IMMUNOLOGICAL STUDIES OF T LYMPHOCYTES IN THE PROTEOGLYCAN-INDUCED ARTHRITIS IN THE MOUSE

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DEDICACE

A mes parents
à Francine
à Sébastien
et à Geneviève

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RESUME

L'utilisation de techniques biochimiques et immunologiques a permis de disséquer la réactivité des cellules T à la molécule de PG. Deux lignées et deux hybridomes de cellules T appartennant tous à la sous-lignée des T helper TH1, ont été isolés. La lignée cellulaire JY.A reconnait seulement le PG fetal humain alors que la lignée JY.D ainsi que les deux hybridomes de cellules T (TH5 et TH14) reconnaissent tous les PG de différentes origines testés à l'exception du PG de la souris et du PG du chondrosarcome du rat. Les trois dernières cellules T reconnaissent un ou des épitopes immunodominants sur le domaine G1 de PG homologue avec la protéine liante. La présence de chaine(s) KS près des épitopes de cellules T influence le niveau de réactivité des deux hybridomes de cellules T. La localisation de l'épitope reconnue par l'hybridome TH5 a été circonscrite à un peptide de vingt huit acides aminés dans la région B de G1. L'injection intra-articulaire de JY.A a des souris naives induit les premiers changements pathologiques associés à l'arthrite rheumatoide.

ABSTRACT

In this study, biochemical techniques were used with immunological techniques to dissect the specific T cell reactivities to the PG molecule. Two T cell lines and two T cell hybridomas that belong to the T helper subset TH1 were isolated. The line JY.A recognized only the human fetal PG while the other line (JY.D) and the two T cell hybridomas (TH5 and TH14) recognized PG of all the different origins tested, except the rat chondrosarcoma and the mouse PG. The three latter T cells were found to recognize immunodominant T cell epitopes on the G1 domain of PG and cross-reacted with link protein. The presence of KS chain(s) close to T cell epitopes restricted the reactivity of the two T cell hybridomas. The location of one T cell hybridoma epitope (TH5) was contained within a peptide of twenty eight amino acids in the B region of G1. T cell line JY.A can induce early pathological change characteristic of rheumatoid arthritis when injected in the knee joint of naive BALB/c mice.

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LIST OF ABBREVIATIONS

AA	Adjuvant arthritis
AS	Ankylosing spondylitis
BSA	Bovine serum albumin
BFPG	Bovine fetal proteoglycan
CAPS	Cyclohexaminopropylsulfonate
CFA	Complete Freund adjuvant
CIA	Collagen-induced arthritis
CNBr	Cyanogen bromide
CS	Chondroitin sulfate
CTL	Cytotoxic T lymphocyte
DFP	Diisofluorophosphanate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylenediamide tetracetic acid
G1	Globular domain 1
GAG	Glycosaminoglycan
HA	Hyaluronate
HAPG	Human adult proteoglycan
HEL	Hen egg lysosyme
HEPES	(N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid])
HFPG	Human fetal proteoglycan
HPLC	High performance liquid chromatography
hsp	Heat shock protein
i.a.	intra-articular
IDDM	Insulin-dependant diabetes mellitus
IFA	Incomplete Freund adjuvant
IL-2	Interleukin-2
IL-4	Interleukin-4
i.p.	intra-peritoneal
JRA	Juvenil rheumatoid arthritis
KS	Keratan sulfate

LP	Link protein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
Mls	Minor lymphocyte stimulating antigen
MPG	Mouse proteoglycan
OA	Osteoarthritis
PBS	Phosphate buffered saline
PG	Proteoglycan aggrecan
PGIA	Proteoglycan-induced arthritis
PMSF	Phenylmethyl sulfonyl fluoride
PVDF	Polyvinylidine difluoride membrane
RA	Rheumatoid arthritis
RCS	Rat chondrosarcoma
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
TCGF	T cell growth factor
TCR	T cell receptor
TLCK	Nα-p-Tosyl-l-lysine chloromethyl ketone
TPCK	N-Tosyl-l-phenylalanine chloromethyl ketone
TSH	Thyroxin stimulating hormone

PART I - REVIEW OF LITERATURE

CHAPTER I : THE IMMUNE SYSTEM

1.1 PHYSIOLOGY OF THE IMMUNE SYSTEM

The physiological role of the immune system is to act as a defence mechanism and keep the body's integrity intact against the aggression of infectious agents such as viruses, bacteria and parasites and to prevent the development of tumor cells.

The immune system relies on the basic principle of discrimination between "self" and "non-self". How is this discrimination possible? It is achieved by basically three prominent features: specificity, memory, and tolerance. When this distinction is made and the foreign material (infectious agent) is recognized, the immune system will mount a response leading to the elimination or control of the infectious agent by the use of effector mechanisms.

1.1.1. THE LYMPHOCYTES

The T and B lymphocytes are the only cells of the immune system that possess the capacity of selective recognition. This is achieved at the molecular level on clonal cells through the presence on their plasma membranes of unique receptors for specific epitopes found on antigens.

1.1.2 ONTOGENY OF THE T CELLS

T lymphocytes ontogeny is in contrast to the maturation of B lymphocytes which takes place in the bone marrow in mammals (Butcher and Weissman, 1990). In the case of the T lymphocytes, hematopoeitic precursors migrate to the thymus where maturation and/or selection of antigen-specific T cells occur.

From the thymus the mature T cells migrate to secondary lymphoid tissues which include the spleen, lymph nodes and mucosal-associated lymphoid tissues where they will eventually mount an immune response when confronted with their specific antigens (Sprent, 1989).

There are several identifiable subsets of T cells (Sprent, 1989). This identification of different T cell subpopulations became possible when purified populations of T cell subsets and T cells from defined differentiation stages became available. These subsets were defined and characterized on the basis of the expression of surface molecules detected by antibodies, by their cytokine secretion profiles and by their effector functions. The two major subsets of mature T cells are the ones that possess either the $CD4^+CD8^-$ or the $CD4^-CD8^+$ phenotypes. Through their T cell receptors (TCR), T cells recognize specific antigenic epitopes only when presented in the context of MHC molecules. Although in both subsets the TCR appears to be very similar, they show different patterns of MHC restriction; $CD8^+$ cell are usually MHC class I restricted and $CD4^+$ cells are MHC class II restricted. In terms of reactivity the $CD4^+$ cells are associated with T helper activity and the $CD8^+$ cell with cytotoxicity, although these functions are no longer considered to be strictly inherent to each subset since examples of cytotoxic $CD4^+$ cells are known (Fleischer and Wagner,1986).

