion

Biological Activities of Costimulator in vitro

The experiments in this chapter describe the copurification through gel filtration, ion exchange chromatography, and isoelectric focusing of three lymphokine activities which were not previously known to be related. These are:

(1) synergy with the T cell mitogen concanavalin A to promote a proliferative response by thymocytes (deficient in helper T cells) cultured at low cell density;

(2) promotion of CTL responses under several suboptimal conditions which reflect a paucity of antigen specific helper cells or macrophages (see below);

(3) restoration of an AFC response against heterologous erythrocytes by T cell-depleted spleen cells. It was subsequently found that these three activities also copurified with T Cell Growth Factor (TCGF) activity (Kendall Smith and Gordon Mills, personal communication). It is not proven that the three activities are a function of the same molecular entity; indeed, this can only be conclusively demonstrated after *in vitro* synthesis of the "costimulator" molecule.

Although the "background" levels in all of these responses are enhanced by the factor, its activity in synergy with mitogens or antigens is far more potent. It is therefore considered to be non-mitogenic. Background

enhancement might be a result of increasing ongoing responses to antigens previously encountered *in vivo*, or unintentionally presented *in vitro*, such as viral antigens or immunogens in FBS.

The most biologically interesting part of this study concerns the ability of the factor to promote CTL responses under conditions where:

a) the responder cells are thymocytes (Figure 55; Tables 21-25), which are deficient in differentiated helper T cells (Cantor and Boyse, 1977; Pilarski, 1977);

b) the responder cells are depleted of Ly 7^+ cells (Table 18) which are known to have helper activity in CTL responses against alloantigens (Pilarski *et al.*, 1980);

c) the stimulator cells have been rendered non-immunogenic by glutaraldehyde fixation (Figure 36) or UV irradiation (Table 19), conditions which can be overcome by the addition of antigen-specific helper T cells (Pilarski, 1979);

d) the number of stimulator cells used is limiting (Figure 37);

e) the responder cells and stimulator cells are depleted of adherent cells, including macrophages (Table 26; H.-S. Teh, personal communication).

Since costimulator is apparently secreted by an antigen- or mitogen-activated helper T cell (Ly $1^+,2^-,7^+$) upon interaction with a macrophage (Chapter IV, Figures 25 and 26; Smith *et al.*, 1979b), the simplest explanation for

the role of costimulator in these responses is that it supplies the signal normally provided by the macrophage and the helper T cell. If macrophages are removed from a responder population, sufficient endogenous costimulator would not be produced, despite the presence of helper T cells. Providing saturating amounts of the helper signal in the form of costimulator then overcomes the requirement for the helper T cell product, and therefore the macrophage. Similarly, the helper T cell would become superfluous.

It should be pointed out that the proposed model predicts that costimulator would overcome an A cell or macrophage requirement in responses where a macrophage is not strictly needed for antigen presentation. These would probably include mitogen responses, where sufficient mitogen binds directly to receptors on T cells, and CTL responses, in which the antigen is presented in cell-bound form, as on a syngeneic or allogeneic spleen or tumor cell. For example, costimulator has been shown to overcome the requirement for macrophages in the Con A response of mouse thymocytes cultured at optimal cell density (Mills *et al.*, 1976). T cell responses to soluble antigens such as PPD may require macrophages to present antigen in the most immunogenic form. In this situation, the prediction would be an enhancement of the response by costimulator, but an ultimate limitation of the response by the number of macrophages present in the responder population. This appears to have been the case in the secondary *in vitro* response against PPD, since the lymph

node cells which had been depleted of macrophages on nylon wool once were more responsive to costimulator and PPD than those which had been passed over nylon wool twice (Table 27). The data presented do not eliminate the possibility that a second signal is normally delivered by a macrophage, and that costimulator acts to boost the number of macrophages or to enhance their function, however.

Mode of Action of Costimulator

Two questions concerning the involvement of costimulator in CTL and antibody responses arise. What is the target of the factor - a helper T cell or the effector cell itself? Does the factor act by increasing the number of antigen-responsive cells by inducing proliferation in the stimulated population, or does it cause further differentiation of helper or effector T cells? The data concerning the CTL response do not distinguish between the factor acting on a helper or a CTL precursor during the *induction* of a response. Some preliminary data suggest that costimulator may help in the generation of helper T cells from thymocyte precursors (data not given). There is fairly strong evidence that the factor can act directly on a cytotoxic lymphocyte however, since CTL can be cloned and kept in culture for many months in the presence of IL2 (TCGF) and no additional antigen (Baker *et al.*, 1978; von Boehmer *et al.*, 1979). Thus it is possible that costimulator can act on precursors of both helper and cytotoxic T cells.

Only *activated* T cells are targets for costimulator.

TCGF is removed from culture medium by spleen cells stimulated by PHA, Con A or alloantigen, but not by normal or LPS-stimulated spleen cells (Smith *et al* 1979a; Coutinho *et al*., 1979; G. Mills, personal communication). In light of this finding, it is predicted that the factor would have no effect on a T cell response to a given antigen prior to exposure of lymphocytes to that antigen. This fits with our observation that preincubation of thymocytes with costimulator for one or two days did not alter the time required for a peak CTL response to develop after allogeneic cells were added (data not shown).

If costimulator has no effect on unstimulated lymphocytes it is perhaps because they do not express the appropriate receptor for the factor. This would ensure that costimulator acted as a second signal for the induction of CTL, the first signal being antigen. Neither signal alone is sufficient to trigger a full response. It has been suggested that the binding of antigen to a T cell receptor (signal 1) induces the appearance of the IL2 receptor, and that IL2 binding to the receptor promotes clonal expansion of the CTL (Smith *et al*., 1979b). Keeping in mind that other differentiative signals may play a role in the induction of the response, the proposed model is in accord with all of the data presented here.

Costimulator is required not only at the beginning of a CTL response *in vitro* (Table 22), but throughout the response (Table 23). If costimulator is withdrawn from

active CTL for a 24 hr period, the killing activity in cultures disappears. The CTL either become inactive, or fail to renew themselves. The active CTL precursors are absolutely dependent upon the factor for clonal expansion, initially, and then for continued renewal. In agreement with the model of Smith *et al.*, (1979b), the "second signal" appears to control the magnitude and duration of a CTL response *in vitro*.

The target cell of costimulator-like factors in the AFC response against heterologous red cells is probably a helper T cell, and not the B cell precursor itself. The failure of costimulator to enhance an LPS response of spleen cells (Figure 33), and to restore the AFC response of BALB/c nu/nu spleen cells (data not shown) is circumstantial evidence that the B cell is not a target for the factor. The aforementioned inability of LPS - activated or normal spleen cells to adsorb costimulator from culture medium further suggests that B cells do not possess a receptor for the factor. The ability of IL2 to restore the AFC response of T-depleted spleen cells (Watson *et al.*, 1979a; 1979c) may reside in their ability to stimulate helper T cell induction rather than the B cell precursors themselves. Thus the degree to which a T cell-deficient population can be stimulated by the factor might be related to the number of residual helper T cells or their precursors. For example, there are CTL precursors in nude spleens, which develop in the presence of alloantigen and TCGF (Gillis *et al.*, 1979).

There may be helper T cell precursors as well, which might differentiate in the presence of IL2. T-helper cells specific for SRBC have been propagated for several months *in vitro* by culturing them with TCGF (Watson, 1979).

This issue has been complicated by the presence of non-IL2 factors in all but the most highly purified IL2 preparations (Watson *et al.*, 1979c). The other type of factor is distinguished from IL2 by its lower pI. It has T cell replacing activity, but unlike IL2, is effective if added late in a response, and may be an antigen-specific factor (J. Watson, personal communication). It is considered likely that this TRF factor interacts with B cells (Watson *et al.*, 1979c) and may be responsible for "late acting T cell-replacing activities" in preparations which also contain IL2 (Schimpl and Wecker, 1975; Hunter and Kettman, 1974).

Specificity of Costimulator

Costimulator activity is not H-2 restricted in the thymocyte proliferation (Figure 31) or CTL assays (Table 25). This is in keeping with its lack of serologically detectable Ia antigens. The factor activity in the thymocyte proliferation (Figure 16) and CTL (Table 24) was not retained by anti-Ia affinity columns which were effective in removing the activity of the Ia^+ AEF (Delovitch and McDevitt, 1978). Most of the antigen-specific helper or suppressor factors (Table 1) which bear Ia determinants are also H-2 restricted (Tokuhisa *et al.*, 1978; Shiozawa *et*

al.,1977; Erb *et al.*, 1976; Tada *et al.*, 1979; Germain *et al.*, 1979). Two exceptions to the correlation between Ia+ factors and H-2 restriction are a helper factor for (T,G)-A--L (Taussig *et al.*, 1975), and a hybridoma-secreted suppressor factor for KLH (Kontiainen *et al.*, 1978).

The question of costimulator's antigen specificity must remain open. There are no conclusive data presented here, or concerning any of the other IL2 factors, which discriminate between the factors being polyclonal but antigen-specific, or truly non-specific. In both cases, it would be predicted that responses to all or nearly all antigens would be helped by preparations of the factor. The only exception would be that a *specific* factor might not be produced against self antigens. Table 25 shows that factor produced by BALB/c (H-2d) mice is as effective in an anti-P815 (H-2d), as in an anti-EL4 (H-2b) CTL response, using CBA (H-2k) thymocyte precursors. Furthermore, BALB/c factor was as potent as CBA factor in the anti-H-2d response. These data suggest that the factor activity is antigen-nonspecific, since an anti-P815 response in unprimed cells promoted by H-2d-produced costimulator would probably constitute an anti-self MHC response. However, it is also possible that the factor is antigen-specific, and contains anti-self specificities, but that self tolerance is maintained at another level of control, such as the number of CTL effector cell precursors. The probability that "abnormal induction" of the CBA precursors by antigen-specific anti-H-2k factor

via the allogeneic effect (Bretscher, 1974) was responsible for the activity of the BALB/c factor is made less likely by the observation that AEF has no activity in CTL responses (J. Shaw, L. Pilarski and T. Delovitch, unpublished results).

The problem of antigen-specificity of Con A induced factors would be quickly solved if affinity columns containing a particular antigen retained factor activity against that antigen, but not others. However, columns containing sheep or horse erythrocyte antigens do not absorb out AFC-enhancing activity against these antigens from Con A supernatants (Bernabe *et al.*, 1979; Harwell *et al.*, 1976; J. Watson, personal communication). Furthermore, *in vivo* priming of factor producing cells to enrich for reactivity against a specific antigen, and *in vivo* negative selection using the Sprent technique to deplete antigen reactive cells increased and decreased, respectively, the factor activity produced to the particular antigen (Bernabe *et al.*, 1979). This apparent paradox was resolved somewhat by the finding that factor activity is specifically adsorbed out by the appropriatetigen-antibody complexes (for example antibody to SRBC bound to SRBC). Antibody alone did not adsorb out activity. The conclusion from these experiments was that Con A factors contain a mixture of diverse molecules, some of which have specificity for idiotypes or other determinants in an antigen-antibody complex which are exposed only after antigen binding, and others of which are nonspecific.

One important question concerning the relevance of these findings to the specificity of IL2 factors remains. Was the activity being detected in the AFC assay actually IL2, or was it the late acting PFC-enhancing factor of low pI uncovered by Watson *et al.* (1979c)? (The Con A factors used by Babbabe *et al.* were not purified.) The low pI factor is sensitive to incubation at room temperature for 30 min with 0.1% 2-Mercaptoethanol. Costimulator (data not shown) and several other IL2 factors (Aarden *et al.*, 1979) are insensitive to this treatment, so this question could be quickly answered. It is not unreasonable to assume that both antigen-specific and nonspecific helper factors are induced by Con A, and that they have different biological roles.

