![Montana State University logo]

The role of T200 (CD45) in leukocyte activation and effector cell function
by Rolf Edmund Taffs

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
© Copyright by Rolf Edmund Taffs (1987)

Abstract:
The membrane glycoprotein T200 (CD45) is widely expressed among functional classes of leukocytes.

Although its precise role is unknown, T200 probably participates in a number of leukocyte activities, including the development and expression of several effector cell functions. In mice, alloantigenic forms of T200 designated Ly-5 have expedited the characterization of structural features of the molecule. These features, along with results of recent studies showing a specific association of T200 with the cytoskeletal protein fodrin, suggest that the role of T200 may involve transmembrane signalling and interaction between the cytoskeleton and the plasma membrane.

The goal of the project was to relate particular structural features of the molecule to its function. Some of these studies were performed to investigate the possibility that Ly-5 alloantigens control in vitro leukocyte activities. Further experiments were conducted to see whether T200 associates directly with the cytoskeleton following treatment of murine cell lines with the polyclonal T cell mitogen concanavalin A (con A).

Using Ly-5 congenic strains of laboratory mice, immunogenetic analysis of a number of leukocyte activities revealed that cytotoxic T lymphocyte (CTL) effector function was influenced by Ly-5 allotype. Also, using electrophoretic analysis of leukocyte membrane and cytoskeletal proteins, T200 molecules were found to undergo an inducible association with the cytoskeleton upon treatment of T cell tumors and cultured CTL with con A.

The conclusions to be drawn from this work are that CTL function may be associated with alloantigenic determinants of T200, and that the mitogenic effect of treatment with con A may be mediated by direct association of T200 molecules with the cytoskeleton. These observations contribute to the body of evidence indicating an important role for T200 in leukocyte activation and effector cell function.

THE ROLE OF T200 (CD45) IN LEUKOCYTE ACTIVATION

AND EFFECTOR CELL FUNCTION

by

Rolf Edmund Taffs

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Microbiology

Montana State University
Bozeman, Montana

October, 1987

ii

APPROVAL

of a thesis submitted by

Rolf Edmund Taffs

This thesis has been read by each member of the
thesis committee and has been found to be satisfactory
regarding content, English usage, format, citations,
bibliographic style, and consistency, and is ready for
submission to the College of Graduate Studies.

_10-8-87_
Date

Sandra J. Ewald
Chairperson, Graduate Committee

Approved for the Major Department

8 October 1987
Date

Norman D. Reed
Head, Major Department

Approved for the College of Graduate Studies

October 9, 1987
Date

Henry L. Parsons
Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute copies of the dissertation in and from microfilm and the right to reproduce and distribute by abstract in any format."

Signature___*Rolf E. Taffs*___

Date_____ *10-8-87*_____

ACKNOWLEDGMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS-<u>Continued</u>

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES-<u>Continued</u>

ABSTRACT

The membrane glycoprotein T200 (CD45) is widely
expressed among functional classes of leukocytes.
Although its precise role is unknown, T200 probably
participates in a number of leukocyte activities,
including the development and expression of several
effector cell functions. In mice, alloantigenic forms of
T200 designated Ly-5 have expedited the characterization
of structural features of the molecule. These features,
along with results of recent studies showing a specific
association of T200 with the cytoskeletal protein fodrin,
suggest that the role of T200 may involve transmembrane
signalling and interaction between the cytoskeleton and
the plasma membrane.

The goal of the project was to relate particular
structural features of the molecule to its function. Some
of these studies were performed to investigate the
possibility that Ly-5 alloantigens control in vitro
leukocyte activities. Further experiments were conducted
to see whether T200 associates directly with the
cytoskeleton following treatment of murine cell lines with
the polyclonal T cell mitogen concanavalin A (con A).
Using Ly-5 congenic strains of laboratory mice,
immunogenetic analysis of a number of leukocyte activities
revealed that cytotoxic T lymphocyte (CTL) effector
function was influenced by Ly-5 allotype. Also, using
electrophoretic analysis of leukocyte membrane and
cytoskeletal proteins, T200 molecules were found to
undergo an inducible association with the cytoskeleton
upon treatment of T cell tumors and cultured CTL with con
A.

The conclusions to be drawn from this work are that
CTL function may be associated with alloantigenic
determinants of T200, and that the mitogenic effect of
treatment with con A may be mediated by direct association
of T200 molecules with the cytoskeleton. These
observations contribute to the body of evidence indicating
an important role for T200 in leukocyte activation and
effector cell function.

INTRODUCTION

Fundamental Observations

A substantial body of research literature supports the theory that functional responses of leukocytes are mediated by plasma membrane molecules. One example is antigen recognition, mediated by the T cell antigen receptor on T lymphocytes and by membrane immunoglobulin on B lymphocytes. Considerable effort has been made to characterize these membrane components. Good reasons exist for seeking the underlying mechanisms of leukocyte activation that control mitosis, secretion, and the expression of other important activities.

No clear and precise knowledge of the mechanisms of activation has been obtained. However, roles for some leukocytes can be distinguished on the basis of their unique plasma membrane components, such as L3T4 and Lyt-2 on T cells. Leukocyte function also is believed to depend on common underlying biochemical mechanisms shared among most or all eukaryotic cells.

Leukocytes express unique arrays of plasma membrane components. One feature that appears to be a common element in these arrays is the expression of the CD45

molecule. This was emphasized in a recent report of a summary conference on human differentiation antigens, held in September of 1986 (1). As reported, the CD45 molecule has been independently identified and partially characterized using a substantial number of monoclonal antibodies (MAb).

These MAb recognize CD45 antigens generally represented on cells of hematopoietic lineage. A number of MAb with specificities for other antigenic determinants on CD45 react with forms of the molecule unique to particular subsets of leukocytes. These indicate the lineage, state of differentiation, or the extent of activation of the cells. Their existence has led to a confusing nomenclature for specifying CD45 on various cell types, so that the molecule is known variously as T200, Ly-5, Art-1, B220, L-CA, or p220 to name a few of the CD45 designations. It is not surprising that workshop participants would seek a unifying nomenclature. The literature to this date still uses the names T200 or Ly-5 for the murine forms of CD45.

The discovery of CD45 was made using alloantisera directed against mouse spleen and lymph node cells (2) and rat thymocytes (3). The initial descriptions of the cellular distribution of these alloantigens indicated that they were restricted largely to thymocytes and T lymphocytes. A more detailed analysis of murine tissues

and tumor cell lines (4) indicated a broader tissue distribution of the murine allotypes, designated Ly-5 antigens. Cells found to express Ly-5 included T and B lymphocytes, monocytes, macrophages, and mast cells.

The same report indicated the distribution of the molecule to be restricted to cells of hematopoietic origin exclusive of erythrocytes and proerythroblasts. Ly-5 alloantisera contain antibodies (Ab) which react with leukocytes in some but not all strains of mice. The reactions of these Ab in various strain combinations led the investigators to propose that a system of allelic genes had been detected (2).

The term T200 was assigned by Trowbridge's group to a high molecular weight antigen recognized by anti-mouse thymocyte serum (5). At the time of its description, it was not known that T200 and Ly-5 Ab recognized the same molecule. The serum they described reacts with leukocytes from all the inbred mouse strains tested. Using alloantisera to Ly-5 and xenogeneic MAb to T200, it was then shown that Ly-5 allotypes define a biochemical polymorphism of the T200 molecule (6). Information obtained from tryptic peptide maps showed that the molecules recognized by these antibodies were structurally related.