Other subsets of T cells are defined on the basis of their different lymphokine production patterns which is an important feature of the $CD4^+$ subset. In the mouse, two major subsets have been characterized: the inflammatory type named TH1 and the antibody-helper type named TH2. TH1 cells are characterized by the release, upon stimulation, of IL-2, IFN-γ and TNFβ, whereas the TH2 subset secretes IL-4, IL-5 and IL-6 (Mosman and Coffman, 1987). Other cytokines are also characteristic of these two subsets

and other T subsets are emerging with different cytokine production patterns (Mosman and Moore, 1991).

1.1.3 THE IMMUNE RESPONSE

An immune response is established when an antigen is "trapped" by the reticuloendothelial system where antigen persists and is presented to $CD4^+$ T cells by MHC class II expressing antigen presenting cells (APC) which include dendritic cells, macrophages and B cells (Unanue, 1989). These cells present the antigen to T cells which will mount the immune response by secreting the appropriate cytokines that will in turn recruit and activate B cells to become plasmocytes that secrete the specific antibodies, and effector T cells including inflammatory and cytotoxic T cells. Other cells are also recruited such as the macrophages and neutrophils. These cells can interact with each other by a network of cytokines and membrane receptor-ligands contacts. The end point of this immune response will be to eliminate the presence of foreign pathogens and tumor cells by effector mechanisms involving antibody reactivity, complement activation and cytotoxic T lymphocytes. This is followed by the establishment of memory cells which can recognize and trigger a more effective response on future exposure to the antigen.

1.2 DISCRIMINATION BETWEEN SELF AND NON-SELF

1.2.1 THE MOLECULES INVOLVED IN T CELL ANTIGEN RECOGNITION

1.2.1.1 THE TCR

The genes encoding the immunoglobulins of B cells and the T cell receptors (TCR) are unique in that they undergo rearrangement that allows the production of a great diversity of receptors and antibodies that recognize different antigens. In the case of T cells, the TCR consists of either αβ or γδ heterodimers (reviewed by Strominger, 1989). The latter form of heterodimer is found on a small proportion of the total T cell population (Lew et al., 1986). It is the predominant T cell type in the mucosal lining (Goodman and Lefrancois, 1988). These cells are believe to be restricted in their responses by non-polymorphic class I MHC-related molecules (class Ib molecules: CD1, TL, and Qa) (Born et al., 1990). Despite some evidence suggesting a possible role in local initiation of the immune response (Born et al., 1990), the physiological function of these cells remains unknown.

Both types of heterodimer have a striking similarity in their domain structures. In the αβ heterodimer, each α (45-60 kD) and β (40-50 kD) chain consists of a constant and a variable domain. The α and γ chains consist of a variable, a joining and a constant domain. The β and δ chains also possess a diversity domain between the variable and joining segments. The variable domain confers antigen specificity . The diversity of the TCR is formed by random recombination during the differentiation of the T cells to the mature state, of one of each of the variable (V), diversity (D), and joining (J) segments (Hedrick, 1989). When these rearrangements for the TCR have occured for

each T cell clone, they become a permanent feature for those T cells. The combinatorial joining events from the V-region, D-region (for β chains), and the J-region gene elements can form approximately 5000 different α-chains and 500 different β-chains that can potentially form 2.5 million TCR combinations (Hedrick, 1989). In contrast to the immunoglobulin mechanism of diversity, somatic mutations have not been found to occur in T cells (Hedrick, 1989). The TCR is noncovalently associated with the CD3 complex on the cell surface (Reinherz et al., 1982). This complex of 5 subunits, is believed to transmit the signal from the TCR to the cytoplasm by mechanisms involving phosphorylation of these subunits (Mustelin and Altman, 1989).

1.2.1.2 MHC CLASS II MOLECULES

Class II molecules of the major histocompatibility complex (MHC) are composed of two non-covalently associated glycosylated polypeptide chains: the α chain (32-34 kD) and the β chain (29-32 kD). The β chain is more glycosylated than the α chain and possesses an intrachain disulfide bond at the N-terminal end (Robinson and Kindt, 1989). Both chains are transmembrane molecules with two external domains of about 90 amino acids each. The N-terminal domain is the peptide-binding region of the molecule. Both chains may interact together by forming a β pleated sheet which forms the "floor" and α helices which form the sides of the peptide-binding cleft (Brown et al., 1988) in a similar manner to class I molecules (Bjorkman et al., 1987a; Bjorkman et al., 1987b). The genetic allelic polymorphism of class II MHC molecules determines the amino acid composition of the peptide binding domain and is responsible for determining the specificity and affinity of peptide binding and T cell