A hybridoma has recently been described which secretes a helper factor for AFC responses upon stimulation with Con A (Schrader *et al.*, 1980). The monoclonal factor is equally effective in promoting responses against SRBC, HRBC, and fluorescein-conjugated polymerized flagellin, and is therefore probably antigen-nonspecific. If the physical characteristics of this factor turn out to correspond with those of IL2, this will provide evidence that the activity resides in a nonspecific mediator. Gillis and Scheid (1980) have recently discovered that a murine T cell lymphoma line, LBRM-33, secretes TCGF upon Con A stimulation. Its product, like that of the hybridoma mentioned above, appears to be antigen-nonspecific.

Comparison of Costimulator's Activities to Those of Other Factors

There is a general consensus that the factors in Table 2, designated IL2, are the same biochemically, and have the same biological activities (Aarden *et al.*, 1979), although not all factors have been tested in each of the four major assay systems used: synergy with mitogens, AFC, CTL, and TCGF responses. The ability of costimulator to replace the requirement for helper cells (Ly 7.2$^+$) in the primary CTL response against alloantigens is probably analogous to the helper T cell replacing activity described by Plate (1976), Sopori *et al.* (1977) and Okada *et al.* (1979), using systems in which helper T cells were separated or removed from CTL precursors. The factors used in these experiments were not purified. Two activities which have been attributed to factors in this group have not been investigated in experiments reported here with purified factor. One is the ability to perpetuate CTL with TCGF (Gillis and Smith, 1977). Another is the ability to promote secondary CTL responses of cells which had been depleted of T helper cells using Ly 1.2 antisera (Wagner and Rollinghoff, 1978). The latter experiment could not be done in our laboratory because Ly 1.2 and 2.2 antisera were not available, and it was not possible to separate helper T cells and CTL effectors using anti-Ly 1.1 and -2.1 antisera. However it is likely that these two activities are due to IL2 itself.

IL1 (LAF) can be distinguished from IL2 by its lack of

activity in the TCGF assay, which measures the ability of factors to promote the long term proliferation of T cell lines (Aarden *et al.*, 1970). Furthermore, the maximum level of activity obtainable in the thymocyte proliferation assay with IL1 is about 10-fold lower than with costimulator (B. Caplan, personal communication).

As mentioned above, allogeneic effect factor (AEF), which acts as a T cell-replacing activity in AFC responses (Delovitch and McDevitt, 1977), has no activity in the CTL or thymocyte proliferation assays (J. Shaw, L. Pilarski and T. Delovitch, unpublished results). No other antigen specific factors have been assayed for costimulator activity.

Fraction 3 costimulator contains interferon activity (G. Mills, personal communication), assayed as the ability to inhibit viral plaques *in vitro* after infection of mouse fibroblasts or tumor lines with virus . However most of the interferon activity is removed by DEAE chromatography, and is very low in fraction 4, The contamination of IL2 preparations with immune interferon was also reported by Simon, Farrar and Kind (1979), who were able to separate the two activities on hydroxylapatite.

Murine granulocyte-macrophage colony stimulating factor (CSF), purified and provided by Dr. R. Stanley (Stanley and Heard, 1977), had no activity in the thymocyte proliferation assay (Shaw *et al.*, 1978b). CSF and IL2 are thought to be separate entities.

VI. Summary: A Model for the Induction and Mode of Action of Costimulator in Mitogen and CTL Responses

A. Nature of the Factor

The lymphokine activity which has been called costimulator resides in highly acidic protein or glycoprotein molecules (pI 3.8 - 4.5) of MW 30,500 and sedimentation coefficient 2.63S. It consists of two 15,000 MW subunits, which remain active after dissociation in SDS (Barry Caplan, unpublished results). It is not retained by anti-Ia affinity columns, and therefore is probably devoid of Ia determinants. These few properties (others are discussed in Chapter III) are sufficient to distinguish costimulator from both IL1 (LAF, Table 3), which has a molecular weight of 18-20,000, and the antigen-specific factors (Table 1) which are generally retained by anti-Ia immunoadsorbants. Costimulator is now known to be indistinguishable from several other factors, and this group has been designated IL2 (Aarden *et al.*, 1979). These include TSF (Chen and Di Sabato, 1976), TMF (Farrar *et al.*, 1978), TRF (Watson *et al.*, 1979b), NSM (Marrack and Kappler, 1977), NSF (Waldman, 1977) and TCGF (Smith *et al.*, 1979b). Although all of these factors have not been systematically compared, direct comparisons between TRF and costimulator (Watson *et al.*, 1979a) and between TRF and TCGF (Watson *et al.*, 1979c) showed that the profiles of activity after isoelectric focusing were superimposable.

Costimulator has been purified over 200 fold from tissue culture medium in which mouse spleen cells were incubated with Con A by gel filtration and isoelectric focusing (Table 6). The factor is highly potent in terms of the molar amounts which promote immune responses *in vitro*. The most purified preparations of costimulator (fraction 5) were maximally active in culture at a protein concentration of 0.05 microgram/ml (Figure 14). Assuming a MW of 30,000, this corresponds to about 10^{-9} M costimulator.

Because there is no evidence that costimulator-like factors are specific for a particular antigen (Chapter V, section C) for the purpose of this discussion it will be assumed that they are non-specific, rather than polyclonal and specific.

B. Induction of IL2 Factors by Mitogen or Cell-Bound Antigens

Costimulator (Figure 25, Table 14), as well as TCGF (Smith *et al.*, 1979a) are produced in the interaction of adherent cells (presumably macrophages) and T cells upon activation with the T cell mitogen Con A. Two pieces of evidence support the view that the factor is the product of a helper T cell. Macrophage-depleted T cells combined with a partially purified macrophage factor (probably IL1), generate IL2 in the presence of Con A (Figure 27). Secondly, the T cell involved has the Ly phenotype of helper T cells for CTL responses, Ly $1^+,2^-,7^+$ (Figures 28 and 29; Pilarski

et al., 1980; Schrader *et al.*, 1980; Gillis and Scheid, 1980). A model depicting the interaction of macrophages and T cells in the induction of IL2 by Con A and alloantigens is shown in Figures 40 and 41, respectively. Although it is generally held that IL2 is produced by allogeneic interactions, it is only assumed that the T cells and macrophages act in a manner analogous to the Con A-driven system. The macrophage becomes activated by a stimulus, and secretes a soluble product, IL1. LPS induces peritoneal macrophages and macrophage cell lines to secrete IL1 (Economou and Shin, 1978; Mizel, 1979; Lachman *et al.*, 1979a, Hoffmann *et al.*, 1979). Another activator is phorbol myristic acetate (Mizel *et al.*, 1978c). Perhaps more biologically relevant is the observation that activated T cells stimulate IL1 production by macrophages. PHA-activated T cells enhance IL1 production by macrophages in a process which requires cell to cell contact (Mizel *et al.*, 1978a). Activated T cells from mice infected with *L. monocytogenes* stimulate IL1 production from "unprimed" macrophages (Farr *et al.*, 1977). The latter type of interaction is H-2 restricted, in that the T cells and macrophages must share the I-A region of H-2. The requirements for cell to cell contact and I-A recognition between T cells and macrophages probably reflect the situation when macrophage-bound antigen is the stimulus for T cells. The requirement for H-2 identity would be bypassed when Con A is the stimulus, since it sticks cells together (Bevan and Cohn, 1975). This would

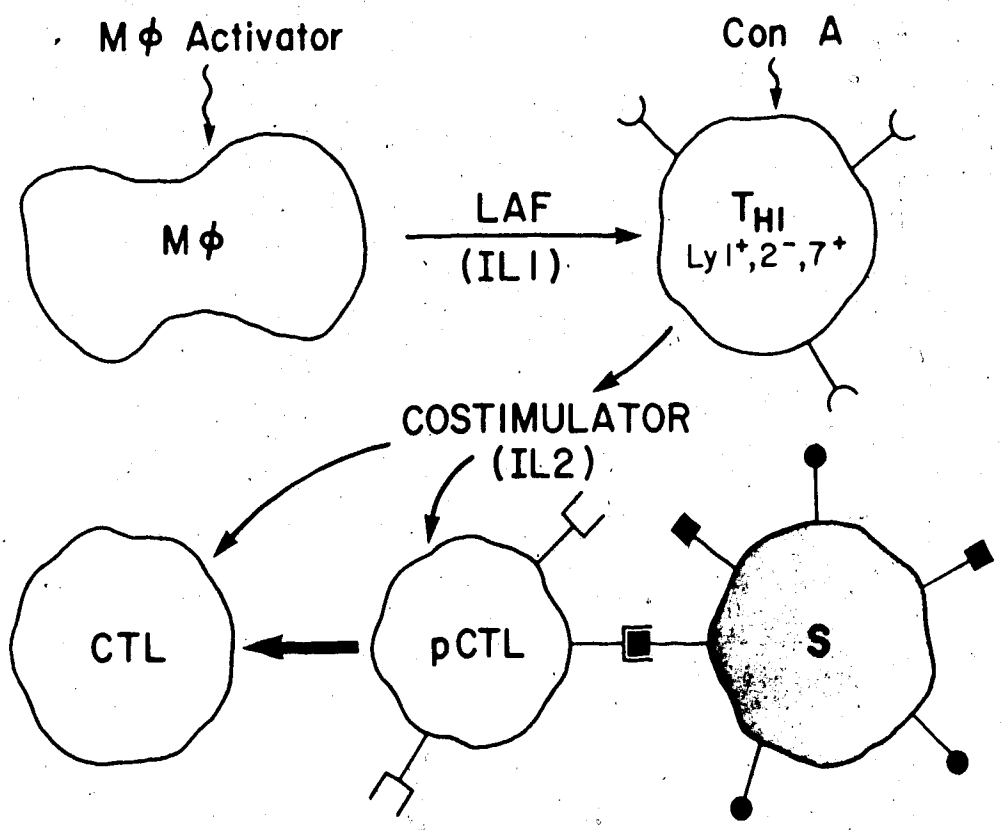


Figure 40. A model for the action of mitogen-induced lymphokines in a CTL response. Activated macrophages secrete LAF (IL1), which enables Con A-stimulated T helper cells (TH1) to secrete costimulator (IL2). The addition of IL2 to CTL-generating cultures enables CTL precursors (pCTL) to respond to an allogeneic stimulator cell (S) in the absence of T helper cells. IL2 may also act directly on CTL to induce proliferation of the stimulated clone.