Corroborating evidence for this relatedness was independently obtained in experiments where

immunoaffinity-purified T200 molecules were seen to immunoprecipitate with Ly-5 alloantiserum (7). This observation indicates that allotypes unique to certain strains of mice carry xenotypic determinants which are unique to the species.

It was also noted that Ly-5 or T200 Ab precipitated molecules of different apparent molecular weight (M_r) on T and B cells (8). Using the technique of 2-dimensional (2-D) peptide mapping, the authors demonstrated the structural relatedness of the T and B cell immunoprecipitates. The similarities between these antigens led to the conclusion that they are isoforms of the same glycoprotein, and thus are members of a family of high molecular weight plasma membrane components.

Although the majority of these studies were undertaken in rodents, it is important to note that CD45 probably has a broad phylogenetic distribution. It has been identified also in humans (9), sheep (10), and chickens (11). The designations of the allotypes (for example Ly-5 antigens in the mouse) are commonly used within the context of species, while the designations of xenotypes (for example T200 antigens) are typically used in referring to CD45 molecules in one or more species under discussion. Within appropriate context, the designations can be used interchangeably. According to the new international nomenclature (1) the antigens are

indicated as having a general (CD45) or restricted (CD45R) cell lineage specificity.

In the murine system, the analysis of these molecules has been facilitated by the production of Ly-5 congenic strains. The basic procedure for producing a congenic strain is to introduce an allotype unique to one strain onto the genetic background of another strain that differs genetically at the locus under investigation. The Memorial Sloan-Kettering Cancer Center in New York is the source of such a congenic strain for the Ly-5 system. The strain is designated C57BL/6-Ly-5.2 and carries the Ly-5.2 allotype on the genetic background of the C57BL/6 strain, which has like most inbred strains of laboratory mice the allotype designated Ly-5.1 (2, 4).

The allotypic variation of CD45 has been described so far only in rodents. Also, the full range of the species distribution of CD45 has not been addressed. However, it may be determined that CD45 is a ubiquitous component of vertebrate immune cells. The phylogenetic range thus far identified and the tissue distribution of the molecule suggest that CD45 may play a fundamental role in leukocyte function.

T200 Biochemistry

Soon after the Ly-5 system was serologically defined, it was discovered that Ly-5 molecules exhibit a number of

molecular weight isoforms (12). These isoforms can be visualized by detergent solubilization of radioiodinated cells followed by immunoprecipitation with Ly-5 alloantisera and electrophoretic analysis. By these methods, the murine isoforms are found to be characteristic of hematopoietic lineage, with T cells exhibiting isoforms of lower M_r (180 kDa) compared to B cells (220 kDa).

A question for several years was whether the basis for the M_r isoforms reside in protein or carbohydrate moieties. It was unknown whether the B cell form is more heavily glycosylated or has a larger protein structure than the T cell form. The 2-D peptide mapping and serological studies indicated a great deal of structural similarity between them.

Lineage specific isoforms had previously been observed but not identified as T200 on T and B cells (13) and on T cells and thymocytes (14). In the latter case the isoforms were seen to differ in their carbohydrate structure. Glycosyl residues are often incorporated into proteins following peptide synthesis but prior to expression of the mature glycoproteins at the cell surface. For the T200 molecule, these carbohydrate additions include mannose and glucosamine residues (15).

In human B and T lymphocytes, evidence was obtained that the high molecular weight isoforms differ in

carbohydrate moieties. Using MAb which specifically

recognize branched or linear polylactosaminyl structures

of glycoproteins, both B and T cells were found to carry

polylactosaminyl residues of the branched type (16). B

cells additionally express the linear type. It was

concluded that differential glycosylation occurs in plasma

membrane molecules, believed at the time to be T200. This

interpretation was corroborated when other investigators

found B and T cell forms of T200 to differ in

susceptibility to changes in electrophoretic mobility

following digestion with neuraminidase (17). It was

thought that these results indicated differences in sialic

acid content between the various molecular weight

isoforms.

A further indication of the complexity of the

carbohydrate differences between T200 isoforms was

obtained by more extensive analysis of the

oligosaccharides released from B and T cells and tumor

cell lines by alkaline borohydride treatment following

pronase digestion (18). Evidence was obtained that

differences in both N- and O-linked glycosylation may

account in part for the molecular weight heterogeneity of

T200 molecules. In addition, B and T cell tumors were

observed to differ to some extent from normal B and T

lymphocytes in their respective glycosylation patterns.

On the other hand, evidence was building for protein

differences between M_r isoforms. The T and B lymphocyte isoforms of T200 have intracellular precursors missing at least some of the carbohydrate structure of the mature glycoprotein. These precursors also differ in size, indicating that they may differ in protein structure (15). As the resolution of these questions progressed, more rigorous biochemical analyses were extended to the allotypic forms of the molecule (19, 20) in addition to the M_r isoforms (8, 21). Both kinds of variants were shown ultimately to differ by the method of 2-D peptide mapping.

Chymotryptic digestion followed by electrophoretic analysis initially showed that the M_r isoforms have the same peptide structure (8). However, under a different set of labeling conditions, unique peptides were found in the 2-D maps for the B and T cell forms (21). These results indicate that the cell specific isoforms share a number of sequences, and that the specific oligopeptides seen to differ between the B and T cell peptide maps are accessible to radiolabeling only under particular treatment conditions.

Biochemical analysis of the protein structure of Ly-5 alloantigens shows that they too differ in protein structure, as revealed by patterns obtained with Cleveland mapping (19). Within each allotype no difference is seen between the B and T cell M_r isoforms by this method.

These findings indicate that the allotypes differ at the level of amino acid sequence, but within a given allotype there is at least some common protein structure. Additional isoforms detected in cloned Ly-23^{+} murine T cells exhibit the same apparent properties (20). In conjunction with analyses demonstrating the differential glycosylation of B and T cell T200 molecules, the results of the protein structural comparisons for the isoforms and the allotypes indicate an extraordinary biochemical complexity in the CD45 family of membrane glycoproteins.

Molecular Genetics

The confirmation of the apparent differences in protein structure between M_r isoforms of a given allotype or between allotypes awaited the biochemical analysis of information molecules encoding the amino acid sequences of CD45. The initial report of the cloning of Ly-5 cDNA (22) presented evidence that the two Ly-5 alleles of the C57BL/6 and congenic C57BL/6-Ly-5.2 strains of mice differed by restriction fragment length polymorphism (RFLP) analysis. The RFLP analysis was accomplished using a specific probe for Ly-5 to screen mouse liver DNA fragments generated by a number of different restriction endonucleases.

RFLP analysis detects gene differences by virtue of the site specificities of the restriction endonucleases

used to cleave genomic DNA. Gene differences are indicated when the gene-specific probe identifies fragments of differing size in DNA digests from different cell sources. The size differences derive from changes in the relative locations of endonuclease cleavage sites. The technique may not distinguish whether structural sequences differ between the alleles, since the changes in locations of cleavage sites may reflect variations in noncoding portions of the DNA. However, the results may imply that the allotypic differences between the proteins are reflected in observable differences at the nucleic acid level.