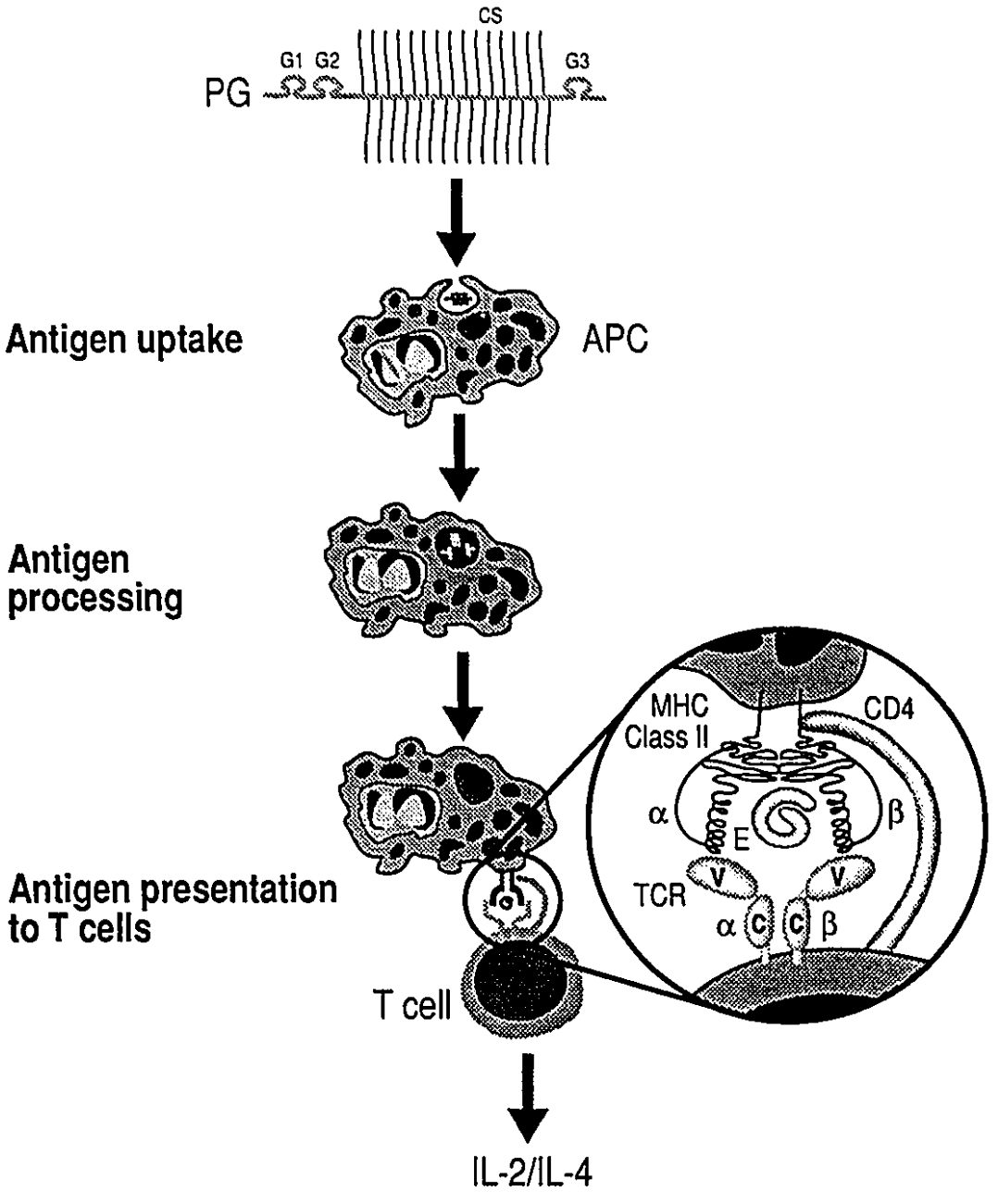

FIGURE 1. Antigen Recognition by T Cells

Schematic representation of antigen presentation to PG-reactive T cells.
APC : Antigen Presenting Cell; **E** : Epitope; **TCR** : T Cell Receptor

recognition. The expression of class II molecules occurs in specialized cells called antigen presenting cells or APC (e.g. macrophages, activated B cells) and is responsible for presentation of exogenous soluble antigens to the T cells. The antigens are usually internalized by the APC and enzymatically cleaved into peptides in the endosomal-lysosomal compartment, a process called antigen processing. It is in the endosomal compartment that antigenic peptides bind to MHC class II molecules (Creswell, 1985). The complex is then shuttled to the surface of the cell membrane and the peptide is presented to the T cell via the T cell receptor (TCR) (Figure 1). This trimolecular interaction is strenghtened by the CD4 molecule on the T cell. With appropriate costimulatory signals, T cells are activated to secrete lymphokines and drive an immune response. Superantigens such as staphylococcal enterotoxins also stimulate T cell clones by forming a trimolecular complex but by binding outside the MHC class II groove; also they do not require processing (Herman et al. 1991).

The MHC class II heterodimers are assembled in the ER and associate with the non-polymorphic invariant chain (Ii) to form a stable trimolecular complex in the cell. The Ii is a type II glycoprotein in which the C-terminal end of the molecule is an extracellular domain . Ii is, however, not required for either the assembly nor the expression of MHC class II at the cell surface since cells lacking (Miller and Germain, 1986; Sekaly et al., 1986) or expressing low levels (Stockinger et al., 1989) of Ii, assemble and display normal levels of class II αβ heterodimers. Ii is capable of inhibiting the binding of cytosolic internal peptides to class II molecules (Roche and Creswell, 1990). Ii is important for the presentation of soluble exogenous antigens since it contain recognition sequences for targetting the αβ-Ii complex to the endosome (Bakke and Dobberstein, 1990; Roche et al., 1992). It is in the endosome that Ii

is cleaved off by proteolytic enzymes to liberate the class II molecule, permiting binding of antigenic peptides to the class II molecule.

1.2.1.3 ACCESSORY MOLECULES

Peripheral T cells express either CD4 or CD8 molecules on their surface (Hedrick, 1989). CD4 will bind to the MHC class II molecules while CD8 binds to MHC class I molecules. These molecules are likely to function as stabilizing elements and/or signal transducers (Finkel et al ,1991). Other molecules are also involved in antigen recognition and T cell activation. These comprise: 1-) The CD2 molecule, a member of the immunoglobulin family, which interacts with the LFA3 molecule on the APC. 2-) CD28 that may have as its ligand the B7 molecule found on activated B cells. It is thought to be responsible for providing the second signal needed for T cell activation. 3-) Members of the integrin family such as the CD11aCD18 (LFA-1) and the VLA-4,5,6 which have as their ligands the ICAM-1,2 and VCAM-1 molecules, respectively on APC (Abbas et al., 1991; Shevach, 1989). All of these have adhesion and/or signal transduction functions in T cells. Another accessory molecule is CD44, a member of the cartilage link protein gene family, that is the principal cell surface receptor for hyaluronate (Aruffo et al., 1990). This molecule is not restricted to T cells but is also present on granulocytes and macrophages cells. It is believed to bind to a series of connective tissue macromolecules such as collagen, fibronectin, high endothelial venule (HEV) addressin and hyaluronate, and can trigger physiological cell function (Miyake et al., 1990; Webb et all., 1990).