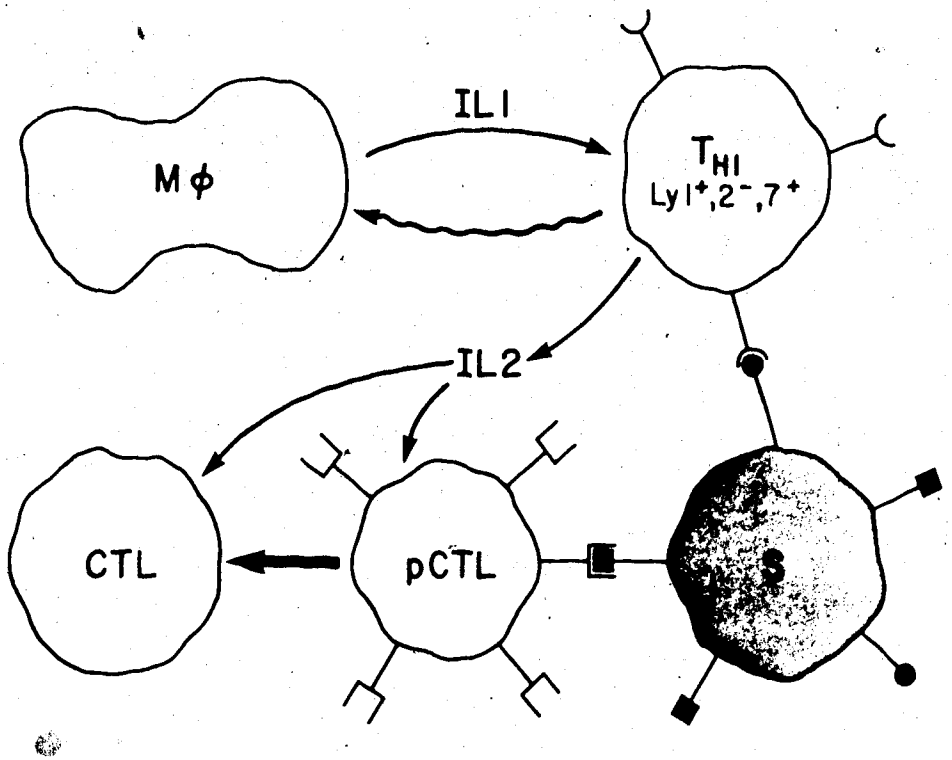


Figure 41. A model for cellular interactions in the induction of a CTL response. A T helper cell (TH1) recognizes antigens on a foreign stimulator cell (S) by means of specific receptors, and activates a macrophage, which secretes IL1. (The TH1-macrophage interaction is I-A restricted.) IL1 induces antigen-stimulated TH1 cells to secrete IL2 in the vicinity of a CTL precursor (pCTL), which also bears receptors for antigens on the stimulator cell. The action of locally released IL2 is thus restricted to antigen-activated pCTL which recognize antigens on the same stimulator cells as the TH1. IL2 may also stimulate the development of antigen-specific helper T cells, and suppressor T cells (not shown).

explain the failure to observe H-2 restriction of macrophages and T cells in IL2 production (Figure 25).

According to the model, the release of IL1 by macrophages enables nearby helper T cells (TH1) which have been activated by either mitogen (Figure 40), or alloantigens on a stimulator cell (S, Figure 41) to secrete IL2.

C. Action of IL2 on Con A-responsive Cells, Helper cells, CTL Precursors and Effectors

IL2 appears to act as a necessary second signal to induce the proliferation of mitogen-or antigen-activated T cells. It replaces the normal requirement for cells which provide a helper signal (Chapter V). For example thymocytes, which are deficient in Ly 1^+ helper T cells (Cantor and Boyse, 1977; Pilarski, 1977) are poor responders to mitogens (Paetkau *et al.*, 1976) unless costimulator is added to the culture medium. IL2 also promotes responses when helper T cells are *inefficiently induced*, as when UV or glutaraldehyde-inactivated stimulator cells are used in CTL responses (Pilarski, 1979). Suboptimal concentrations of stimulator cells may lead to insufficient help. IL2 also substitutes for helper T cells in AFC responses against SRBC (Watson *et al.*, 1979a).

IL2 could act on a number of target T cells to promote these responses; it does not appear to act on B cells (Chapter V, section C; Watson *et al.*, 1979c).

Mitogen-activated T cells, but not B cells, adsorb IL2 activity from culture medium. (Smith *et al.*, 1979a). It is thought that upon activation, T cells express a surface receptor for IL2 which was previously either cryptic or not present. However, the IL2-responsive T cells have not been identified in terms of function or surface markers. The most obvious target for the factor in CTL responses are the killer cell precursors and effectors (pCTL and CTL in Figures 40 and 41). Lalande *et al.* (1980) have recently used a fluorescence-activated cell sorter to separate alloantigen-stimulated from unstimulated cells after a 12 hr MLC. The stimulated cells gave a good CTL response in the absence of further antigenic stimulus if crude Con A-induced factors (containing IL2) were added to the culture medium. The response was equivalent in magnitude to that of stimulated cells which received further antigen. This result implies that sensitivity to IL2 can be induced in as little as 12 hr, and from that point, no more signal 1 is required, at least for some time. The question as to whether IL2 acts on the CTL precursor or a helper T cell is still unanswered.

The finding of Gillis and Smith (1977) that CTL can be cloned and perpetuated *in vitro* if maintained in TCGF suggests that the factor may act directly on CTL effectors. The question as to whether IL2 acts as a differentiative *and* a proliferative signal in CTL generation from precursors must be left open. Recent results indicate that a differentiative step necessary for the expression of CTL

function occurs in the absence of DNA synthesis (MacDonald and Lees, 1979). Whether an inductive signal other than IL2 is required for this early step (perhaps provided by an antigen-specific T cell product), with IL2 acting to expand a differentiated clone remains to be seen.

The ability of IL2 to replace the helper T cell in an AFC response suggests that it may act in helper T cell amplification or induction. The ability of IL2 to maintain continuous cultures of T cells with antigen-specific helper activity has recently been established (Watson, 1979). It may act to induce CTL helper T cells (if they are different from AFC helpers), or in the generation of suppressor T cells.

D. Generation of an Antigen-specific Response with a Non-specific Helper Factor

One of the arguments against the physiological relevance of non-specific helper factors has been the objection to a mechanism of induction which would allow the triggering of lymphocyte clones responsive to antigens unrelated to the one that induce the helper factor. These potentially include self antigens. The objection would be overcome if the nonspecific factor were released by an antigen-specific helper T cell which is in close contact with a CTL precursor responsive to the same set of alloantigens (although not necessarily the same determinant). This is the situation depicted in Figure 41.

Implicit in this model are the ideas that IL2 activity is concentration-dependent, and that it is adsorbed by proximal, activated target cells. As the distance from the secreting helper cell increases, the concentration and effect of the factor decreases. The inducing capabilities are thus restricted to precursor cells which are specifically attracted to the same antigen, whether it is an allogeneic stimulator cell or a syngeneic macrophage bearing antigen. Thus the CTL precursor and the costimulator-secreting cell are brought together through "linked associative recognition" of antigens on a stimulator cell.

Work by Pilarski (1977) has shown that helper T cells for CTL are antigen-specific. However, they can generate CTL responses from thymocyte precursors to antigens other than the ones they bear specific receptors for if the two antigens are physically linked, as on an F1 stimulator cell. This is analagous to the hapten/carrier experiments (Chapter I) in which carrier primed T cells were effective in generating responses to haptens for which there were no helper cells only if the hapten and carrier were linked. The experiment of Pilarski is compatible with an antigen non-specific mediator, which is released by an antigen specific helper T cell. The specificity of the mediator, in effect, is controlled by the specificity of the secreting cell, in a constellation of cells in which the helper and precursor T cells are linked through recognition of a

stimulator cell.

REFERENCES

Aarden, L.A., et al., 1979. Revised nomenclature for antigen-nonspecific T cell proliferation and helper factors. J. Immunol., 123:2928-2929.

Abramson, S., R.G. Miller, and R.A. Phillips 1977. The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J. Exp. Med., 158:1567-1579.

Ada, G.L., and P. Byrt 1969. Specific inactivation of antigen-reactive cells with ^{125}I-labelled antigen. Nature, 222:1291-1292

Andrews, P., 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J., 91:222-233.

Arala-Chaves, M.P., L. Hope, J.H. Korn, and H. Fudenberg 1978. Role of adherent cells in immune responses to phytohemagglutinin and concanavalin A. Eur. J. Immunol., 8:77-81.

Armerding, D., Z. Eshhar, and D.H. Katz 1977. Activation of T and B lymphocytes in vitro. VI. Biochemical and physicochemical characterization of the allogeneic effect factor. J. Immunol., 119:1468-1474.

Askonas, B.A., A.J. Cunningham, H.W. Kreth, G.E. Roelants, and A.R. Williamson 1972. Amplification of B cell clones forming antibody to the 2,4 dinitrophenyl groups. Eur. J. Immunol., 2:494-498.

Askonas, B.A., and J.R. North 1976. The life style of B cells - cellular proliferation and the invariancy of IgG. Cold Spring Harbor Sympos., 41:749-759.

Atkins, R.C., and W.L. Ford 1975. Early cellular events in a systemic graft-vs.-host reaction. I. The migration of responding and nonresponding donor lymphocytes. J. Exp. Med., 141:664-680.

Bach, F.H., H. Bock, K. Graupner, E. Day, and M. Klostermann

1969. Cell kinetic studies in mixed leukocyte cultures: an *in vitro* model of homograft reactivity. Proc. Nat. Acad. Sci. USA, 62:377-384.

Bach, F.H., M.B. Widmer, M.L. Bach, and J. Klein 1972. Serologically defined and lymphocyte defined components of the major histocompatibility complex in the mouse. J. Exp. Med., 136:1430-1444.

Baker, P.E., S. Gillis, and K.A. Smith 1979. Monoclonal cytolytic T-cell lines. J. Exp. Med., 149:273-278.

Barnstable, C.J., E.A. Jones, and W.F. Bodmer 1979. Genetic structure of major histocompatibility regions. International Rev. Biochem., 22:151-225.

Baum, L.L., and L.M. Pilarski 1978. *In vitro* generation of antigen-specific helper T cells that collaborate with cytotoxic T cell precursors. J. Exp. Med., 148:1579-1591.

Beller, D.I., and E.R. Unanue 1977. Thymic maturation *in vitro* by a secretory product from macrophages. J. Immunol., 118:1780-1787.

Benacerraf, B., I. Green, and W.E. Paul 1967. The immune response of guinea pigs to hapten-poly-L-lysine conjugates as an example of the genetic control of the recognition of antigenicity. Cold Spring Harbor Symp., 32:569-575.

Bennink, J.R., and P.C. Doherty 1978. Different rules govern help for cytotoxic T cells and B cells. Nature, 276:829-831.

Bernard, O., N. Hozumi, and S. Tonegawa 1979. Sequences of mouse immunoglobulin light chain genes before and after somatic changes. Cell, 15:1133-1144.

Bernabe, R.R., C. Martinez-Alonso, and A. Coutinho 1979. The specificity of nonspecific concanavalin A-induced helper factor[redacted] J. Immunol., 9:546-552.

B[redacted]76. Cytotoxic T cell response to h[redacted]y antigens - the role of H-2. Cold Spring [redacted]519-527.

Bevan, M., and M. Cohn 1975. Cytotoxic effects of antigen-and mitogen-induced T cells on various targets. J. Immunol., 114:559-565.

Binz, H., and H. Wigzell 1975a. Shared idiotypic determinants on B and T lymphocytes reactive against the same antigenic determinants. I. Demonstration of similar or identical idiotypes on IgG molecules and T-cell receptors with specificity for the same alloantigens. J. Exp. Med., 142:197-211.

Binz, H., and H. Wigzell 1975b. Shared idiotypic determinants on B and T lymphocytes reactive against the same antigenic determinants. III. Physical fractionation of specific immunocompetent T lymphocytes by affinity chromatography using anti-idiotypic antibodies. J. Exp. Med., 142:1231-1240.

Binz, H., and H. Wigzell 1977a. Idiotypic, alloantigen-reactive T lymphocyte receptors and their use to induce specific transplantation tolerance. Prog. Allergy, 23:154-198.