In the same study and a subsequent report (23), these investigators similarly analyzed a number of other inbred mouse strains, showing that the RFLP patterns accurately distinguish the Ly-5 alleles corresponding to the serological identities of the strains. Interestingly, the RFLP analysis of the strain designated ST/bJ shows unique RFLP patterns indicative of a third Ly-5 allele, consistent with the observation in this strain of unusual serological reaction patterns obtained with Ly-5 alloantisera. The RFLP analysis thus supports the view that the Ly-5 gene of the ST/bJ strain represents a unique allele.

The ability to distinguish alleles of the Ly-5 system by RFLP analysis led to the determination of a number of

allelic variants in a survey of natural (noninbred)

populations of mice (24). In the absence of complete

nucleic acid sequence data for the alloalleles in

noninbred mice, it is not possible to state definitively

which of these RFLP differences represent amino acid

sequence variations in Ly-5 molecules.

However, the results of the survey show that the Ly-5

system may be considerably more variable among mice than

previously indicated by serological analysis based solely

on testing inbred mouse strains (24). The physiological

significance of this extensive allotypic variation or the

role that the allotypic determinants may play in

biochemical processes is essentially unknown. A portion

of the work reported in the present study has a bearing on

these questions.

Evidence now exists at the nucleic acid level that

the M_r isoforms also differ in protein structure. It was

found that a size difference between Ly-5 mRNA molecules

from murine B and T cell leukemia lines corresponds to the

difference in apparent molecular weight of the B and T

cell forms of Ly-5 glycoproteins (22). The nature of the

difference in transcript size which reflects cell

type-specific differences in protein structure of Ly-5 was

examined by S1 nuclease mapping (25). By hybridization

to Ly-5 cDNA probes and S1 nuclease digestion of

unprotected single stranded sequences, an additional

sequence not present in T cell transcripts was identified near the 5´ end of the B cell mRNA, probably adjacent to the leader sequence. This indicates that the B and T cell glycoprotein isoforms not only differ in amino acid sequence, but arise by selective use in these cells of specific portions of the Ly-5 gene. The results imply that B and T cells have in common a large portion of their amino acid sequence but differ at the N-terminal end of the protein.

It was shown recently that in B cell cDNA prepared by primer extension, there exist sequences corresponding to at least two exons located near the 5´ end of the gene which are not detected in T cell mRNA (26). It was also indicated by this study that other exons elsewhere in the gene may be used alternatively by both B and T cells.

Similar results were independently obtained (27) indicating that alternative mRNA splicing involves exons encoded near the 5´ end of the Ly-5 gene. The selective use of exons results in the differential expression of alternative amino acid sequences in specified domains of the protein. This type of control allows isoforms encoded by a single gene to have both common and unique amino acid sequences. Restriction endonuclease mapping of overlapping clones spanning over 60 kilobases around the Ly-5 locus supports the hypothesis that a single Ly-5 gene encodes the various isoforms of Ly-5 molecules (25).

Similar schemes for the flow of biological information in this genetic system have been proposed for the rat (28) and human (29) forms of T200.

Important information regarding the structure of Ly-5 glycoproteins and their disposition in the leukocyte plasma membrane also is revealed by nucleic acid analysis. Earlier results obtained at the protein level using protease treatments of BW5147 murine thymoma cells indicate that the major portion of the T200 molecule is exposed at the cytoplasmic side of the plasma membrane (30). In the mouse, the cDNA sequence data are consistent with the model that Ly-5 molecules are transmembrane glycoproteins having an N-terminal domain of 370 amino acid residues located on the outside surface of the T cell plasma membrane (25). A single hydrophobic transmembrane sequence of 22 residues is indicated.

The same study showed that the remaining 730 residues constitute a cytoplasmic domain unusually large for an integral membrane protein. This agrees with the conclusions drawn from an earlier examination of the rat T200 cDNA sequence (31). For murine T200, partial amino acid sequence data have confirmed the identity and locations of residues assigned on the basis of the cDNA sequence (32). This study confirmed that the cDNA in question encodes Ly-5 molecules and validated the conclusions drawn from the nucleic acid sequence data.

Multiple cysteine residues were identified in the extracellular domain in both rat (31) and mouse (25) T200. The locations of most or all of these residues appear to be conserved. However, the external domains show substantially less overall amino acid sequence homology than the highly conserved cytoplasmic domains which approach 90% homology between the mouse, rat, and human forms of T200 (29). The amino acid sequence homology was deduced from comparison of the DNA sequences.

In discussion of the broader implications of the sequence data for human T200, it was hypothesized by others that the cytoplasmic domain of the molecule is probably critical to the function of T200, indicated by the apparent homology observed among species (29). These authors suggested that this function involves interaction with structural elements inside the cell. They also proposed that the variability in the external structure of T200 on particular functional sets of leukocytes in some way modulates interactions of the cells with other cells or with extracellular matrix components.

Direct evidence that these hypotheses are true has not yet been obtained, and it is the lack of this information which led to the work presented in this study. It may be discovered in the near future that the primary function of the external domain involves specific association with adjacent membrane molecules and not with

extracellular matrix components or molecules on the
surface of other cells. In either case, it is intriguing
that a membrane molecule may couple unique regulatory
domains on the outer surface of the plasma membrane to a
common functional domain inside the cell.

Cellular Activation Processes

In both rat and mouse, the cytoplasmic domains of
T200 contain multiple serine residues (25,31). Two of
these serines occur within peptide sequences thought to be
potential sites for phosphorylation by the enzyme known as
protein kinase C (PKC) (25). Previous investigation of
the phosphorylation of Ly-5 molecules demonstrated the
existence of phosphoserine in the cytoplasmic domain (33),
interpreted by that author as indicating a possible
association with cytoskeletal components. In addition,
biochemical analysis showed that Ly-5 can serve as a
substrate for PKC (34). PKC is generally considered to be
an important phosphorylating enzyme mediating processes
involved in the activation of T lymphocytes, as reviewed
elsewhere (35).

It is important to relate these observations to the
mechanisms by which various chemical treatments or
ligation of membrane receptors can activate particular
sets of leukocytes. Activation is taken to mean some
detectable change in cell appearance or behavior, whether

this is observed morphologically, biochemically, or by changes in expression of a cellular function. The importance of these in vitro observations is indicated only by the extent to which they resemble genuine physiological processes.

As an example, tumor-promoting phorbol esters have been shown to augment expression of T cell receptor β chain and IL-2 mRNAs in the EL-4 T cell lymphoma line (36). Regulation of gene transcription is a way that cells can control their metabolic commitment to specific activities. The underlying mechanism for this control is partly revealed by the observations that phorbol diester receptor copurifies with and directly activates PKC (37, 38). The involvement of the cytoskeleton may be indicated by the rapid translocation of PKC activity from the cytosol to the plasma membrane upon treatment of cells with phorbol ester (39).

Further evidence of the functional relevance of PKC to cell activation is the cytoplasmic alkalinization (a prerequisite to mitosis) that occurs with phorbol ester treatment (40). Also, calcium flux in response to chemical and physiological mitogenic signals is probably important to the inductive event (41). The kinase activity of the epidermal growth factor receptor is controlled by PKC, induced by both phorbol ester treatment and epidermal growth factor as the activator (42).