1.2.2 THE SELECTION OF LYMPHOCYTIC CLONES

It is in the thymus, during ontogeny of the T cells, that positive and negative selection of T cells occurs. These crucial events lead to the formation of the peripheral T cell repertoire. In positive selection, the T cell clonotypes, with restriction specificities, are selected to give rise to an emerging repertoire for the recognition of small peptides associated with self-MHC molecules (Teh et al., 1988; Sha et al., 1988). In reality not all the T cells that possess reactivity to other MHC molecules species are useful. In negative selection, which also occurs in the thymus, clones of T cells with high affinity to self MHC molecules, and perhaps against self-peptides associated with MHC molecule presented by thymic APC, are eliminated by apoptosis and therefore never reach the periphery (Kappler et al., 1987). Some clones of the primary repertoire survive the double selection procedure. These T cell clones, which possess the ability to recognize self-peptides having escaped negative selection, can be made self-tolerant by at least three mechanisms, clonal anergy, T cell suppression and peripheral clonal deletion.

1.2.3 TOLERANCE

After proper selection of T cell clones has occured in the body during ontogeny of the T cells in the thymus, those that reach the periphery can perform their functions. The reactivities of the T cells are however tightly regulated by different mechanisms. If autoreactive T cells have escaped thymic clonal deletion, these cells will be kept self-tolerant by strict regulatory mechanisms which include T suppressor cells, antigen specific suppression (Jenson and Kapp, 1985) and anergy (Schwartz, 1990).

Until further progress is made in our understanding of T suppressor receptors and genes, the existence of a T cell suppressor (Ts) subset will remain unclear, although the notion that some T cells can inhibit the function of other T cells is now unquestioned. This activity is found mainly in $CD8^+$ T cells, but their growth and differentiation may be dependant on $CD4^+$ T cells. Suppressor T cells were thought to be restricted by a region of the class II MHC called the I-J region, thought to be located between the I-A and I-E loci based on the genetic studies of various intra-MHC recombinant strains and antisera that could absorb suppressor factors derived from these strains of mice (Murphy et al., 1976; Tada et al., 1976). However sequencing of the entire mouse MHC class II genome has not demonstrated any DNA sequence that could code for an "I-J" molecule in that area (Kronenberg et al., 1983). The inhibitory effects of suppressor T cells seem to be mediated by secretory proteins. Even though they recognize antigens in a specific maner they may act in a non-specific manner such as by the secretion of large amounts of TGF-β, which is a powerful inhibitor of both B and T cell proliferation (Lotz et al., 1990), or by expression of cytolytic activity.

CHAPTER II : AUTOIMMUNITY

2.1 MECHANISMS OF AUTOIMMUNITY

The ability of the immune system to discriminate between self and non-self is possible because it is tolerant to its own antigens and can react to foreign antigens. This signifies that the immune system has the potential of generating T cell clones that recognize both self and foreign antigens. As discussed earlier, this self-tolerance can be due to three principal mechanisms: clonal deletion, clonal anergy and suppressor T cells.

The self-reactive cells that have escaped clonal deletion can become self-tolerant by clonal anergy, induced when T cells encounter self-antigens without second signals and can no longer respond to the antigen (Schwartz, 1990). It is believed that autoimmunity results from the failure of the mechanisms responsible for maintaining self-tolerance. This can be due to incomplete deletion of self-reactive clones or to disregulation of normally anergic self-reactive lymphocytes. Many interacting factors are believed to contribute to the development of autoimmunity, such as genetic factors and microbial infections. The following section will discuss the principal factors thought to be involved in the development of autoimmune diseases.

2.1.1 IMMUNE CELLS

The cells that play a pivotal role in autoimmunity are those involved in the recognition of antigens, namely the B and T cells. T cells may be solely responsible for the abnormality because even in disorders mediated by autoantibodies, T helper cells are needed for the production of the high affinity antibodies, as in experimental myasthenia gravis (Lennon et al., 1976). For this reason and because T cells play a central role in the regulation of all

immune responses much attention has focused on the role of T cells in autoimmunity. This concept was proved correct in instances where T cells reactive to specific tissue autoantigens were isolated from animal models of autoimmune diseases and shown to transfer the diseases to naive recipients (Schwartz and Datta, 1989). T cells specific for autoantigens have been also isolated from patients with such diseases, though their principal role in these diseases in humans has not yet been demonstrated (Schwartz and Datta, 1989).

Other cell types are also involved in the initiation and perpetuation of these diseases. APC and cells from the reticuloendothelial system may be involved in the initial step when autoantigen is presented to T cells. In autoimmune disease like rheumatoid arthritis, the cells that normally synthesize the macromolecules which form the tissue, can also play a part in the tissue damage. These cells can secrete enzymes responsible for the degradation of matrix molecules. Usually the net balance between synthesis and catabolism is in favor of synthesis as seen in growth or in a status-quo of the total mass of the molecules forming the matrix with normal turnover. But in autoimmune disease this balance can shift towards net loss of tissue due to excessive catabolism of the resident cells under the influence of cytokines. The influx of inflammatory cells, such as macrophages and neutrophils, can also contribute to increased catabolism and net loss of tissues by their secretion of degradative enzymes and oxygen radicals.

Some autoimmune diseases and their experimental counterparts in animals, are believed to be principally mediated by $CD4^+$ T cells (exemplified by rheumatoid arthritis) while $CD8^+$ CTL cells have been shown to contribute to the pathogenecity of other diseases such as in insulin-dependent diabetes mellitus (IDDM) (Bach, 1991). B cells can contribute to the pathology of some autoimmune diseases, such as myasthenia gravis (Lennon et al., 1976), where

autoantibodies and the formation of immune complexes are involved in the effector phase by causing tissue injury. A majority of the B cells that account for the production of natural autoantibodies are derived from a small subset of B cells that express the $CD5^+$ marker at their surface (Gadol and Ault., 1986). However these B cells are not the only B cell type involved in the development of autoimmune disease (Wofsy and Chiang, 1987). In antibody mediated autoimmune diseases tissue injury can be caused by complement-mediated lysis of cells, by recruitment and activation of inflammatory cells and phagocytosis of antibody-coated cells.