Binz, H., and H. Wigzell 1977b. Antigen-binding, idiotypic T lymphocyte receptors. New York and London: Plenum Press, Contemporary Topics in Immunobiology, Volume 7, edited by O. Stutman, pp113-177.

Blanden, R.V., 1972. T cell response to viral and bacterial infection. Transplant. Rev., 19:56-88.

Blyden, G., and R.E. Handschumacher 1977. Purification and properties of human lymphocyte activating factor (LAF). J. Immunol., 118:1631-1638.

Boyse, E.A., M. Miyazawa, T. Aoki, and L.J. Old 1968. Ly-A and Ly-B: two systems of lymphocyte isoantigens in the mouse. Proc. Roy. Soc. B., 170:175-193.

Brack, C., M. Hirama, R. Lenhard-Schuller, and S. Tonegawa 1978. A complete immunoglobulin gene is created by somatic recombination. Cell, 15:1-14.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the

principle of protein-dye binding. Analyt. Biochem., 72:248-254.

Brand, A., D.G. Gilmour, and G. Goldstein 1976. Lymphocyte differentiating hormone of bursa of fabricius. Science, 193:319-320.

Bretscher, P., 1972. The control of humoral and associative antibody synthesis. Transplant. Rev., 11:217-267.

Bretscher, P., 1974. Two signal model for B cell induction. Transplant. Rev., 23:37-48.

Bretscher, P., 1977. An integration of B and T lymphocytes in immune activation. *In* B and T Cells in Immune Recognition. (F. Loor and G.E. Roelants, eds.), John Wiley and Sons, New York.

Bretscher, P., and M. Cohn 1970. A theory of self-nonself discrimination. Science, 169:1042-1049.

Britton, S., 1972. When allogeneic mouse spleen cells were mixed *in vitro* the T cells secrete a product which guides the maturation of B cells. Scand. J. Immunol., 1:89-98.

Buchmuller, Y., and J. Mauel 1979. Studies on the mechanisms of macrophage activation. II. Parasite destruction in macrophages activated by supernates from concanavalin A stimulated lymphocytes. J. Exp. Med., 150:359-370.

Burgess, A.W., D. Metcalf, and S. Russel 1978. Regulation of hematopoietic differentiation and proliferation by colony stimulating factors. *In* Differentiation of Normal and Neoplastic Hematopoietic Cells. (B. Clarkson, P.A. Marks, and J.E. Till, eds.), Cold Spring Harbor Labs., pp339-357.

Burnet, M., 1959. The Clonal Selection Theory of Acquired Immunity. Vanderbilt University Press, Nashville, Tenessee.

Byrt, P., and G.L. Ada 1967. An *in vitro* reaction between labelled flagellin or haemocyanin and lymphocyte-like cells from normal animals. Immunol., 17:503-516.

Cantor, H., and R. Asofsky 1970. Synergy among lymphoid cells mediating the graft-versus-host response. II. Synergy in graft versus -host reactions produced by BALB/c lymphoid cells of differing anatomic origin. J. Exp. Med., 131:235-246.

Cantor, H., and E.A. Boyse 1975a. Functional subclasses of T lymphocytes bearing different Ly antigens. I. Generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. J. Exp. Med., 141:1376-1389.

Cantor, H., and E.A. Boyse 1975b. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly^+ cells in the generation of killer activity. J. Exp. Med., 141:1390-1399.

Cantor, H., and E.A. Boyse 1976a. Separation of helper T cells from suppressor T cells expressing different Ly components. II. Activation by antigen: after immunization, antigen-specific suppressor and helper activities are mediated by distinct T cells subclasses. J. Exp. Med., 143: 1391-1401.

Cantor, H., and E.A. Boyse 1976b. Regulation of cellular and humoral immune responses by T-cell subclasses. Cold Spring Harbor Symp., 41:23-32.

Cantor, H., and E.A. Boyse 1977. Lymphocytes as models for the study of mammalian cellular differentiation. Immunol. Rev., 33:105-124.

Cerottini, J.C., and K.T. Brunner 1977. Mechanism of T and K cell-mediated cytolysis. *In* B and T Cells in Immune Recognition. (F. Loor and G.E. Roelants, eds.), John Wiley and Sons, New York.

Cerottini, J.C., A.A. Nordin, and K.T. Brunner 1970. Specific *in vitro* cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. Nature, 228:1308-1309.

Chen, D.-M., and G. DiSabato 1976. Further studies on the thymocyte stimulating factor. Cell. Immunol., 22:211-224.

Chen, D.-M., and G. DiSabato 1977. Some effects of thymocyte-stimulating factor. Cell. Immunol., 30:195-203.

Chiorazzi, N., S.M. Fu, and H.G. Kunkel 1979. Induction of polyclonal synthesis by human allogeneic and autologous helper factors. J. Exp. Med., 149:1543-1548.

Claman, H.N., E.A. Chaperon, and R.F. Triplett 1966. Thymus-marrow cell combinations. Synergism in antibody production. Proc. Soc. Exp. Biol. Med., 122:1167-1171.

Cline, M.J., 1975. The White Cell. Harvard University Press, Cambridge, Mass.

Coutinho, A., 1975. The theory of the "one nonspecific signal" model for B cell activation. Transplant. Rev., 23:49-65.

Crumpton, M.J., and D. Snary 1979. Cell surface-antigens--with special reference to histocompatibility antigens. International Review of Biochem., 23:127-176.

Cunningham, A.J., 1976. Evolution in microcosm: the rapid somatic diversification of lymphocytes. Cold Spring Harbor Symp., 41:761-770.

Cunningham, A., and A. Szenberg 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunol., 14:599-600.

Cunningham, B.A., R. Henning, R.J. Milner, K. Reske, J.A. Ziffer, and G.M. Edelman 1976. Structure of murine histocompatibility antigens. Cold Spring Harbor Symp., 41:351-362.

Delovitch, T.L., J. Biggin, and F.-Y. Fung 1978. *In vitro* analysis of allogeneic lymphocyte interaction. II. *I*-region control of the activity of a B-cell-derived H-2 restricted allogeneic effect factor and its receptor during B-cell activation. J. Exp. Med., 147:1198-1212.

Delovitch, T.L., and H.O. McDevitt 1977. *In vitro* analysis of allogeneic lymphocyte interaction. I. Characterization and cellular origin of an Ia-positive helper factor--allogeneic effect factor. J. Exp. Med., 146:1019-1032.

Delovitch, T.L., and U. Sohn 1979. *In vitro* analysis of allogeneic lymphocyte interaction. III. Generation of a helper allogeneic effect factor (AEF) across an *I-J* subregion disparity. J. Immunol., 122:1528-1534.

Diener, E., and V. Paetkau 1972. Antigen recognition: early surface-receptor phenomena induced by binding of a tritium-labelled antigen. Proc. Nat. Acad. Sci. USA, 69:2364-2368.

DiSabato, G., M. Altin, and D.-M. Chen 1977. Thymocyte-stimulating factor from peripheral blood cells. Cell. Immunol., 30:204-215.

DiSabato, G., D.-M. Chen, and M. Altin 1978. Production of Thymocyte-Stimulating Factor by murine spleen lymphocytes: Cellular and biochemical mechanisms. Cell. Immunol., 35:340-352.

DiSabato, G., D.-M. Chen, and J.W. Erickson 1975. Production by murine spleen cells of an activity stimulating the PHA-responsiveness of thymus lymphocytes. Cell. Immunol., 17:495-504.

DiSabato, G., E.K. Morass, and M. Altin 1979. Effect of thymocyte-stimulating factor-containing supernatants on the surface antigens of murine thymocytes. Cell. Immunol., 44:252-261.

Doherty, P.C., R.V. Blanden, R.M. Zinkernagel 1976. Specificity of virus immune effector T cells for H-2K or H-2D compatible interactions: implications for H antigen diversity. Transplant. Rev., 29:89-124.

Domzig, W., and M.-L. Lohmann-Matthes 1979. Antibody dependent cellular cytotoxicity (ADCC) against tumor cells. II. The promonocyte identified as effector cell. Eur. J. Immunol., 9:267-272.

Dryer, W.J., and J.C. Bennet 1965. The molecular basis of antibody formation: a paradox. Proc. Nat. Acad. Sci. USA, 54:864-869.

Dutton, R.W., R. Falkoff, J.A. Hirst, M. Hoffmann, J. Kappler, J.R. Kettman, J.F. Lesley, and D. Vann 1971. Is

there evidence for a non-antigen specific diffusable chemical mediator from the thymus derived cell in the initiation of the immune response? Prog. Immunol., 1:355-368.

Economou, J. S., and H.S. Shin 1978. Lymphocyte-activating Factor. I. Generation and physicochemical characterization. J. Immunol., 121:1446-1452.

Eichmann, K., 1975. Idiotype suppression. II. Amplification of a suppressor T cell with anti-idiotypic activity. Eur. J. Immunol., 5:511-517.

Eichmann, K., and K. Rajewsky 1975. Induction of T and B cell immunity by anti-idiotypic antibody. Eur. J. Immunol., 5:661-666.

Erb, P., and M. Feldmann 1975. The role of macrophages in the generation of T helper cells. III. Influence of macrophage derived factors in helper cell induction. Eur. J. Immunol., 5:759-766.

Erb, P., M. Feldmann, and N. Hogg 1976. Role of macrophages in the generation of T helper cells. IV. Nature of genetically related factor derived from macrophages incubated with soluble antigens. Eur. J. Immunol., 6:365-372.

Erb, P., B. Meier, and M. Feldmann 1979. Is genetically related macrophage factor (GRF) a soluble immune response (Ir) gene product? J. Immunol., 122:1916-1919.

Eshhar, Z., D. Amerding, T. Waks, and D.H. Katz 1977. Activation of T and B lymphocytes *in vitro*. V. Cellular locus, metabolism, and genetics of induction and production of the allogeneic effect factor. J. Immunol., 119:1457-1467.

Farr, A.G., M.E. Dorf, and E.R. Unanue 1977. Secretion of mediators following T lymphocyte-macrophage interaction is regulated by the major histocompatibility complex. Proc. Natl. Acad. Sci. USA, 74:3542-3546.

Farrar, J.J., W.J. Koopman, and J. Fuller-Bonar 1977. Identification and partial purification of two synergistically acting helper mediators in human mixed

leukocyte culture supernatants. J. Immunol., 119:47-54.

Farrar, J.J., P.L. Simon, W.J. Koopman, and J. Fuller-Bonar 1978. Biochemical relationship of Thymocyte Mitogenic Factor and factors enhancing humoral and cell-mediated immune responses. J. Immunol. 121:1353-1360.

Feldmann, M., and A. Basten 1972. Cell interactions in the immune response *in vitro*. III., Specific collaboration across a cell impermeable membrane. J. Exp. Med., 136:49-67.

Feldmann, M., P.C.L. Beverly, J. Woody, and I.F.C. McKenzie 1977. T-T interactions in the induction of suppressor and helper T cells: analysis of membrane phenotype of precursors and amplifier cells. J. Exp. Med., 145:793-801.

Finke, J.H., C.G. Orosz, and J.R. Battisto 1977. Splenic T-killer cells can be generated by allogeneic thymic cells in conjunction with assisting factor. Nature, 167:253-254.

Gately, C.L., M.K. Gately, and M.M. Mayer 1975. The molecular dimensions of mitogenic factor from guinea pig lymph node cells. J. Immunol., 114:10-16.