In B lymphocytes, a rapid translocation of PKC from cytosol to plasma membrane cell fractions occurs in response to phorbol ester, lipopolysaccharide (a polyclonal B cell mitogen), or crosslinking of surface immunoglobulin (sIg) (43). Several membrane proteins on B cells become phosphorylated in response to phorbol ester or crosslinking of sIg (44). Some of these specifically phosphorylated proteins also associate with the cytoskeleton.

Phosphorylation of membrane receptors in response to phorbol ester has also been studied in T lymphocytes (45). PKC-mediated phosphorylation of T cell antigen receptor components leads to down-regulation of the antigen receptor complex in response to phorbol ester. This suggests a regulatory role for the phosphorylation events. Also, interleukin 2 (IL-2) receptor expression is increased by treatment with anti-T3 antibody, phorbol ester, or phytohemagglutinin (PHA) (46). This is correlated with a redistribution of PKC from the cytosol to the membrane fraction of the cells, revealing that the translocation and activation of PKC is a common signal shared by IL-2 induction and antigen stimulation of T cells.

The outcome of PKC activation by phorbol ester treatments of T cells includes phosphorylation of serine and threonine residues on the IL-2 receptor (47).

However, the serine residue in the human IL-2 receptor thought to be the predominant PKC-phosphorylated site can be changed by site-directed mutagenesis without apparent alteration in IL-2 binding, receptor function, or modulation of the receptor by phorbol ester (48). A similar situation exists in the transferrin receptor model (49). These observations do not indicate that PKC activation is unimportant to these processes, but rather that no absolute correlation exists between specific substrate phosphorylation by PKC and a phosphorylation-dependent regulatory event in response to an inductive signal.

Therefore, it is possible that in other situations including the phosphorylation of T200, the relationship between PKC-mediated processes and the observed substrate phosphorylation is merely coincidental. For example, the phosphorylation and subsequent proteolytic modification of specific cytoskeletal proteins in phorbol ester-treated human neutrophils (50) may not have a direct bearing on responses in these cells thought to require cytoskeletal reorganization. These correlative observations need to be appraised in light of more definitive biochemical analysis, but a key role for PKC-mediated phosphorylation in a number of surface receptor-mediated events and cytoskeletal reorganization is indicated.

Response of Lymphocytes to Concanavalin A

With the previous considerations in mind, it is
noteworthy that other activators of T lymphocytes bring
about apparently specific phosphorylation of receptor or
membrane components. The specific phosphorylation of a 20
kDa constitutive component of the T cell antigen receptor
is seen in accessory cell-dependent, antigen-specific
activation of cloned murine cytotoxic T lymphocytes (CTL)
(51). The same event occurs in response to concanavalin A
(con A). This provides indirect evidence that the
mechanism of activation by this polyclonal T cell mitogen
may have physiological relevance.

A recent communication in abstract form (52)
indicated that among several con A-binding proteins on
CTL, a specific increase in phosphorylation is observed in
only two, with M_r of 15-17 kDa and 205 kDa. The increase
in phosphorylation of both proteins is seen in response to
phorbol ester, anti-T cell receptor antibody, and con A
treatments. Under conditions which abrogate PKC
activity, the increase in phosphorylation is not observed.
The proteins in CTL plasma membranes which bind con A have
been analyzed biochemically by immunoprecipitation (53)
and functionally by cytotoxicity testing (54). In the
cytotoxicity tests, con A treatment reversibly inhibits

CTL-mediated killing (54). However, only some of these CTL membrane con A receptors have been identified by immunoprecipitation (53). These receptors include T200 molecules.

Previously it was shown that high molecular weight membrane components of surface radioiodinated T lymphocytes bound con A, though these glycoproteins were not precisely identified in the electrophoretic analyses (14, 55). It has been postulated that T200 molecules are likely candidate sites for the binding and mitogenic effect of con A on T lymphocytes (56). The specifically phosphorylated 205 kDa protein observed in con A-treated CTL (52) therefore may be the T200 glycoprotein. The hypothetical identification has not yet been confirmed by immunoblotting or immunoprecipitation.

The chemical analysis of con A and its apparent effects on responding cells are relevant to the topic of the functional activities of con A receptors. A general discussion of the chemical structure of con A and a number of parameters governing its binding to oligosaccharide moieties of glycoproteins were included in a recent review of lectins (57). The association constants for con A and various carbohydrate structures found on proteins were determined by analyzing the effect of specific structural changes in the asparagine-linked glycosidic residues of glycopeptides (58).

The two most important parameters of con A association with receptor-like membrane molecules are probably carbohydate specificity and valence. Con A binds principally to terminal mannosyl residues of glycoproteins and can thus be removed by the competitive binding of α-methyl-D-mannopyranoside. Con A has an apparently lower affinity for glucosyl residues, with very low affinities for the other terminal residues tested. Differences in binding attributable to changes in carbohydrate composition at positions other than the oligosaccharide termini have less impact on binding affinities (58).

Valence was addressed in a study of special importance to the work presented here. The study compared con A with a succinylated derivative designated succinyl-con A (59). At neutral pH, con A is a tetravalent molecule composed of four similar subunits having a combined nominal molecular weight of 112 kDa. A highly succinylated derivative was determined to contain 10 succinyl groups per subunit and was found at neutral pH to consist of a divalent dimer of half the size of the underivatized molecule. Comparing functional activities of these chemicals, it was discovered that at low lectin concentrations (3-6 ug per ml) both forms were highly mitogenic, but as the lectin concentration was increased, only the succinylated form was capable of inducing the

incorporation of [^{3}H]thymidine.

Con A can induce cap formation in lymphocytes; in contrast, cells treated with the succinylated derivative do not show cap formation, though addition of anti-con A antibody will cause caps to form (59). Cap formation by other ligands can be inhibited by the presence of con A but not succinylated con A (59). These observations indicate the relationship of lectin valence, ligand cross linking, and cap formation. The results show that the divalent molecule can deliver a mitogenic signal in the absence of the capping and cap-inhibiting property apparent in the tetravalent form of the lectin (59). They may further suggest some relationship between receptor capping or cap inhibition and the failure to observe mitogenesis at high doses of tetravalent con A (10 ug per ml or greater). The underlying mechanisms of these phenomena have not been fully described, though a portion of the present work has a bearing on these observations.

There is evidence from the responses of human lymphocytes to con A that lectin concentrations hyperoptimal for proliferation (assessed by [^{3}H]thymidine incorporation) induce greater numbers of blasting cells synthesizing DNA than seen at the optimal concentration (60). The blasts show growth arrest at varying stages in the cell cycle, but few cells undergo mitosis or cell death. The authors concluded that the incorporation assay

in the presence of large amounts of con A does not successfully measure the DNA synthesis occurring in the cultures, which can be assessed in individual cells observed by flow cytometry, as judged by morphological criteria.

It is important for later discussion to describe the results obtained by a somewhat different analysis which compared proliferation response curves of mitogen-treated lymphocyte cultures (61). The high-dose unresponsiveness of these cells to con A mitogenesis is reversible by removing con A from the cultures via competitive binding and reculturing the cells in the absence of con A. When the treatment with supraoptimal concentrations of con A is followed by reculture in the presence of the lectin at the optimal mitogenic concentration, a pronounced increase in proliferative response is observed, relative to the level seen in cultures maintained in the continuous presence of the optimal con A concentration.