2.1.2 AUTOANTIGENS, ANTIGENIC MIMICRY AND SUPER ANTIGENS IN AUTOIMMUNITY

Knowing that the immune system can make the distinction between self and non-self, based on epitope discrimination, a molecular mechanism should explain the events that initiate and perpetuate the disease. Antigenic mimicry involves cross-reactive epitopes shared by microbial antigens with homologous amino acid sequences found in mammalian proteins not sharing the same function as the microbial antigen (Oldstone, 1987). It is believed that microbial epitopes that mimic self-peptides could break the tolerance acquired naturally against the self-peptides. How this occurs is not known, but it may reside in the inbalance, provoked when infection occurs, of the tolerance mechanisms (awakening the self-reactive T cell clones) or, as in the case of autoantibody-mediated diseases, in T cells that recognize a foreign epitope could provide help to B cell that would in turn secrete antibodies specific to a self epitope.

There are a number of examples where antigen mimicry has been implicated in autoimmune diseases. In the majority of cases, the evidence is for cross-reactive antibody epitopes between microbial pathogens and self-molecules as in the case of thyroid autoimmunity (Graves disease) where antibodies cross-react to the thyroxin stimulating hormone (TSH) receptor and the *Yersinia enterocolitica* binding site to TSH (Heywa et al., 1986) or in examples of cross-reactive epitope between *Yersinia pseudotuberculosis* and HLA-B27 (Chen et al., 1987) or with *Klebsiella* and HLA-B27 (Ogasawa et al., 1986).

These examples are only suggestive of the possible involvement of antigen mimicry in autoimmune disease. An experimental demonstration of this concept has been observed in adjuvant arthritis. In susceptible strains of rats, a rheumatoid-like arthritis is induced by injection of killed *Mycobacterium tuberculosis* in the form of complete Freund's adjuvant. The pathology is characterized by a T cell-dependant destruction of peripheral joints which leads to chronic deforming arthritis. The disease can be adoptively transfered by a $CD4^+$ T cell clone specific to a mycobacterial antigen (Holoshitz et al., 1983). This antigen is contained in a nonapeptide of the N-terminal region of the 65 kD heat shock protein of this organism (van Eden, et al.. 1988). This T cell clone cross-reacts to cartilage PG preparations derived from various species (van Eden et al. 1985). But the identity of the cross-reacting epitope remains to be established because crude PG preparations were used and there is a lack of a similar sequence in PG (van Vollenhaven et al., 1988).

2.1.3 EQUILIBRIUM BETWEEN TOLERANCE AND AUTOIMMUNIZATION

How do T and B cell clones, that recognize self-epitopes and have escaped clonal deletion but do not react with self-tissue under normal circumstances due to a state of anergy, suddenly become reactive to self-antigen? Antigenic mimicry by pathogenic microbes can constitute at least an initial basis for the development of the pathology. Yet, it is unclear whether this is sufficient to start an immune reaction against self-antigen. Many putative autoantigens are sequestered and poorly available to the immune system because they reside in avascular sites such as articular cartilage (Poole et al., 1988), or are not accessible because of the blood-brain barrier. Some cryptic epitopes may be revealed to the immune system after an injury or an infection that recruit APC which present self-antigen that otherwise would not be presented to immune cells.

Antigen presenting cells may in fact play an essential role in breakage of tolerance. As in the case of clonal deletion of T cells in the thymus, dendritic cells that serve as antigen presenting cells are responsible for the destruction of T cells that recognize self-antigen associated with their MHC molecules (Kappler et al., 1987; Ramsdell and Fowlkes, 1990).

Furthermore, the lack of second signals or costimulatory signals provided by APC is known to induce tolerance in the T cell. This is believed to be the major mechanism for the control of T cell reactivity against self-antigen for autoreactive T cells that have escaped clonal deletion (Jenkins, 1992). It is possible that under specific circumstances some autoreactive T cells may overcome their anergic state when encountering APC's that can provide

the proper costimulatory signals along with presentation of self-antigen. The CD28 antigen on T cells is believed to be the molecule signaling the co-stimulation of T cells in the mouse (Jenkins, 1992). Stimulation of this molecule by specific antibody on anergized T cells has been shown to effectively overcome the anergic status of the T cells (Jenkins, 1992).

2.1.4 GENETIC PREDISPOSITION

The development of autoimmune diseases is under the influence of genetic control. One of the most important groups of genes involved in autoimmunity are the MHC genes because of the role of their gene products in the establishment of the T cell repertoire and in the induction of the immune responses to protein antigens. These molecules are highly polymorphic and therefore some individuals will have MHC molecules which are associated with resistance whilst others are associated with susceptibility to certain autoimmune diseases.

There are examples of both human and animal autoimmune disease associations with MHC molecules. The strongest association is seen between ankylosing spondylitis and the MHC class I allelle HLA-B27 (Calin, 1989).

Some recent studies have demonstrated that MHC genes could be related to both susceptibility and resistance to autoimmune disease. An example of this is seen in insulin dependent diabetes mellitus (IDDM) in which 95% of caucasians with this disease have either one or both HLA-DR3/DR4 haplotypes (Rotter et al., 1983). The susceptibility to the human disease is determined by the structure of the DQβ chains, particularly at position 57 which is located in the antigen binding cleft (Todd et al., 1988). In fact, in humans and in the NOD mouse model of IDDM it has been observed

that the presence of aspartic acid (ASP) at position 57 in the β chain of the human DQ haplotypes as well as its murine homologue I-A, seems to confer resistance to the disease (Acha-Orbea et al., 1987; Todd et al., 1988).

Genetic factors are also important in susceptibilty to systemic lupus erythematosus (SLE). MHC-linked genes and other undefined genes seem to play a role. In humans the haplotypes HLA-DR2 and DR3 are associated with the highest risk for development of this disease (Mackworth-Young and Schwartz, 1988).

In rheumatoid arthritis (RA), the strongest association is seen with HLA-DR4 and to a lesser extent with DR1 and DRw10 haplotypes (Stastny. 1976; Schiff et al., 1982; Winchester, 1989). A common feature of these alleles is that they possess an almost identical amino acid sequence in the third diversity region of the DR β1 chain (Winchester, 1989). The location of this sequence is in the polymorphic region of the MHC class II molecule and, as in the case of position 57 of the DQ β chain in IDDM, it participates in the formation of the antigen binding cleft. This points to the importance of the formation of the trimolecular (TCR-peptide-MHC II) complex in the disease.