Gately, M.K., and M.M. Mayer 1974. The molecular dimensions of guinea pig lymphotoxin. J. Immunol. 112:168-177.

Gerhart, S., G. Mills, V. Monticone, and V. Paetkau 1976. Quantitative analysis of the proliferative activity induced in murine thymocytes by concanavalin A. J. Immunol., 117:1314-1319.

Germain, R.N., M. Pierres, S.-T. Ju, M. Dorf, and B Benacerraf 1979. Properties of T-suppressor factor specific for L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT). Abstracts of 2nd International Lymphokine Workshop, #109.

Gershon, R.K., and K. Kondo 1971. Infectous immunological tolerance. Immunol., 21:903-914.

Gery, I., R,K. Gershon, and B.H. Waskman 1971. Potentiation of cultured mouse thymocyte responses by factors released by peripheral leukocytes. J. Immunol., 107:1778-1780.

Gery, I., and R. Handschumacher 1974. Potentiation of the T lymphocyte response to mitogens. III. Properties of the mediator(s) from adherent cells. Cell Immunol., 111:162-169..

Gery, I., and B.H. Waksman 1972. Potentiation of the T lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). J. Exp. Med., 136:143-155.

Gillis, S., M.M. Ferm, W. Ou, and K. Smith 1978a. T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol., 120:2027-2032.

Gillis, S., and M. Scheid 1980. Identification and phenotypic characterization of T-cell growth factor (TCGF) producing T-lymphoma lines. Abstracts, 9th Annual ICN-UCLA Symposia on Molecular and Cellular Biology. (Journal of Supramolecular Structure Supplement 4, 1980).

Gillis, S., and K.A. Smith 1977. *In vitro* generation of tumor-specific cytotoxic lymphocytes. Secondary allogeneic mixed tumor lymphocyte culture of normal murine spleen cells. J. Exp. Med., 146:468-482.

Gillis, S., and K.A. Smith 1977. Long term culture of tumor-specific cytotoxic T cells. Nature, 268:154-156.

Gillis, S., N.A. Union, P.E. Baker, and K.A. Smith 1978b. The *in vitro* generation of nude mouse cytolytic T-lymphocytes. J. Exp. Med., 149:1460-1476.

Gillis, S., N.A. Union, P.E. Baker, and K.A. Smith 1979. The *in vitro* generation and sustained culture of nude mouse cytolytic T-lymphocytes. J. Exp. Med., 149:1460-1476.

Goodman, J.W., 1977. Antigen receptors of T and B lymphocytes. *In* B and T Cells in Immune Recognition. pp 191-210. (F. Loor, and G.E. Roelants, eds.), John Wiley and Sons, Toronto.

Goldstein, G., 1978. Polypeptides regulating lymphocyte differentiation. *In* Differentiation of Normal and Neoplastic Hematopoietic Cells. (B. Clarkson, P.A. Marks and J.E. Till, eds.), Cold Spring Harbor Labs.

Gorczynski, R.M., R.G. Miller, and R.A. Phillips 1972. initiation of antibody production to sheep erythrocytes *in vitro*: replacement of the requirement for T-cells with a cell-free factor isolated from cultures of lymphoid cells. J. Immunol., 108:547-551.

Gordon, J., and L.D. MacLean 1965. A lymphocyte-stimulating factor produced *in vitro*. Nature, 208:795-796.

Gorer, P.A., and H. Schutze 1938. Genetical studies on immunity in mice. II. Correlation between antibody formation and resistance. Journal of Hygiene, 38:647-662.

Greaves, M., and G. Janossy 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev., 11:87-130.

Green, M.I., A. Pierres, M.E. Dorf and B. Benacerraf 1977. The *I-J* subregion codes for determinants on suppressor factor(s) which limit the contact sensitivity to picryl chloride. J. Exp. Med., 146:293-296.

Hammerling, G.J., S.J. Black, C. Berek, K. Eichmann, and K. Rajewsky 1976. Idiotypic analysis of lymphocytes *in vitro*. II. Genetic control of T helper responsiveness to anti-idiotypic antibody. J. Exp. Med., 143:861-869.

Harwell, L., J.W. Kappler, and P. Marrack 1976. Antigen-specific and nonspecific mediators of T cell/B cell cooperation. III. Characterization of the nonspecific mediator(s) from different sources. J. Immunol., 116:1379-1384.

Herzenberg, L.A., L.A. Herzenberg, S.J. Black, M.R. Loken, K. Okumura, W. van der Loo, B.A. Osborne, D. Hewgill, J.W. Goding, G. Gutman, and N.L. Warner 1976. Surface markers and functional relationships of cells involved in murine B-lymphocyte differentiation. Cold Spring Harbor Symp., 41:33-45.

Hiserodt, J.C.,G.J. Tiangco, and G.A. Granger 1979. The LT system in experimental animals. I. Rapid release of high levels of lymphotoxin (LT) activity from murine lymphocytes during the interaction with lectin-treated allogeneic or xenogeneic target cells *in vitro*. J. Immunol., 123:311-316.

Hochman, J., D. Inbar, and D. Givol 1973. An active antibody fragment (Fv) composed of the variable portions of heavy and light chains. Biochem., 12:1130-1135.

Hoessli, D.C., A.P. Jones, and B.H. Waksman 1977. Potentiation of the T lymphocyte response to mitogens. IV. Serum-free production and testing of macrophage soluble products. Cell. Immunol. 30:310-320.

Hoffmann, M.K., S. Koenig, R.S. Mittler, H.F. Oettgen, P. Ralph, C. Galanos, and U. Hammerling 1979. Macrophage factor controlling differentiation of B Cells. J. Immunol., 122:497-502.

Hoffmann, M.K., and J. Watson 1979. Helper T cell-replacing factors secreted by thymus-derived cells and macrophages: cellular requirements for B cell activation and synergistic properties. J. Immunol., 122:1371-1375.

Honjo, T., S. Packman, D. Swan, and P. Leder 1976. Quantitation of constant and variable region genes for mouse immunoglobulin lambda chains. Biochem., 15:2780-2785.

Hood, L., J.H. Campbell, and S.C.R. Elgin 1975. The organization, expression, and evolution of antibody genes and other multigene families. Ann. Rev. Genetics, 9:305-353.

Howard, J.C., 1979. Immunological tolerance. Int. Rev. Biochem., 22:227-262.

Howie, S., and M. Feldmann 1977. *In vitro* studies on H-2 linked unresponsiveness to synthetic polypeptides. III. Production of an antigen-specific T helper cell factor to (T,G)-A--L. Eur. J. Immunol., 9:417-421.

Howie, S., C.R. Parish, C.S. David, I.F.C. McKenzie, P.H. Maurer, and M. Feldmann 1979. Serological analysis of antigen-specific helper factors specific for (T,G)-A--L and GAT. Eur. J. Immunol., 9:501-506.

Hozumi, N., and S. Tonegawa 1976. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. Proc. Nat. Acad. Sci. USA, 73:3628-3632.

Hubner, L., Muller, G., Schimpl, A., Wecker, E. 1978. Partial characterization and purification of murine T cell-replacing factor, TRF - II. Biochemical characteristics. Immunochem., 15:33-39.

Hunter, P., and J.R. Kettman 1974. Mode of action of a supernatant activity from T cell cultures that nonspecifically stimulates the humoral immune response. Proc. Nat. Acad. Sci. USA, 71:512-516.

Hunig, Th., A. Schimpl, and E. Wecker 1977. Mechanism of T-cell help in the immune response to soluble protein antigens. I. Evidence for In Situ generation and action of T-cell-replacing factor during the anamnestic response to dinitrophenyl Keyhole Limpet Hemocyanin In Vitro. J. Exp. Med., 145:1216-1227.

Hunig, Th., A. Schimpl, and E. Wecker 1977. Mechanism of T-cell help in the immune response to soluble protein antigens. II. Reconstitution of primary and secondary *in vitro* immune responses to dinitrophenyl-carrier conjugates by T-cell-replacing factor. J. Exp. Med., 145:1228-1236.

Jacobs, D., 1979. Synergy between T cell-replacing factor and bacterial lypopolysaccharide (LPS) in the primary antibody response *in vitro*: a model for lipopolysaccharide adjuvant action. J. Immunol., 122:1421-1426.

Jandinski, J., H. Cantor, T. Takakuma, D.L. Peavy, and C.W. Pierce 1976. Separation of helper T cells from suppressor T cells expressing different Ly components. I. Polyclonal activation: suppressor and helper activites are inherent properties of distinct T-cell subclasses. J. Exp. Med., 143:1382-1390.

Janis, M., and F.H. Bach 1970. Potentiation of *in vitro* lymphocyte reactivity. Nature, 225:705-706.

Jerne, N.K., 1955. The natural-selection theory of antibody formation. Proc. Nat. Acad. Sci. USA, 41:849-856.

Jerne, N.K., 1971. The somatic generation of immune recognition. Eur. J. Immunol., 1:1-9.

Julius, M.H., E. Simpson, and L.A. Herzenberg 1973. A rapid

method for the isolation of functional thymus derived cells. Eur. J. Immunol., 3:645-649.

Kapp, J.A., C.W. Pierce, S. Schlossman, and B. Benacerraf 1974. Genetic control of immune responses *in vitro*. V. Stimulation of suppressor T cells in non-responder mice by the tetrapolymer L-glutamic acid^{60}-L-alanine^{30}>-L-tyrosine^{10} (GAT). J. Exp. Med., 140:648-659.

Kapp, J.A., C.W. Pierce, J. Theze, and B. Benacerraf 1978. Expression of IR genes in T cells. *In* IR Genes and Ia Antigens. (H. McDevitt, Ed.), Academic Press, New York.

Kasakura, S., and L. Lowenstein 1965. A factor stimulating DNA synthesis derived from the medium of leukocyte cultures. Nature, 208:794-795/

Katz, D.H., 1977. Lymphocyte Differentiation, Recognition, and Regulation. Academic Press, New York.

Kilburn, D.G., F.O. Talbot, H.-S. Teh, and J.G. Levy 1979. A specific factor which enhances the cytotoxic response to a syngeneic tumor. Nature, 277:474-476.

Kindred,B., 1979. Nude mice in immunology. Prog. Allergy, 26:137-238.

Kindred, B., and R.B. Corley 1977. A T cell-replacing factor specific for histocompatibility antigens in mice. Nature, 268:531-532.

Kishimoto, T. T. Hirano, T. Kuritani, Y. Yamamura, P. Ralph, and R.A. Good 1978. Induction of IgG production in human B lymphoblastoid cell lines with normal human T cells. Nature, 271:756-758.

Klinman, N.R., 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. J. Exp. Med., 136: 241-260.

Klinman, N.R., and J.L. Press 1975. The B cell specificity repertoire: its relationship to definable subpopulations. Transplant. Rev., 24:41-83.

Kohler, G., 1976. Frequency of precursor cells against the enzyme beta-galactosidase. An estimate of the BALB/c strain antibody repertoire. Eur. J. Immunol., 6:340-346.

Kohler, G., and C. Milstein 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256:495-497.

Kohler, G., and C. Milstein 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol., 6:511-519.

Kontiainen, S., E. Simpson, E. Bohrer, P.C.L. Beverley, L.A. Herzenberg, W.C. Fitzpatrick, P. Vogt, A Torano, I.F.C. McKenzie, and M. Feldmann 1978. T cell lines producing factor. Nature, 274:477-480.

Koopman, W. J., J.J. Farrar, and J. Fuller-Bonar 1978. Evidence for the identification of lymphocyte activating factor as the adherent cell-derived mediator responsible for enhanced antibody synthesis by nude mouse spleen cells. Cell. Immunol. 35:92-98.