These results establish the reversibilty of the apparent high-dose inhibition of con A-induced mitogenesis. The release from mitogenic inhibition by removal of excess con A is also observed in cloned helper T cells, thought to respond to con A by an IL-2-mediated autocrine mechanism (62). Whether the observed inhibitions of capping (59), progression through the cell cycle (60), or uptake of DNA precursors (59, 60, 62) are

all mediated through the same membrane receptor is
unknown. Based on the studies of the cap-inhibiting
properties of con A, it seems likely that one or more of
these con A-binding proteins are immobilized by the
tetravalent lectin, leading to a reversible inhibition of
several processes. This suggests a relationship between
membrane-mediated proliferative signals and
cytoskeletally mediated events.

Data obtained by numerous methods of T cell
activation and mitogenesis are consistent with a general
hypothesis that functional responses, mediated by membrane
components, have as immediate sequelae measurable changes
in the levels of specific activation components. When
these components can be measured, they may be regarded as
markers of cell activation. The appearance of the markers
may reflect common biochemical processes known to be
involved in a number of inducible cellular events.

The relationship of the activation marker to the
underlying process is exemplified by PKC-mediated
phosphorylation of discrete molecules and the concomitant
translocation or reorganization of associated membrane and
cytoskeletal elements. It seems that in most cases the
precise roles of the components and the nature of their
interaction are poorly understood. This lack of
understanding limits the experimental basis for modeling
leukocyte actvation mechanisms.

Roles of T200 Antigens

Considering the current knowledge of the biochemistry of T200, it is remarkable that the function of such a unique and ubiquitous leukocyte membrane component remains so obscure. As discussed by others (63), the similarities among differing forms of T200, and the apparent complexity of biological information contained in T200 molecules across various cell types and species, strongly suggest that at least some biochemical features of T200 have consequences of critical importance for lymphocyte function. As yet, no report has indicated the precise biochemical role or roles of this molecule.

Indirect evidence exists that particular isoforms of T200, detected as apparent markers of leukocyte differentiation, have functional activities associated with the cell types on which they are found. In T lymphocytes, a number of these forms have been identified. Among these are the CT antigens acquired late in differentiation by CTL (64-67). CT antigen expression is correlated with the acquisition of lytic activity. The CT antigens are carbohydrate in nature, and are inducible by IL-2. They probably participate in lytic function, revealed by the ability of anti-CT MAb to block target cell lysis.

In their discussion of the possible role of molecules
expressing CT antigens (66), the authors briefly reviewed
numerous studies relating cell surface receptor function,
transmembrane signalling, and proteolytic activation of
PKC. They suggested that the calcium-dependent
proteolytic activity that copurifies with T200 (68) may
have a direct functional role in the acquisition or
expression of cytotoxic activity in CTL.

The proteolytic activity that copurifies with T200
(68) was identified in the laboratory where the present
investigation was conducted. The authors discussed the
close relationship between the inhibition of CTL killing
by Ab to T200, the requirement for calcium in the lytic
process, and the pivotal role that limited proteolysis
appears to play in lymphocyte activation. Whether T200 is
in fact autoproteolytic or is tightly associated with a
proteolytic enzyme in lymphocytes remains to be shown.
However, it is significant that the requirements for the
observed proteolysis of T200 and the requirements for
effector cell function are remarkably alike.

There has been considerable effort made by many
researchers to investigate the role of T200 using various
cell types and antibodies. A number of recent studies in
addition to the work on CT antigens have implicated T200
as a component of the biochemical mechanism of T
lymphocyte activation and effector cell function. Results

obtained from inhibition tests using alloantisera and monoclonal antibody directed against T200 strongly suggest that this glycoprotein participates in events leading to the development and function of T lymphocytes (69-72). These studies demonstrated modulating activities of various anti-T200 MAb or alloantisera in functional lymphocyte assays, as was shown for CT antigens (64). The assays were performed under conditions where effector cells were not lysed by the treatment with Ab, nor inhibited simply by the presence of irrelevant Ab typically included in such experiments as a negative control.

In particular, results from the laboratory in which the present work was conducted used anti-T200 MAb (69) and Ly-5 alloantisera (70) to assess the participation of T200 molecules in the allogeneic mixed lymphocyte culture (MLC) response, lectin and oxidative mitogenesis assays, and the generation and effector cell function of CTL. Alloantisera were shown to cause concentration dependent, allotype specific inhibition in a number of these assays, of which CTL generation, CTL-mediated lysis, and the mitogenic response to con A are most pertinent to the present study. These and other investigations (64, 71, 72) have clearly shown that antibody treatments are capable of altering in vitro T lymphocyte responses.

The possible roles of T200 molecules in other functions of leukocytes have been investigated. Regulatory T cells of the T4[+] phenotype arising in autologous MLC have activity as inducers of T8[+] suppressor T cells and express a unique antigenic form of T200 designated 2H4 (73). T cells expressing the 2H4 antigen lose their suppressor-inducer activity when treated with anti-2H4 MAb, taken to indicate a direct involvement of 2H4 antigens in T4[+] cell function.

An antigenic form of T200 designated p220 was shown to be a marker functionally associated with the expression of IL-2 receptor molecules (74). Treatment of post-thymic T cells with MAb to p220 increases the early expression of IL-2 receptors on those cells. The molecule may provide less mature T cells an additional regulatory component for IL-2 driven differentiation. The apparent expression of this antigen on a number of other cell types (B cells and NK cells for example) is enigmatic. It could be that a closely related cross-reactive form exists on these cells, with inapparent structural differences. Alternatively, p220 may regulate or be regulated by lymphokine receptor expression on other cells besides immature T cells.

It is notable that B lymphocytes uniquely express specific T200 determinants designated B220, with an M_r of 220 kDa, found on both murine (75) and human (76) B cells.

Although the role of the B220 isoform and its precise

relationship to p220 are unknown, certain features of B

cell T200 suggest a similar functional role. It has been

noted that IL-2, considered in general to be a T cell

lymphokine, alters the lectin-binding properties of T200

molecules on B cells (77).

MAb to Ly-5 alloantigens have been shown to inhibit

the polyclonal IgG response, but not the IgM or

proliferative responses, of B cells treated with

lipopolysaccharide (78). The same MAb were used to

inhibit the in vitro plaque-forming cell response of B

cells to different types of antigens in a manner

suggesting an involvement of the T200 molecules in the

recognition of or response to signals provided by

accessory cells (79).

These observations indicate that T200 on B cells may

be intimately involved in differentiation events mediated

by soluble factors, or may somehow regulate the expression

of receptors for those factors. Collectively, the

experiments reviewed here are taken to mean that T200 is a

likely participant in biochemical processes of

physiological significance in immune cells. While this

indirect type of evidence is meaningful to the question of

the participation of T200 in such processes, it is

generally not possible from experimental results such as

these to obtain a clear and precise picture of the

mechanisms underlying the activation event or effector cell function.

Differences in the selection of the model system, assay, and antibody may have a dramatic impact on the results obtained in this type of investigation. It was indicated by one study that a rat anti-mouse T200 MAb had no effect on con A-induced mitogenesis of murine splenocytes (80), in contrast to results obtained using alloantisera (70). The apparent difference could be rationalized in a number of ways, for instance by the fact that different antigenic determinants are recognized by xenotypic and allotypic antibodies. The difference may actually reveal important distinctions between particular antigenic determinants in terms of their specific involvement in one or another process. However, these explanations are largely a matter of conjecture and often are difficult to validate by conventional techniques.