These findings suggest at least two possible mechanisms. Because of certain amino acid sequences of some MHC class II haplotypes, these might fail to bind self protein with high affinity which would normally cause clonal deletion of autoreactive T cells in the thymus. T cells reactive with self antigen may involve a molecular mimicry mechanism where autoantigen associated with the MHC molecule resemble the combination produced by a viral peptide with the MHC. It was observed that some HLA-DR4 subhaplotype amino-acid sequences have homology with Epstein-Barr virus sequence (Roudier et al., 1988, 1989).

Because these MHC class II alleles are also found in healthy individuals, and conversely, other alleles commonly present in normal individuals are also found in some patients with autoimmune disease, it is likely that genes other than those coding for MHC class I and II contribute to disease development. Breeding analysis of mouse strains that develop autoimmune diseases indicate that as many as twenty genes may contribute to different diseases (Schwartz and Datta, 1989). Some of the non-MHC genes that may influence autoimmunity are the complement genes C2 and C4 in humans (Kemp et al., 1987; Schifferli et al., 1986) and C5 in the mouse; also the two cytokines tumor necrosis factor (TNF) and lymphotoxin (LT) (Schwartz and Data, 1989) which are located within the MHC locus (Trowsdale et al., 1991). In the mouse, there is also the minor lymphocyte stimulating antigen (Mls-1) that has been found to be associated with some autoimmune diseases (Herman et al., 1991). Their actions reside in the modification of the T cell repertoire. Their genes possess a sequence of an integrated mammary tumour retrovirus, and produce molecules that act as superantigens deleting T cell clones that express certain Vβ TCR (Herman et al., 1991).

Hormonal influences are another factor to consider. In SLE, females are affected ten times more than males (Amhed et al., 1985a). It is also the case in the SLE animal model in the (NZB x NSW)F1 mice where the disease develops in females and can be retarded by androgen treatment (Amhed et al., 1985b). Other autoimmune diseases tend to be more frequent in females, but it is not always clear if it is due to a hormonal influence. There is also a predominance of RA in female with a ratio of 2:1 over males, for which the role of hormone has been implicated (Amhed et al., 1985a). However, the exact influence of sex hormones in this disease is still not clear (Heath and Fortin, 1992).

2.2 AUTOIMMUNE DISEASES

Although there are different diseases that possess the characteristics of autoimmune diseases, they all have similarities in the immune mechanisms that lead to the immunopathology of self destruction. Knowing the mechanisms that underline a particular disease may help in the understanding of another. There are several human autoimmune diseases that have an animal equivalent. These clinical and experimental autoimmune diseases can be classified as organ specific or systemic autoimmune diseases based on the involvement of the target tissues. Following is a brief description of representative autoimmune diseases in humans and their animal equivalents or models.

The organ specific autoimmune diseases are characterized by diabetes mellitus, thyroiditis, which occur in two forms (Hashimoto's thyroiditis and Grave's disease), multiple sclerosis and myasthenia gravis. Of the systemic autoimmune diseases, SLE and the RA are examples. These representative autoimmune disease are briefly discussed below, except for RA which is the subject of Chapter 4.

2.2.1 TYPE I DIABETES MELLITUS

IDDM or type I diabetes or juvenile diabetes is a disorder of glucose metabolism due to a deficiency of insulin. This deficiency arises from the destruction of the insulin-producing β cells of the islets of Langerhans in the pancreas. This occurs as a result of several immunological mechanisms in which autoantibodies, cytokines and CTL-mediated lysis have been found to contribute to the destruction of the insulin producing β islet cells. This produces insulinitis characterized by pancreatic lesions involving cellular

necrosis and lymphocytic infiltrations (Rossini et al., 1985). Immunological characteristics are the infiltration of both $CD4^+$ and $CD8^+$ T cells, high expression of MHC class II molecules on islet cells, and the presence of both anti-β islet cell and anti-insulin antibodies in the blood. There is some association in the white Caucasian with the HLA-DR3 and DR4 haplotypes (Rotter et al., 1983). More recently susceptibility has also been linked to HLA-DQ genes (Todd et al., 1987). Several molecules derived from β islet cells have been proposed as the autoantigen. These include the 64 kD glutamic acid decarboxylase (Baekkeskov et al., 1990), the 65 kD hsp that may trigger IDDM in NOD mice (Elias et al., 1990), the 38 kD protein that is extracted from β islet cells granules (Roep et al., 1991), and peripherin (Boitard et al. 1989). However the nature of the antigens that initiate the islet-specific immune response is not known, nor whether these proposed autoantigens are involved in the initial triggering event of the anti-β islet cells response or whether these reactions represent an epiphenomenon.

Two representative animal models of spontaneous IDDM have been described extensively, one in the inbred BB rat strain and the other in the non-obese diabetic (NOD) mouse strains, both of which develop a spontaneous T cell-mediated insulinitis (Rossini et al, 1985). The disease can be transfered by lymphocytes from diabetic mice (Bendelac et al., 1987). There is also a remarkable similarity between MHC gene linkage in the mouse and that in humans. In the NOD mouse model, the expression of I-A molecules is critical for the outcome of the disease; in transgenic NOD mice with I-E molecules, the incidence of the disease is reduced, therefore confering a protective effect (Nishimato et al., 1987). Sequencing of cDNA clones encoding I-A β chains of NOD mice has revealed that the first external domains (amino terminal) differ from those of MHC-matched ($H\text{-}2^d$) strains

(Acha-Orbea and McDevitt, 1987). This occurs in the conserved region between position 248 and 252 with radical amino acid changes. But in particular residue 57 of I-A^d β chains in the mouse as well as the DQ β chain in human (the analog of mouse I-A β chain) appears to be of importance in the outcome of the disease (Todd et al., 1988). An Asp at position 57 is associated with resistance whereas a substitution with Ser is observed in susceptible NOD mouse strains.