Koopman, W. J., J. Farrar, J. Oppehheim, J. Fuller-Bonar, and S. Dougherty 1977. Association of a low molecular weight helper factor(s) with thymocyte proliferative activity. J. Immunol., 119:55-59.

Kreth, H.W., and A.R. Williamson 1973. The extent of diversity of anti-hapten antibodies in inbred mice: anti-NIP (4-hydroxy-5-iodo-3-nitro-phenacetyl) antibodies in CBA/H mice. Eur. J. Immunol., 3:141-147.

Lachman, L.B., M.P. Hacker, G.T. Blyden, and R.E. Handschumacher 1977. Preparation of lymphocyte-activating factor from continuous murine macrophage cell lines. Cell. Immunol., 34:416-419.

Lachman, L.B., M.P. Hacker, and R.E. Handschumacher 1977. Partial purification of human lymphocyte-activating factor (LAF) by ultrafiltration and electrophoretic techniques. J. Immunol., 119:2019-2023.

Lachman, L.B., J.O. Moore, and R.S. Metzgar 1978. Preparation and characterization of lymphocyte-activating

factor (LAF) from acute monocytic and myelomonocytic leukemia cells. Cell. Immunol., 41:199-206.

Lafferty, K.J., and J. Woolnough 1977. The origin of the allograft reaction. Immunol. Rev., 35:231-262.

Lafferty, K.J., H.S. Warren, J.A. Woolnough, and D.W. Talmage 1978. Immunological induction of T lymphocytes: Role of antigen and the lymphocyte costimulator. Blood Cells, 4:395-404.

Lalande, M.E., M.J. McCutcheon, and R.G. Miller 1980. Quantitative studies on the precursors of cytotoxic lymphocytes. VI. Second signal requirements of specifically activated precursors isolated 12 h after stimulation. J. Exp. Med., 151:12-19.

Langman, R.E., 1978. Cell-mediated immunity and the major histocompatibility complex. Rev. Physiol. Biochem. Pharmacol., 81:1-37.

Larsson, E.-L., B. Mannervik, and H. Blomgren 1979. Characterization of a human mitogenic factor (MF) released by phytohemagglutinin-activated lymphocytes. Cell. Immunol., 44:424-432.

Lawton, A.R., R. Asofsky, M.B. Hylton, and M.D. Cooper 1972. Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to mu chains. J. Exp. Med., 135:277-297.

Lee, K.C., 1980. On the origin and mode of action of functionally distinct macrophage subpopulations. Molecular and Cellular Biochemistry, in press.

Lee, K.-C., C. Shiozawa, A. Shaw, and E. Diener 1976. Requirement for accessory cells in the antibody response to T cell-independent antigens *in vitro*. Eur. J. Immunol., 6:63-68.

Lenhard-Schuller, R., B. Hohn, C. Brack, M. Hirama, and S. Tonegawa 1978. DNA clones containing mouse immunoglobulin kappa chain genes isolated by *in vitro* packaging into phage lambda coats. Proc. Nat. Acad. Sci. USA, 75:4709-4713.

Levin, A.S., H.H. Fudenberg, J.E. Hopper, S.K. Wilson, and A. Nisonoff 1971. Immunofluorescent evidence for cellular control of synthesis of variable regions of light and heavy chains of immunoglobulins G and M by the same gene. Proc. Nat. Acad. Sci. USA, 68:169-171

Lindahl, K.F., and K. Rajewsky 1979. T cell recognition: genes, molecules and functions. Int. Rev. Biochem., 22A:97-150.

Lonai, P., Y. Ben-Neriah, L. Steinman, and D. Givol 1978. Selective participation of immunoglobulin V region and major histocompatibility complex products in antigen binding by T cells. Eur. J. Immunol. , 8:827-832.

Lyons, C.R., T.F. Tucker, and J.W. Uhr 1979. Specific binding of T lymphocytes to macrophages. V. The role of Ia antigens on macrophages in the binding. J. Immunol., 122:1598-1600.

MacDonald, H.R., and R.K. Lees 1979. Primary generation of cytolytic T lymphocytes in the absence of DNA synthesis. J. Exp. Med., 150:196-201.

Mackler, B.F., R.A. Wolstencroft, and D.C. Dumonde 1972. Concanavalin A as an inducer of human lymphocyte mitogenic factor. Nature New Biol., 239:139-142.

Marrack (Hunter), P.C., and J.W. Kappler 1975. Antigen-specific and non-specific mediators of T cell/B cell cooperation. I. Evidence for their production by different cells. J. Immunol., 114:1116-1125.

Marrack, P., and J.W. Kappler 1977. Anti-Ia inhibits the activity of B cells but not a T cell-derived helper mediator. Immunogenetics, 4:541-555.

Marshak-Rothstein, A., P. Fink, T. Gridley, D.H. Raulet, M.J. Bevan, and M.C. Gefter 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. J. Immunol., 122:2491-2497.

Matzinger, P., and G. Mirkwood 1978. In a fully H-2 incompatible chimera, T cells of donor origin can respond to minor histocompatibility antigens in association with either

donor or host H-2 type. J. Exp. Med., 148:84-92.

Max, E.E., J.G. Seidman, and P. Leder 1979. Sequences of five potential recombination sites encoded close to an immunoglobulin kappa constant region gene. Proc. Nat. Acad. Sci. USA, 76:3450-3454.

McDevitt, H.O., T.L. Delovitch, and J.L. Press 1977. Functional and genetic analysis of Ia antigens. Cold Spring Harbor Symp., 41:489-496.

McKenzie, I.F.C., J. Gardiner, M. Cherry, and G.D. Snell 1977. Lymphocyte antigens: Ly-4,Ly-6, and Ly-7. Transplant. Proc., 9:667-669.

McKenzie, I.F.C., T. Pang, and R.V. Blanden 1977. The use of H-2 mutants as models for the study of T cell activation. Immunol. Rev. 35:181-230.

McKenzie, I.F.C., and M. Potter 1979. Murine lymphocyte surface antigens. In press.

Metcalf,D., and M.A.S. Moore 1971. Haemopoietic Cells. North-Holland Research Monographs. Front. Biol. vol. 24.

Miller, J.F.A.P., and M.A. Vadas 1976. Antigen activation of T lymphocytes: influence of major histocompatibility complex. Cold Spring Harbor Symp., 41:579-588.

Miller, R.G., and M. Dunkley 1974. Quantitative analysis of the chromium release cytotoxicity assay for cytotoxic lymphocytes. Cell. Immunol., 14:284-302.

Mills, G., V. Monticone, and V. Paetkau 1976. The role of macrophages in thymocyte mitogenesis. J. Immunol., 117:1325-1330.

Mishell, R.I., and R.W. Dutton 1967. Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med., 126:423-442.

Mitchell, G.F., and J.F.A.P. Miller 1968. Immunological activity of thymus and thoracic-duct lymphocytes. Proc. Nat.

Acad. Sci. USA, 59:296-303.

Mitchison, N.A., 1971. The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. Eur. J. Immunol., 1:18-27.

Mizel, S.B. 1979. Physicochemical characterization of lymphocyte-activating factor (LAF). J. Immunol., 122:2167-2172.

Mizel, S.B., J.J. Oppenheim, and D.L. Rosenstreich 1978a. Characterization of lymphocyte-activating factor (LAF) produced by the macrophage cell line, P388D1. I. Enhancement of LAF production by activated T lymphocytes. J. Immunol., 120:1497-1503.

Mizel, S.B., J.J. Oppenheim, and D.L. Rosenstreich 1978b. Characterization of lymphocyte-activating factor (LAF) produced by a macrophage cell line, P388D1. II. Biochemical characterization of LAF induced by activated T cells and LPS. J. Immunol., 120:1504-1508.

Mizel, S.B., and D.L. Rosenstreich 1979. Regulation of lymphocyte-activating factor (LAF) production and secretion P388D1 cells: identification of high molecular weight precursors of LAF. J. Immunol., 122:2173-2178.

Mizel, S.B., D.L. Rosenstreich, and J.J. Oppenheim 1978c. Phorbol myristic acetate stimulates LAF production by the macrophage cell line, P388D1. Cell Immunol., 40:230-235.

Moore, M.A.S., and D. Metcalf 1970. Ontogeny of the haemopoietic system: yolk sac origin of *in vivo* and *in vitro* colony forming cells in the developing mouse embryo. Brit. J. Haematol., 18:279-296.

Moorhead, J.W., 1977. Soluble factors in tolerance and contact sensitivity of 2,4-dinitrofluorobenzene in mice. J. Immunol., 119:315-321.

Mosier, D.E., and L.W. Coppleson 1968. A three-cell interaction required for the induction of the primary immune response *in vitro*. Proc. Nat. Acad. Sci. USA, 61:542-547.

Mozes, E., and J. Haimovich 1979. Antigen specific T-cell helper factor cross reacts idiotypically with antibodies of the same specificity. Nature, 278:56-57.

Muller, G., L Hubner, A. Schimpl, and E. Wecker 1978. Partial characterization and purification of murine T cell-replacing factor, TRF - I. Purification procedures and gel electrophoretic analysis. Immunochem., 15:27-32.

Naor,D., and D. Sulitzeanu 1967. Binding of radioiodinated bovine serum albumin to mouse spleen cells. Nature, 214:687-688.

Nossal, G.J.V., and J.W. Schrader 1975. B lymphocyte-antigen interactions in the initiation of tolerance or immunity. Transplant. Rev., 23:138-158.

Okada, M., T. Kishimoto, T. Igarashi, T. Teranishi, and Y. Yamamura 1978. LPS- or 8Br-cyclic AMP-induced production of T cell-activating factor(s) in macrophage tumor cell line J774.1. J. Immunol., 120:1097-1101.

Okada, M., G.R. Klimpel, R.C. Kuppers, and C.S. Henney 1979. The differentiation of cytotoxic T cells in vitro. I. Amplifying factor(s) in the primary response is Lyt 1+ cell dependent. J. Immunol., 122:2527-2533.

Orr, H.T., J.A. Lopez de Castro, P. Parham, H.L. Ploegh, and J.L. Strominger 1979a. Comparison of amino acid sequences of two human histocompatibility antigens, HLA-A2 and HLA-B7: location of putative alloantigenic sites. Proc. Nat. Acad. Sci. USA, 76:4395-4399.

Orr, H.T., D. Lancet, R.J. Robb, J.A. Lopez de Castro, and J.L. Strominger 1979b. The heavy chain of human histocompatibility antigen HLA-B7 contains an immunoglobulin-like region. Nature, 282:266-270.

Owen,J.J.T., 1979. Developmental aspects of the lymphoid system. Int. Rev. Biochem., 22:1-28.

Padlan, E.A., D.R. Davies, S. Rudikoff, and M. Potter 1976. Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. Immunogenetics, 13:945-949.

Paetkau, V.H., 1975. Structure and organization of immunoglobulin genes. PAABS REVISTA, 4:211-219.

Paetkau, V.H., 1979. Thymocyte stimulating factors. *In* Molecular Mediators of Cellular Immunity. (J.W. Hadden and W.E. Stewart II., eds.) Humana Press, Inc., Clifton, New Jersey. In press.

Paetkau, V., G. Mills, S. Gerhart, and V. Monticone 1976. Proliferation of murine thymic lymphocytes *in vitro* is mediated by the concanavalin A-induced release of a lymphokine (costimulator). J. Immunol. 117:1320-1324.