Investigators have found evidence that differences in T200 antigenic determinants recognized by various MAb may account for apparent discrepancies of this sort. In antibody-mediated inhibition of natural killer (NK) cell cytotoxic activity, it was shown that MAb binding to a distinct region of T200 molecules on NK cells were inhibitory (81). Other MAb of the same subclass but recognizing a different region of T200 were incapable of inhibiting NK cell cytotoxic function.

Other NK cell inhibition studies previously had indicated the functional involvement of T200 molecules in the activity of murine NK cells (82) and their human counterpart (83). These studies, and several others from a number of independent research groups, have provided strong evidence that T200 is in some way involved in NK cell-mediated cytotoxicity. Forms of T200 unique to NK cells have been described (84), so in a general way, the results obtained with NK cells are similar to what is seen in other classes of leukocytes regarding the functional involvement of T200 molecules.

The overall observation is that antigenic determinants on T200 molecules can be bound by various Ab in a way that may have functional significance. The fact that MAb do not each inhibit every (or in some instances, any) cellular functions actually lends credibility to the notion that cell lineage or differentiation markers identified as epitopes on T200 molecules are part of a highly regulated system.

Such a system may have arisen owing to a need to have a common regulatory mechanism for different membrane receptors. Functional receptors, such as the receptors for antigen or for lymphokines, are required in specific cellular compartments at particular times or developmental stages. A single structural component (such as T200?) might be used to couple the recognition of different

signals by their receptors to a common intracellular response mechanism.

In this hypothetical scheme, there could be a common molecule, with features like those observed in the structure of the T200 molecule, which undergoes precise modifications in certain of its domains. These modifications, possibly like those accounting for the various T200 M_r isoforms, would engage or control a particular receptor in an activation process. The modifications would involve regions uniquely associated with specific functional activities of the isoforms.

In this model, there could also be regions of the molecule that the differing forms had in common, such as a single type of cytoplasmic domain. The impact of the binding of Ab to a particular site would depend on the relevance of that determinant to the functional task of the molecule. Though no such scheme has been demonstrated in leukocytes, it is attractive to speculate that T200 might play such a role in leukocyte responses. The model would account for the unusual features of T200 and if proven, would tell a fascinating story in the cell biology of leukocytes.

Possible Roles of T200 Allotypes

An interesting aspect of the antigenic heterogeneity of T200 molecules is the allotypic variation observed in

rodents (discussed in detail earlier in this communication). There are indications from other genetic systems in mice and other species that specific allotypic differences control immune responses and result in heterogeneity of reactions to soluble antigens and pathogenic microorganisms. Alloantigens encoded by genes of the major histocompatibility complex are the best known of these. In a number of systems, molecular analysis is unraveling key features of the biochemical processes responsible for allotypic variation.

However, there are few systems in which the precise relationship is known between the genetic control of host resistance to a particular pathogen and the detailed biochemical features of the host gene product responsible for the attained level of immune response. The precise description of the relationship would depend on detailed studies in very diverse areas, each with its own requisite expertise. It is likely that the time has come in which significant progress in these complex areas of molecular and cellular immunology will be made, though at the present time there are generally only a few systems in which the basic relationships have been described.

Investigation of the role of T200 in antibody-dependent cell-mediated cytotoxicity (ADCC) employed Ly-5 alloantisera and Ly-5 congenic strains of mice in ADCC inhibition tests (85). The results indicate

the involvement of T200 molecules in murine ADCC, adding
to the information discussed earlier regarding the role of
T200 suggested by inhibition tests. During the ADCC
study, it was observed that C57BL/6 (B6) mice usually had
a somewhat lower cytotoxic response to Ab-coated sheep
erythrocytes (SE) than C57BL/6-Ly-5.2 (B6-Ly-5.2) congenic
mice (unpublished data).

This observation led to speculation that alleles of
the Ly-5 system might operate in some unknown way as
immune response genes, controlling the level of response
in ADCC or other leukocyte activities. There are several
genes of immunological interest on chromosome 1 of the
human and the mouse which are thought to reside close to
the locus controlling T200 molecules, as discussed briefly
elsewhere (29). In the mouse, some of these genes control
leukocyte membrane alloantigens. There also are
functionally defined genes which control immune responses
to microbial pathogens or proliferative alloreactivity.
In some of these systems, the nature of the gene products
is unknown. It remains a mystery that considerable
allelic variation exists at the Ly-5 locus for which no
regulatory function has been identified, despite a
substantial body of literature indicating that particular
determinants on the molecule are associated with specific
leukocyte functions.

Among murine chromosome 1 loci of immunological relevance, two (Mls and the gene controlling Fc receptor allotypes) have been described which might have a relationship to ADCC reactions or other responses discussed in the present work. Work I will present in this thesis compared ADCC activity and other immune responses of the B6 (Ly-5.1 allotype) and B6-Ly-5.2 congenic strains of mice. A rather large segment of chromosome 1 (possibly 3 centimorgans) has been transferred from the donor strain (SJL) to the B6 background. Whether Mls or Fc receptor (FcR) allotype genes in this congenic strain are derived from the donor strain or the from the background strain is unknown.

The Mls locus was described by functional assay to be a system of allelic genes mapping to chromosome 1 which control significant proliferative responses in MLC (86). There are substantial proliferative responses in murine allogeneic MLC in the absence of murine major histocompatibility complex differences, known to control much of this type of alloreactivity. Systematic study of MHC-identical strain combinations led to the conclusion that a system of allelic genes was responsible for the phenomenon.

The reactions controlled by Mls alleles are manifested by reciprocal MLC reactivity in some strain combinations, and unidirectional responses in other

combinations. The physiological role of Mls genes is a matter of current debate. Because gene products of the various alleles have not been identified, it remains to be shown how they control apparent alloreactivity. However, they might have a considerable impact on cell-mediated immune responses in ways that have not been described.

A gene determining FcR allotype is also located on chromosome 1. This system of allelic genes was discovered by serological testing and was shown to be linked to the Mls locus (87). Ab bound to FcR arm the ADCC effector cell, enabling specific recognition of membrane target antigens on pathogens, and leading to the destruction of the pathogens by ADCC. This killing activity is controlled largely by the amount of Ab available for arming and the specificity of the Ab for a potential target.

FcR allelic variation could have an effect on the process of ADCC. The structural difference between allotypes (identified by alloantibodies) might influence ADCC response due to a difference in the amount of Ab attached to the effector cell. It could also be that the receptor allotype determines how well the FcR-Ab structure serves in antigen recognition.

To my knowledge, there is no report that places any gene controlling an immune response or encoding a leukocyte membrane antigen within or near the region of

chromosome 1 that carries the Ly-5 locus. In the absence of information, allotypic variation observed in ADCC response of Ly-5 congenic mice might still be caused by some other allelic gene linked to Ly-5. There could also be an unlinked allelic gene still retained in the congenic strain that controls this response, though the extent of backcrossing makes this possibility unlikely.

By influencing an activation mechanism or the ability to engage a surface-bound Ab-mediated response, the Mls or Fc receptor genes could dramatically influence the ADCC response of a mixed population of effector cells. Any examination of the role of Ly-5 alloantigens would have to consider these loci, which might similarly account for the apparent allotypic heterogeneity.