2.2.2 THYROIDITIS

In Hashimoto's thyroiditis, autoantibodies are produced against thyroglobulin and intracellular microsomal proteins. This leads to the destruction of the thyroid follicular cells, perhaps through complement-fixing IgG autoantibodies. It results in the under-production of thyroid hormone. The gland is also infiltrated with $CD4^+$ and $CD8^+$ T cells, as well as with macrophages and plasma cells (Volpe, 1987). There is also a genetic basis to Hashimoto's disease with an increased association with the HLA-DR5 haplotype (Volpe, 1987). In Grave's disease, the autoantibodies, are directed against the TSH receptor and instead of mediating destruction of tissue they mimic the effect of TSH. This results in over stimulation of the thyroid cells to secrete excessive amounts of thyroxine leading to high metabolism. Autoreactive $CD4^+$ T helper cells infiltrate the gland.

There are animal models of thyroiditis. The best studied animal model of Hashimoto's thyroiditis is in the mouse, where the specific epitope of the autoantigen and the immunogenetics have been fully characterized. The epitope which causes disease has been delineated to a 5-10 kD tryptic fragment of thyroglobulin in CBA mice (Salemero et al., 1987). This fragment contains

a T helper cell epitope and the disease is tranferable to syngeneic naive recipients with epitope-reactive T cell lines and clones (Maron et al., 1983; Romdall et al., 1987). It was shown that iodination of thyroglobulin was important in inducing T cells to react to the autoantigen (Champion et al., 1987). MHC genes were also found to have a prominent role in this disease (Volpe, 1987).

2.2.3 MYASTHENIA GRAVIS

Myasthenia gravis is an autoimmune disorder of neuromuscular transmission in which autoantibodies against acetylcholine receptors block the binding of acethylcholine to its receptor (Lindstrom, 1985). This effect is probably due to steric hindrance caused by the autoantibodies that bind to immunodominant epitopes of the α chain of the receptor (Lindstrom, 1985). T helper cells that recognize an epitope located on denatured α subunit have also been identified (Hohlfeld et al., 1987). Animal models of experimentally induced myasthenia gravis have been extensively studied in inbred strains of rats and mice. Induction of the disease is made possible by injecting, in adjuvant, acethylcholine receptor purified from the electric organs of the eel *Torpedo californica*. The disease is associated with both T cell responses and antibody production to the antigen (Lennon et al., 1976). Immunogenetics is also important since the susceptibility to the disease is associated with a sequence in the external domain of the β chain of I-a molecules (Christadoss et al., 1982).

2.2.4 MULTIPLE SCLEROSIS AND EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

The brain and the central nervous system (CNS) are immune privileged tissues of the body. This privilege is largely due to the brain-blood barrier that limits access of the immune system to the brain. Multiple sclerosis is a chronic and relapsing autoimmune disease of the CNS. The pathological features consist of the demyelination of the white matter with characteristic plaques. The histopathology is characterized by infiltration of macrophages and of both T helper and cytotoxic T cells (Waksman, 1985). Besides reactivity to self MHC antigen, the two most important autoantigens believed to be involved in this disease are the myelin basic protein and the proteolipid protein (Hafler et al., 1987). However these antigens have not consistently been found to be T cell antigens in this disease (Hafler et al., 1987).

A well described representative animal model of multiple sclerosis called experimental allergic encephalomyelitis (EAE) is produced in susceptible strains of rats and mice when brain, spinal cord tissue or myelin basic protein (MBP) are injected in these animals. Preclinical changes involved perivascular infiltration of inflammatory cells in the brain. This is a T cell-mediated disease associated with production of antibodies to MBP. Antibodies to MBP and myelin may facilitate demyelination which is not induced by antibodies alone (Waksman, 1985). The disease developes in PL/J and B10.PL strains of mice with the H-2^u haplotype and in the SJL/J (H-2^s) strain, when immunized with MBP. The N-terminal 11 residues constitute the encephalogenic epitope for the H-2^u strain (Zamvil et al., 1986) whereas the epitope resides in position 89-101 for the SJL/J mice (Sabal et al., 1988). It was also found that T cells from SJL/J mice were reactive to a peptide (139-151)

of the proteolipid protein which was encephalitogenic (Whitham et al., 1991). In the H-2^u mice the disease was induced by T cells with restricted usage of the T cell receptor that allowed the use of specific anti-TCR antibody therapy (Urban et al., 1988; Acha-Orbea et al., 1988) or T-cell receptor peptide therapy (Vandenbark et al., 1989; Howell et al., 1989). The induction of the disease could also be prevented by non-T cell stimulatory MBP peptides that bind to the I-A^u molecules and competitively inhibit T cell reactivity to MBP (Urban et al., 1989).

2.2.5 SYSTEMIC LUPUS ERYTHEMATOSUS

Ninety percent of patients are young women. The principal clinical symptoms are rashes, characterised by the butterfly rash on the face, glomerulonephritis and arthritis (Hahn, 1980). Hormones, principally estrogens, are believed to influence the disease, and explain its preponderance in young females (Alarcon-Segovia, 1989). Important pathological features are the presence of large amounts of antinuclear antibodies. These antibodies have been demonstrated to be polyspecific for the disease. Monoclonal antibodies produced by hybridomas derived from patients with lupus are also polyspecific (Schwartz and Data, 1989). They will cross-react with both single and double stranded DNA, synthetic polynucleotide, membrane-associated and cytoskeletal proteins, histones and DNA-histone complexes, and also with other negatively charged structures such as phospholipids, proteoglycans, dextran sulfate and other molecules (Brinkman et al, 1990). The sugar-phosphate backbone may constitute the shared common determinant. It is however not believed that these cross-reactive antibodies play a major role in the process (Brinkman et al., 1990).