Paul, W.E., and B. Benacerraf 1977. Functional specificity of thymus-dependent lymphocytes. Science, 195:1293-1300.

Paul, W.E., E.M. Shevach, D.W. Thomas, S.F. Pickeral and A.S. Rosenthal 1976. Genetic restriction in T-lymphocyte activation by antigen-pulsed peritoneal exudate cells. Cold Spring Harbor Symp., 41:571-578.

Pickel, K., U. Hammerling, and M.K. Hoffmann 1976. Ly phenotype of T cells releasing T cell replacing factor. Nature, 264:72.

Pilarski, L.M. 1977. A requirement for antigen-specific helper T cells in the generation of cytotoxic T cells from thymocyte precursors. J. Exp. Med., 145:709-725.

Pilarski, L.M., A.R. Al-Adra, and I.F.C. McKenzie 1980. Surface markers on the T cells that regulate cytotoxic T cell responses. II. Distribution of Ly 6.1 and Ly 7.2 on the effector cells of help and suppression. Submitted for publication.

Pilarski, L.M., P.A. Bretscher, and L.L. Baum 1977. Helper T cells are required for the polyclonal stimulation of cytotoxic T cells by Concanavalin A. J. Exp. Med., 145:1237-1249.

Pilarski, L.M. 1979. Antigen-specific helper T cells are essential for cytotoxic T cell responses to metabolically inactivated stimulator cells. Eur. J. Immunol., 9:454-460.

Plate, J.M.D., 1976. Soluble factors substitute for T-T cell collaboration in generation of T killer lymphocytes. Nature, 260:329-331.

Plate, J.M.D. 1977. Two signals required for the initiation of killer T-cell differentiation. Cell. Immunol., 32:183-192.

Rabbits, T.H., 1979. The organization of immunoglobulin genes. Int. Rev. Biochem., 23:49-69.

Rabellino, E.M., G.D. Ross, H.T.K. Trang, N. Williams, and D. Metcalf 1978. Membrane receptors of mouse leukocytes. II. Sequential expression of membrane receptors and phagocytic capacity during leukocyte differentiation. J.Exp. Med., 147:434-445.

Raff, M.C., 1970. Role of the thymus-derived lymphocytes in the secondary humoral immune response in mice. Nature, 226:1257-1258.

Rajewsky, K., and K. Eichmann 1977. Antigen receptors of T helper cells. New York and London: Plenum Press, Contemporary Topics in Immunobiology, Volume 7, edited by O. Stutman, pp 69-112.

Ramseier, H., and J. Lindenmann 1972. Aliotypic antibodies. Transplant. Rev. 10:57-96.

Rief, A.E., and J.M.V. Allen 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissue. J. Exp. Med., 120:413-433.

Roelants, G.E., and B.A. Askonas 1971. Cell cooperation in antibody induction: the susceptibility of helper cells to lethal radioactive antigens. Eur. J. Immunol., 1:151-155.

Rubin, A.S., A.B. MacDonald, and A.H. Coons 1973. Specific heterologous enhancement of immun responses. V. Isolation of a soluble enhancing factor from supernatants of specifically stimulated and allogeneically induced lymphoid cells. J. Immunol., 111:1314-1325.

Rubin, A.S., A.B. MacDonald, and A.H. Coons 1974. Specific

heterologous enhancement of immune responses. VI. Partial purification of a nonspecific enhancing factor from supernates of allogeneically stimulated human lymphocyte cell lines. J. Exp. Med., 139:439-444.

Sakano, H., K. Huppi, G. Heinrich, and S. Tonegawa 1979. Sequence at the somatic recombination sites of immunoglobulin light-chain genes. Nature, 280:288-294.

Sakano, H., J.H. Rogers, K. Huppi, C. Brack, A Traunecker, R. Maki, R. Wall, and S. Tonegawa 1979. Domains and the hinge region of an immunoglobulin heavy chain are encoded in separate DNA segments. Nature, 277:627-633.

Schendel, C., and F.H. Bach 1974. Genetic control of cell-mediated lympholysis in mouse. J. Exp. Med., 140:1534-1546.

Schimpl, A., and E. Wecker 1973. Stimulation of IgG antibody response *in vitro* by T cell- replacing factor. J. Exp. Med., 137:547-552.

Schimpl, A., and E. Wecker 1975. A third signal in B cell activation given by TRF. Transplant. Rev., 23:176-188.

Schrader, J.W., B. Arnold, and I. Clark-Lewis 1980. A Con A-stimulated T-cell hybridoma releases factors affecting haematopoietic colony-forming cells and B-cell antibody responses. Nature, 283:197-199.

Schwartz, R.H., and W.E. Paul 1976. T-lymphocyte-enriched murine peritoneal exudate cells. II. Genetic control of antigen-induced T-lymphocyte proliferation. J. Exp. Med., 143:529-540.

Scott, J.W., and J.H. Finke 1978. The generation of cytotoxic lymphocytes requires soluble helper factor produced in response to lymphocyte defined antigens. Fed. Proc., 37:1557. (Abstract)

Secher, D.S., 1979. Structure of immunoglobulins. Int. Rev. Biochem., 23:1-48.

Seidman, J.G., A Leder, M.H. Edgell, F. Polsky, S.M.

Tilghman, D.C. Tiemeier, and P. Leder 1978. Multiple related immunoglobulin variable-region genes identified by cloning and sequence analysis. Proc. Nat. Acad. Sci. USA, 75:3881-3885.

Seon, B.-K., Y. Yagi, and D. Pressman 1973. Comparative chemical study of alpha and mu chains from a single patient. J. Immunol., 111:1285-1298.

Shaw, J., B. Caplan, V. Paetkau, L.M. Pilarski, T.L. Delovitch, and I.F.C. McKenzie 1980. Cellular origins of costimulator and its activity in cytotoxic T lymphocyte responses. J. Immunol., in press.

Shaw, J., V. Monticone, G. Mills, and V. Paetkau 1978. Effects of costimulator on immune responses *in vitro*. J. Immunol., 120:1974-1980.

Shaw, J., V. Monticone, and V. Paetkau 1978. Partial purification and molecular characterization of a lymphokine (costimulator) required for the mitogenic response of mouse thymocytes *in vitro*. J. Immunol., 120:1967-1973.

Shearer, G.M., R.G. Rehn, and G.A. Barbarino 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes. Effector cell specificity to modified cell surface components controlled by the H-2K and H-2D serological regions of the major histocompatibility complex. J. Exp. Med., 141:1348-1364.

Shearer, G.M., T.G. Rehn, and A.M. Schmitt-Verhulst 1976. Role of the murine major histocompatibility complex in the specificity of *in vitro* T-cell-mediated lympholysis against chemically-modified autologous lymphocytes. Transplant. Rev., 29:222-248.

Shiozawa, C., E. Diener, and B. Singh 1979. B-cell triggering by carrier specific T-cell derived helper factor. Submitted for publication.

Shiozawa, C. B. Singh, S. Rubinstein, and E. Diener 1977. Molecular control of B cell triggering by antigen-specific T cell-derived helper factor. J. Immunol., 118:2199-2205.

Siegel, L.M., and K.J. Monty 1966. Determination of

molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparation of sulfite and hydroxlamine reductases. Biochim. Biophys. Acta., 112:346-362.

Silver, J., J.M. Cecka, M. McMillan, and L. Hood 1976. Chemical characterization of products of the H-2 complex. Cold Spring Harbor Symp., 41:369-377.

Simon, P. L., J.J. Farrar, and P.D. Kind 1977. The xenogeneic effect. III. Induction of cell-mediated cytotoxicity by alloantigen-stimulated thymocytes in the presence of xenogeneic reconstitution factor. J. Immunol., 118:1129-1131.

Simon, P.L., J.J. Farrar, and P.D. Kind 1979. Biochemical relationship between murine immune interferon and a killer cell helper factor. J. Immunol., 122:127-132.

Simpson, E., and R.D. Gordon 1977. Responsiveness to H-Y antigen: Ir gene complementation and target cell specificity. Imm. Rev., 35:59-75.

Sjoberg, O., J. Andersson, and G. Moller 1972. Reconstitution of the antibody response *in vitro* of T cell-deprived spleen cells by supernatants from spleen cell cultures. J. Immunol., 109:1379-1385.

Smith, K.A., S. Gillis, and P.E. Baker 1979a. The production and action of T cell growth factor. *In* Proceedings of the 2nd International Lymphokine Workshop. (A. de Weck, ed.), Adacemic Press, New York.

Smith, K.A., S. Gillis, and P. E. Baker 1979b. The role of soluble factors in the regulation of T-cell immune reactivity. *In* Proceedings of the 13th International Leukocyte Culture Conference. In press.

Sober, H.A. (ed.) 1968. Handbook of Biochemistry. The Chemical Rubber Co. Cleveland. Pp c3-c35.

Sopori, M.L., B.J. Alter, F.H, Bach, and M.L. Bach 1977. Cell-free factors substitute for "signal 2" in generating cytotoxic reactions. Eur. J. Immunol., 7:823-825.

Sprent, J., 1978a. Restricted helper function of F1-parent bone marrow chimeras controlled by K-end of H-2 complex. J. Exp. Med., 147:1838-1842.

Sprent, J., 1978b. Role of the H-2 complex in induction of T helper cells *in vivo*. I. Antigen specific selection of donor T cells to sheep erythrocytes in irradiated mice dependent upon sharing of H-2 determinants between donor and host. J. Exp. Med., 148:478-489.

Springer, T., G. Galfre, D.S. Secher, and C. Milstein 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol., 9:301-306.

Stanley, E.R., M. Cifone, P.M. Heard, and V. Defendi 1976. Factors regulating macrophage production and growth: identity of colony-stimulating factor and macrophage growth factor. J. Exp. Med., 143:631-647.

Stanley, E.R., and P. Heard 1977. Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. J. Biol. Chem., 252:4305-4312.

Storrie, B., G Goldstein, E.A. Boyse, and U. Hammerling 1976. Differentation of thymocytes: evidence that induction of the surface phenotype requires transcription and translation. J. Immunol., 116:1358-1362.

Strominger, J.L., D.L. Mann, P Parham, R. Robb, T. Springer, and C. Terhorst 1976. Structure of HLA-A and B antigens islolated from culture human lymphocytes. Cold Spring Harbor Symp., 41:323-329.

Swain, S.L., and R.W. Dutton 1980. Production of Con A-induced helper T cell factor requires a T cell and an Ia-positive non-T cell. J. Immunol., 124: 437-444.

Swain, S.L., and P.R. Panfili 1979. Helper cells activated by allogeneic H-2K or H-2D differences have an Ly phenotype distinct from those responsive to I differences. J. Immunol., 122:383-391.

Tada,T., and T. Takemori 1974. selective roles of thymus-derived lymphocytes in the antibody response. I.

differential suppressive effect of carrier-primed T cells on hapten-specific IgM and IgG antibody responses. J. Exp. Med., 140:239-252.

Tada, T. M. Taniguchi, and T. Takemori 1975. Properties of primed suppressor T cells and their products. Transplant. Rev., 26:106-129.

Tada, T., M. Taniguchi, and C.S. Takemori 1976. Properties of the antigen-specific suppressive T cell factor in the regulation of antibody responses in the mouse. IV. Special subregion assignment of the gene(s) that code for the suppressive T cell factor in the H-2 histocompatibility complex. J. Exp. Med., 144:713-725.