Immunogenetic and Biochemical Analysis

Approach

The aim of the research project in the most general terms was to obtain information relating the structures of T200 molecules to their biochemical function in leukocytes. The opportunity existed to pursue an analysis of the apparent difference in ADCC response in Ly-5 congenic mice. Constraints outlined in detail below led to the experimental design ultimately used. Since there are some limitations to immunogenetic analysis it was reasonable to broaden the scope of the study and search

for clues regarding the existence and the nature of other
structure-function relationships of T200 molecules.

Based on the implications of results obtained by
other investigators, a project designed to examine a
possible role for the cytoplasmic domain of T200 was
proposed, in order to advance the current understanding of
the biochemistry of T200 and to serve as a complementary
analysis consistent with the major goals of the research
endeavor.

It is important to understand the rationale behind
the decision to examine the role of the cytoplasmic domain
of T200. The T200 glycoprotein is one of the major con
A-binding proteins on CTL (53) and several membrane
components which bind con A are thought to be functionally
involved in CTL mediated cytotoxicity (54). Ly-5
alloantisera inhibit ADCC responses (85), block
generation and effector function of allogeneic CTL in
murine MLC (70), and inhibit con A-induced mitogenesis
(70). Con A, a T cell mitogen, has been shown to induce
changes in cytoskeletal and membrane protein organization
coincident with cap formation in lymphocytes (88, 89).

T200 molecules probably undergo an inducible
phosphorylation in response to con A (52), which may
indicate that they participate directly in cytoskeletal
reorganization brought about by a mitogenic signal in
leukocytes. By virtue of the size, structure, and

conservation of sequence in the cytoplasmic domain, and other significant observations noted here, the question was raised whether T200 becomes associated with the cytoskeleton of cells activated by a mitogenic signal.

Previous work by Bourguignon's group demonstrated that T200 becomes associated with the cytoskeletal fraction when Thy-1 molecules on murine T lymphoma BW5147 cells are capped (90). Fodrin was shown by these authors to form a stable complex with T200 in plasma membrane fractions purified from BW5147 cells (90) and was found by others to accumulate underneath con A patches and caps on murine and human lymphocytes (89).

Other leukocyte membrane proteins are induced by ligands to undergo cytoskeletal associations, including sIg (91, 92) and the FcR for IgE (93). These cell surface receptors undergo a decrease in nonionic detergent solubility upon ligand binding and have been recovered from detergent-insoluble residues of ligand-treated cells by incubation of the residues under conditions favoring the depolymerizatin of actin (92, 93). It was decided that a similar procedure could be used to examine the possible cytoskeletal association of T200 molecules in cells treated with con A.

Design Considerations

Because many immune response differences in various strains of laboratory mice are believed to be under genetic control, the observation of a difference in ADCC response was of considerable interest to me, since no apparent immune response differences have yet been attributed to allotypes of T200. No studies have shown that a genetically controlled (or in other words, functionally allotypic) immune response to a particular pathogen maps to the Ly-5 locus or region.

A genetic analysis of these strains was proposed to systematically test the hypothesis that Ly-5 allotype controls ADCC or other in vitro responses. Assays were selected so that leukocytes from mice of known Ly-5 allotype could be tested for activities thought to involve T200 molecules. The goal of the study was to determine whether allelic genes linked to the Ly-5 locus might control ADCC activity or other responses in which T200 may participate.

The genetic background of the Ly-5 congenic mice is derived from Mls disparate strains and might also represent disparate FcR allotypes. I was unable to determine by pedigree alone whether such genetic differences might confound the results of the present work. The donor strain of the chromosome segment

containing the Ly-5 locus in the congenic B6-Ly-5.2 strain carries an allele for Mls functional allotype capable of stimulating B6 strain lymphocytes in a unidirectional manner (86). As this allelic difference could result in ambiguous MLC reactions or exert unknown influences on other reactions, the possibility of a confounding genotype effect had to be addressed.

Additionally, Fc receptor allotypic variation in the experimental population might have a bearing on the observed ADCC responses, though unlike the Mls system, the Fc receptor allotypic variation and its impact on the strain combinations used in this work are unknown. However, since the Fc receptor gene is linked to Mls, it was possible to propose an experimental approach that could resolve the issue of their possible contributions to the responses measured in this study. This was done by attempting to produce Mls reactions among the different strains under study. Because the FcR allotype gene and Mls are linked, a negative alloreactivity of the kind controlled by the Mls locus would provide at least some indirect evidence that no FcR allotype differences were present in these strains.

Other investigators have indicated the importance of testing littermates segregating for putative immune response genes when the response is suspected of being dependent on haplotype or allotype (94). Such analyses

reduce the risk of encountering confounding environmental or genetic effects, and thus provide the most rigorous control for variation not due to the gene or chromosome segment under investigation. Although the discussion by those authors pertains specifically to the analysis of the genetic control of NK cell activity, the underlying principles are of critical importance to the experimental design used to test the influence of allotype on many immune responses.

Consequently, I developed a plan to use littermates of an F_2 generation of mice segregating for alleles of the Ly-5 locus. These segregants were produced simply by mating [B6 X B6-Ly-5.2]F_1 mice. The Ly-5 segregants were typed for Ly-5 alloantigens to classify the littermate mice according to genotypic group, and their responses in anti-SE ADCC and various other in vitro assays were assessed independently for differences which might be attributed to Ly-5 allotype.

In a technical bulletin provided to users of the B6-Ly-5.2 strain, the sole supplier of these mice indicated that animals obtained from their colony may carry murine hepatitis virus. Serological confirmation of the presence of this and other murine viral pathogens was obtained in a survey of the local colony, conducted to determine the suitability of these mice for introduction to the Animal Resources Center at Montana State

University. This serological screening revealed a major impediment to the unambiguous interpretation of the present study.

The animal care facility was designed in part to adequately protect and care for animals used locally in biomedical research. Significant concerns regarding the importation of animals carrying endogenous pathogens led to the decision that the strains of mice involved in this genetic analysis would have to be obtained from viral pathogen-free sources or else the project would have to be terminated, since for technical reasons it was no longer feasible to maintain animals outside the Animal Resources Center.

Communications with representatives of the Memorial Sloan-Kettering Cancer Center indicated that their attempts to rederive virus-free stocks of B6-Ly-5.2 mice had been unsuccessful. Their method was to prevent transmission of the virus by perinatal transfer of mouse pups to virus-free foster mothers. This method cannot ensure against vertical transmission of the pathogen or infection by contact with contaminated materials. Therefore, the strain was rederived locally by a different method.

The need to rederive virus-free mice stemmed from the rather substantial risk these mice posed to other animals in the facility. It may have been possible to provide

adequate quarantine measures so that the other local stocks of uninfected mice would be protected from viral pathogens. However, quarantine does nothing to alleviate the problems associated with immune response testing of mice carrying viral pathogens.

The presence of these pathogens could introduce the kind of systematic error which might completely invalidate the genetic analysis undertaken in this study. Rederivation of the B6-Ly-5.2 parental strain would significantly reduce the risk of obtaining results confounded by uncontrolled effects due to the presence of viral pathogens. Strain-related differences in production of Ab have been shown to occur in response to mouse hepatitis virus (95). In addition, this virus is known to perturb lymphokine production and NK activity in B6 mice (96). This was the basis for the decision to proceed with the rederivation.

The newly rederived B6-Ly-5.2 strain and commercially available B6 mice uncontaminated by murine hepatitis virus were tested or used to produce Ly-5 segregant mice. Experiments were planned following rederivation to confirm results obtained using the segregants from the original stocks. A more specific description of the rederivation procedure is outlined in the discussion of experimental methods.

In overview, tests for allotype effect on selected immune responses in vitro were designed to reveal which of them might be influenced by Ly-5 allotype. Any observed difference probably could be attributed to the allotypic determinants residing on the extracellular domain of the T200 molecule. However, there is no direct evidence of identity between the cytoplasmic domains of the Ly-5.1 and Ly-5.2 molecules. The homology observed in this domain between species suggests that aspects related to its functional role preclude even minor variations. There is still the possibility that an observed allotype effect might be mediated by structural differences in the cytoplasmic domains of the Ly-5 allotypes.

The indications that T200 may be involved in mediating membrane-cytoskeleton interactions imply that the cytoplasmic domain of the molecule is crucial to its role in leukocyte function. The planned genetic analysis of immune responses was incapable of resolving the role of the cytoplasmic domain. However, membrane molecules capable of interacting with cytoskeletal elements have unique properties, making it possible to examine the control of these molecular associations.

Membrane glycoproteins lacking any direct or indirect attachments to the cytoskeleton generally are solubilized by nonionic detergents. To reveal cytoskeletal association of membrane receptors, polymers of

cytoskeletal elements can be depolymerized to release the associated membrane components from the nonionic detergent-insoluble residues of cells (92, 93).

I had no reason to believe that T200 molecules would be different in this regard, if in fact they associate with the submembranous cytoskeletal matrix. This follows from the observation that fodrin, considered to be an actin-associated protein, specifically associates with T200 molecules (90, 97). Therefore, I thought it reasonable to suspect that actin depolymerization would lead to the recovery of cytoskeletally associated T200 in a soluble form, allowing immunoprecipitation and electrophoretic analysis. Actin filaments can be readily depolymerized since many factors are known to influence the equilibrium between the polymerized and depolymerized forms, as recently reviewed (98).

I decided to look for an induced association of T200 with submembranous matrix components. By treating T cell lymphoma lines with con A and comparing detergent soluble and insoluble fractions it might be possible to demonstrate and characterize this association. Untreated cells and cells treated with lectin would provide samples suitable for comparison. Appropriate tests might then be devised to relate the cytoskeletal association to the mitogenic activity of the lectin. Because leukocyte tumor cell lines may possess aberrantly arranged cytoskeletal

elements (99), I decided that the tests would include cultured CTL, which retain specific functional activity and are not transformed.

Preview of Experiments

The results I present here show evidence that Ly-5 alloantigenic determinants may control a limited range of immune responses, probably related to particular biochemical differences between allotypic determinants. Though most of the responses measured in the study are not influenced by Ly-5 allotype, it could be argued that the view obtained from results of these experiments is biased against the possibility that Ly-5 allotype influences early events or responses measured outside the optimal response range. These alternative possibilities are tested only in a very limited way.

More specifically, in murine ADCC response, I found the serendipitous observation of a lower activity in the B6 strain compared to the B6-Ly-5.2 strain to be a reproducible observation, by systematic testing of a presumptive genotype effect. I found that the apparent response difference declines in segregating littermate mice, indicating that the parent strain difference may not be due to Ly-5 allotype. I confirmed these results in rederived mice.

Results from tests of Ly-5 allotype effect in a number of other leukocyte-mediated in vitro immune responses are also presented. These tests include NK cell activity, responses to polyclonal T cell mitogens, proliferative response in allogeneic MLC, and CTL-mediated lysis of murine tumor target cells. Of these, the last study alone exhibited a significant allotype effect in the Ly-5 segregant mice, apparent in a limited response range of the cytotoxic activity of allospecific CTL to P815 tumor target cells.

I present results of experiments designed to examine the role of the cytoplasmic domain of T200, which revealed that an inducible association of T200 with cytoskeletal components occurred in response to con A. Data shown from immunoprecipitations of detergent-soluble membrane fractions of cells revealed a modulation of membrane T200 molecules in response to con A. Inducible cytoskeletal associations were demonstrated by evidence obtained from immunoprecipitation of detergent-insoluble fractions of con A-treated cells. That the cytoskeletal association was specific and involved actin is implied by the methods I used to induce the associations, the cell lines I selected for the study, and the conditions I used to recover cytoskeletally associated T200 molecules. Further, I found out that T200 is a major component which undergoes an inducible association in response to lectin.

As a whole, the observations I made in the immunogenetic and biochemical analyses presented here have considerable relevance to the processes of leukocyte activation, surface receptor capping, and cytotoxic effector cell function. These results extend the current understanding of the role of T200 molecules in leukocyte activation and function. These studies thus lend new insight to the functional aspects of the cytoplasmic and extracellular allotypic domains of T200 molecules.

MATERIALS AND METHODS

Mice

Mice of the strains designated BALB/c, C57BL/6 (B6),
A.SW, and SJL were obtained from Jackson Laboratories, Bar
Harbor, ME, and maintained at the Animal Resources Center
at Montana State University. C57BL/6-Ly-5.2 (B6-Ly-5.2)
congenic mice were obtained from Dr. E. A. Boyse, Memorial
Sloan-Kettering Cancer Center, New York, NY, following 22
generations of backcrossing. Siblings segregating for
Ly-5 alleles (Ly-5 segregants) were produced by mating
heterozygous [B6 X B6-Ly-5.2]F_1 (hereafter simply
designated F_1) mice. The strain used for foster mothers
in the rederivation, informally designated virus antibody
free (VAbF) CD1, was obtained from Charles River Breeding
Laboratory, North Wilmington, MA, and carries the formal
trademark designation Crl:CD-1(ICR)BR. Age-matched mice
were used at 6-9 weeks of age for ADCC and NK assays, 7
weeks of age or older for mitogen stimulation, MLC, or CTL
assays, and 8 weeks of age or older for alloimmunizations
and in vitro antigen stimulation.

Rederivation

VAbF CD1 mice were maintained in protective isolation using a sterile positive pressure flexible film isolator throughout the rederivation procedure. Following Caeserean section of the B6-Ly-5.2 mothers just prior to their anticipated parturition, the uterus was aseptically transferred into the isolator and the B6-Ly-5.2 pups were placed with the CD1 foster mothers. Following rederivation, mice were kept in the isolator for 4-5 weeks before removal to microisolator cages. Subsequent animal care and manipulative procedures were performed in laminar flow units using aseptic technique.

Typing

Ly-5.1 antiserum was produced by immunizing SJL mice with A.SW spleen, lymph node, and thymus cells as described (2). Ly-5.2 antiserum was produced in a similar manner by immunizing [B6 X A.SW]F_1 mice with SJL cells. Antisera were tested for specificity using thymocytes from B6, SJL, A.SW, and B6-Ly-5.2 mice in indirect immunofluorescence assays (IFA). The specificities were confirmed by electrophoretic analysis of immunoprecipitates from B6 and SJL thymocytes using methods outlined below. Monoclonal antibodies (MAb)