Takei, F., J.G. Levy, and D.G. Kilburn 1976. *In vitro* induction of cytotoxicity against syngeneic mastocytoma and its suppression by spleen and thymus cells from tumor-bearing mice. J. Immunol., 116:288-293.

Takei, F., J.G. Levy, and D.G. Kilburn 1977. Characterization of suppressor cells in mice bearing syngeneic mastocytoma. J. Immunol., 118:412-417.

Takei, F., J.G. Levy, and D.G. Kilburn 1978. Characterization of a soluble factor that specifically suppresses the *in vitro* generation of cells cytotoxic for syngeneic tumor cells in mice. J. Immunol., 120:1218-1224. p 1200-1215

Talmage, D.W., J.A. Woolnough, H. Hemmingsen, L. Lopez, and K.J. Lafferty 1977. Activation of cytotoxic T cells by nonstimulating tumor cells and spleen cell factor(s). Proc. Natl. Acad. Sci. USA, 74:4610-4614.

Taniguchi, M., T. Saito, I. Takei, and T. Tada 1979. Characterization of antigen-specific suppressive factor produced by an I-J positive cell hybridoma. Abstracts 2nd Int. Lymphokine Workshop.

Taniguchi, M., T. Tada, and T. Tokuhisa 1976. Properties of the antigen-specific suppressive T-cell factor in the regulation of antibody response of the mouse. III. Dual gene control of the T-cell-mediated suppression of the antibody response. J. Exp. Med., 144:20-31.

Taussig, M.J., 1979. Characterization of an antigen-specific suppressor factor from a hybrid T-cell line. Abstracts 2nd Int. Lymphokine Workshop.

Taussig, M.J., E. Mozes, and R. Isac 1974. Antigen-specific thymus cell factors in the genetic control of the immune response to poly-(tyrosyl, glutamyl)-poly-D, L-alanyl--poly-lysal. J. Exp. Med., 140:301-312.

Taussig, M.J., A.J. Munro, R. campbell, C.S. David, and N.A. Staines 1975. Antigen-specific T-cell factor in cell cooperation. Mapping within the I region of the H-2 complex and ability to cooperate across allogeneic barriers. J. Exp. Med., 142:694-700.

Teh, H.-S., E. Harley, R.A. Phillips, and R.G. Miller 1977. Quantitative studies on the precursors of cytotoxic lymphocytes. I. Characterization of a clonal assay and determination of the size of clones derived from single precursors. J. Immunol., 118:1049-1056.

Teh, H.-S., M. Letarte, R.A. Phillips, and R.G. Miller 1978. Characterization of the target cell antigen in I-region mediated lympholysis. Cell. Immunol., 37:397-408.

Teh, H.-S., R.A. Phillips, and R.G. Miller 1978. Quantitative studies on the precursors of cytotoxic lymphocytes. IV. Specificity and cross-reactivity of cytotoxic clones. J. Immunol., 120:425-428.

Tokuhisa, T., M. Taniguchi, K. Okumura, and T. Tada 1978. An antigen-specific I region gene product that augments the antibody response. J. Immunol., 120:414-421.

Tonegawa, S., 1976. Reiteration frequency of immunoglobulin light chain genes: further evidence for somatic generation of antibody diversity. Proc. Nat. Acad. Sci. USA, 73:203-207.

Treves, A.J., 1978. *In vitro* induction of cell-mediated immunity against tumor cells by antigen-fed macrophages. Immunol. Rev., 40:205-226.

Uhr, J.W., J.C. Cambier, F.S. Ligler, J. Kettman, E.S. Vitetta, I. Zan-Bar, and S. Strober 1978. Murine IgD and B

lymphocyte differentiation. *In* Differentiation of Normal and Neoplastic Hematopoietic cells. Cold Spring Harbor Labs., pp 505-518.

Uhr, J.W., D. Capra, E.S. Vitetta, and R.G. Cook 1979. Organization of the immune response genes. Science, 206:292-297.

Uhr, J.W., E.S. Vitetta, J. Klein, M.D. Poulik, D.G. Klapper, and J.D. Capra 1976. Structural studies of H-2 and TL alloantigens. Cold Spring Harbor Symp., 41:363-368.

Unanue, E.R., 1978. Role of macrophages in the immune response. Immunol. Rev., 40:227-255 .

Unanue, E.R., J.-M. Kiely, and J. Calderon 1976. The modulation of lymphocyte functions by molecules secreted by macrophages. II. Conditions leading to increased secretion. J. Exp. Med., 144:155-166.

Uotila, M., H.N. Rode, and J. Gordon 1978. Blastogenic factor: its role in the mixed leukocyte culture reaction. Eur. J. Immunol., 8:133-138.

Valbuena, O., K.B. Marcu, M. Weigert, and R.P. Perry 1978. Multiplicity of germline genes specifying a group of related mouse kappa chains with implications for the generation of immunoglobulin diversity. Nature, 276:780-784.

von Boehmer, H., W. Hass, and N.K. Jerne 1978. Major histocompatibility complex-linked immune-responsiveness is acquired by lymphocytes of low-responder mice differentiating in thymus of high-responder mice. Proc. Nat. Acad. Sci. USA, 75:2439-2442.

von Boehmer, H., H. Hengartner, M. Nabholz, W. Lernhardt, M.H. Schreier, and W. Haas 1979. Fine specificity of a continuously growing killer cell clone specific for H-Y antigen. Eur. J. Immunol., 9:592-597.

Wabl, M.R., L. Forni, and F. Loor 1978. Switch in immunoglobulin class production observed in single clones of committed lymphocytes. Science, 199:1078-1080.

Wagner, H., 1973a. Synergy during *in vitro* cytotoxic allograft responses. I. Evidence between thymocytes and peripheral T cells. J. Exp. Med., 138:1379-1397.

Wagner, H. 1973b. Cell-mediated immune response *in vitro*. IV. Metabolic studies on cellular immunogenicity. Eur. J. Immunol. 3:84-89.

Wagner, H., M. Feldmann, W. Boyle, and J.W. Shrader 1972. Cell-mediated immune response *in vitro*. III. The requirement for macrophages in cytotoxic reaction against cell-bound and subcellular alloantigens. J. Exp. Med., 136:331-343.

Wagner, H., and M. Rollinghoff 1978. T-T-cell interactions during *in vitro* cytotoxic allograft responses. I. Soluble products from activated Ly1+ T cells trigger autonomously antigen-primed Ly23+ T cells to cell proliferation and cytolytic activity. J. Exp. Med., 148:1523-1538.

Waldmann, H., 1975. T cell dependent mediator in the immune response. III. The role of Non-specific Factor (NSF) in the *in vitro* immune response. Immunol., 28:497-507.

Waldmann, H., 1977. Conditions determining the generation and expression of T helper cells. Immunol. Rev., 35:121-145.

Waldmann, H., and J. Howard 1979. Cellular basis for the immune response. Int. Rev. Biochem., 22:29-95.

Waldmann, H., and A. Munro 1974. T cell dependent mediator in the immune response. II. Physical and biological properties. Immunol., 27:53-64.

Waldmann, H., and A. Munro 1975. The interrelationship of antigenic structure, thymus independence and adjuvanticity. IV. A general model for B-cell induction. Immunol., 28:509-522.

Waldmann, H., P. Poulton, and C. Desaymard 1976. Antigen-non-specific T-cell factor in B-cell activation. Origin, biological properties, and failure to show a relationship to H-2. Immunol., 30:723-733.

Watson, J., 1973. The role of humoral factors in the

initiation of *in vitro* primary immune responses. IIa. characterization of factors that replace thymus derived cells. J. Immunol., 111:1301-1308.

Watson, J., 1974. The nature of the signals required for the induction of antibody synthesis. *In* The Immune System. (E.E. Sercarz, A.R. Williamson, and C.F. Fox, eds.)., pp 511-532.

Watson, J., 1979. Continuous proliferation of murine antigen specific helper T lymphocytes in culture. J. Exp. Med., 150:1210-1519.

Watson, J., L.A. Aarden, and I. Lefkovits 1979b. The purification and quantitation of helper T cell-replacing factors secreted by murine spleen cells activated by Concanavalin A. J. Immunol., 122:209-215.

Watson, J., L.A. Aarden, J. Shaw, and V. Paetkau 1979a. Molecular and quantitative analysis of helper T cell-replacing factors on the induction of antigen-sensitive B and T lymphocytes. J. Immunol., 122:1633-1638.

Watson, J., S. Gillis, J. Marbrook, D. Mochizuki, and K.A. Smith 1979c. Biochemical and biological characterization of lymphocyte regulatory molecules. J. Exp. Med., 150:849-861.

Weissman, I., V. Papaioannou, and R. Gardener 1978. Fetal hematopoietic origins of the adult hematolymphoid system. *In* Differentiation of Normal and Neoplastic Hematopoietic Cells. (B. Clarkson, P. Marks, and J.E. Till, eds.). Cold Spring Harbor Labs., pp 33-47.

Wigzell, H., 1970. Specific fractionation of immunocompetent cells. Transplant. Rev., 5:76-104.

Wilson, D.B., J.L. Blyth, and P.C. Nowell 1968. Quantitative studies on the mixed lymphocyte interaction in rats. III. Kinetics of the response. J. Exp. Med., 128:1157-1181.

Wolfenstein-Todel, C., E.C. Franklin, and R.A. Rudders 1974. Similarities of the light chains and the variable regions of the heavy chains of the IgG2 lambda and IgA1 lambda myeloma proteins from a single patient. J. Immunol., 112:871-876.

Woody, N.N., M. Feldmann, P.C.L. Beverly, and I.F.C. McKenzie 1977. Expression of alloantigen Ly-5 and Ly-6 on cytotoxic effector cells. J. Immunol., 118:1739-1743.

Woolnough, J.A., and K.J. Lafferty 1979. Generation of homogeneous populations of alloreactive T cells *in vitro*. Aust. J. Exp. Biol. Med. Sci., 57:127-139.

Zinkernagel, R.M., C.N. Callahan, A. Althage, S. Cooper, J.W. Streilein, and J. Klein 1978. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. J. Exp. Med., 147:897-911.

Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, P.A. Klein, and J. Klein 1978. On the thymus in the differentiation of H-2 self-recognition by T cells: evidence for dual recognition? J. Exp. Med., 147:882-896.

PUBLICATIONS BASED WHOLLY OR PARTLY ON THIS WORK

Shaw, J., V. Monticone, and V. Paetkau 1978. Partial purification and molecular characterization of a lymphokine (Costimulator) required for the mitogenic response of mouse thymocytes *in vitro*. J. Immunol., 120:1967-1973.

Shaw, J., V.Monticone, G. Mills, and V. Paetkau 1978. Effects of costimulator on immune responses *in vitro*. J. Immunol., 120:1974-1980.

Watson, J., L.A. Aarden, J. Shaw, and V. Paetkau 1979. Molecular and quantitative analysis of helper T cell replacing factors on the induction of antigen-sensitive B and T lymphocytes. J. Immunol., 122:1633-1638.

Paetkau, V., J. Shaw, G. Mills, and B. Caplan 1980. Cellular origins of costimulator (Interleukin 2). Immunological Reviews (In Press).

Shaw, J., B. Caplan, V. Paetkau, L.M. Pilarski, T.L. Delovitch, and I.F.C. McKenzie 1980. Cellular origins of costimulator and its activity in cytotoxic T lymphocyte responses. J. Immunol. (In Press).

Paetkau, V., J. Shaw, B. Caplan, G.B. Mills, and K.-C. Lee 1980. Interleukin 2 in cell-mediated immune responses. J. Supramolecular Structure (In Press).