There exists a SLE mouse model which develops in certain strains of mice. It has enabled a better understanding of this disease in terms of genetic and immunological mechanisms. Spontaneous severe lupus like glomerulonephritis occur in F1 (NZB X NZW) hybrid progeny while parent mice do not develop this disease except for the NZB strain which develops another autoimmune disease called hemolytic anemia (Schwartz and Data, 1989). However disease does not occur in F1 mice of other NZB crosses except with the SWR strain. Anti-DNA antibodies have been extensively studied in these animal models of SLE without being shown to have a definitive role in the disease (Schwartz and Data, 1989). Another mouse strain is the congenic MRL for which two congenic strains exist the MRL-*lpr/lpr* and MRL-+/+. The former develop lymphoadenopathy and severe lupus while the latter only develops a mild form of lupus nephritis late in life. The *lpr* gene has been transferred into other strains of mice without developing disease, although they produce autoantibodies. This indicates that the MRL background is needed for the disease and that possibly the *lpr* gene acts by increasing the severity of the disease (Schwartz and Data, 1989). A third animal model is the BXSB mice which is a recombinant inbred line of C57/BL6 female and SB/Le male. An accelerator gene (*Yaa* gene) has been linked with the Y chromosome inherited from the SB/Le mouse (Schwartz and Data, 1989). Susceptibility determining factors have also been associated to polymorphic MHC genes as well as TCR genes (Pisetsky, 1991). Immunological mechanisms implicate both T and B cells. These involve abnormal B cell polyclonal activation and accumulation of double negative T cells. However disturbance in the $CD5^+$ B cells population seems not to be the reason for autoantibody production (Pisetsky, 1991). The exact role of these lymphocytes in this disease is still not elucidated.

CHAPTER III : ARTICULAR CARTILAGE

3.1 SYNOVIAL JOINTS

These comprise the majority of important articulations in the body. They are composed of a capsule, a synovial membrane, the synovial fluid and articular cartilage. The fibrous capsule which is composed primarily of collagen fibers, envelopes the synovial joint and is continuous with the periosteum and perichondrium. This capsule in combination with ligaments, such as the collateral and the cruciate ligaments of the knee the latter being inside the joint cavity, confer joint stability. When these ligaments are disrupted,the joint becomes less restrained in its mobility and severe cartilage degeneration can result (Bird et al., 1978).

Lining the fibrous capsule is the synovial membrane which consists of a thin layer of synovial cells (one to four cell layers thick) that envelops all the intra-articular surfaces except the cartilage itself and is in direct contact with the synovial fluid. This membrane is composed of two major cell types: type A cells with a macrophage-like function; and type B cells which have a fibroblast-like function (Sledge, 1989). The synovium's function is to keep the joint cavity healthy with phagocytic cells and cells which synthesise components essential for the synovial fluid such as hyaluronate (Sledge, 1989). This synovial membrane is formed on a subintimal layer of connective tissues which is well vascularized (Sledge, 1989).

The synovial fluid is composed of elements derived from synovial cell products: glycoproteins, hyaluronate, small molecules from the subintimal blood supply that crosses the synovial membrane and also molecules diffusing from subchondral bone. The functions of the synovial fluid are

ones of nutrition, lubrication, and the maintenance of the intra-articular pressure (Sledge, 1989).

The articular cartilage of synovial joints is a hyaline cartilage that covers the articulating surfaces of bone. Because of its organization and molecular composition this specialized connective tissue possesses unique biophysical properties that include resistance to compressive and tensile forces. It acts as a shock absorber while providing with synovial fluid, almost frictionless articulation under high pressure (Urban et al, 1979).

3.2 STRUCTURE AND COMPOSITION OF ARTICULAR CARTILAGE

The articular cartilage is composed of chondrocytes and matrix. The latter occupies most of the cartilage volume. The particular biomechanical properties of the cartilage are due to the matrix which is composed of 65-80 % water retained by the highly negatively charged glycosaminoglycans. The swelling of this tissue is restrained by the collagenous fibrilar network resulting in a high hydrostatic pressure (Poole, 1993).

The chondrocytes are the cells responsible for the synthesis and degradation of the extracellular matrix. The chondrocyte as well as the extracellular matrix are not homogeneous throughout the tissue. In fact based on the distribution of chondrocyte and matrix with respect to the depth of the tissue, articular cartilage can be divided into four zones (Meachim and Stockwell, 1979). Underlying the smooth regular shape of the surface of the cartilage is found a superficial zone where cellularity is the highest with cells flattened parallel to the surface. The collagen fibrils are also oriented parallel to the cartilage surface. It is the zone where the collagen fibers are at their densest while aggrecan content is at its lowest but decorin is maximal

(Rosenberg et al., 1985; Poole et al., 1986a; Poole, 1993). Below this zone the cells are distributed more evenly within the tissue. A well organized network of type II collagen fibrils is formed with the interlacing of hyaluronate associated with the large aggregating proteoglycan which is considerably increased in amount as compared to the superficial zone. This mid-zone occupies about 40-45% of the articular cartilage (Meachim and Stockwell, 1979). In the deep zone, which represents 40-45% of the tissue, the cells are arranged in columns in which groups of 3-4 cells are arranged in stacks. The largest collagen fibrils are oriented perpendicular to the articular surface. Underneath this zone is the zone of calcified cartilage and subchondral bone.

3.3 THE MACROMOLECULES COMPOSING THE MATRIX.

3.3.1 COLLAGENS

Fifteen different types of collagen molecules have been described to date (Linsenmayer, 1991). Genetically distinct, these molecules are biochemically related to each other. Their structures consist of triple helical domains composed of repeating amino-acid units of glycine-X-Y, where X and Y are usually proline and hydroxyproline respectively but can also be any amino acid. Their function is to support the tissue and provide tensile properties. Type II collagen is the principal species found in the cartilage and accounts for up to 95 % of all collagen found in the tissue (Poole, 1993). It consist of a triple helix of three identical polypeptides, each called a [α1(II)] chain wounded around a common axis. Synthesized by the chondrocyte as a procollagen that has extended non-helical domains at each end of the molecule, it is processed in the extracellular matrix by cleaving off, with proteolytic enzymes, these two

propeptide domains leading to a collagen molecule with short non-helical telopeptides domains at each end of the triple helix . These amino and carboxy-terminal propeptides appear to be involved in the control of the biosynthesis of collagen by in part preventing intracellular fibril formation (Nimni and Harkness, 1988). When released from the triple helix, the C-terminal C-propeptide (CP-II), is thought to be involved in the calcification process taking place in the growth plate (Hinek et al., 1987;Kujawa et al., 1989; Poole and Rosenberg, 1986b; van der Rest et al., 1986). It is a calcium binding protein with subunits of Mr 35 kD. The collagen monomers that are generated, are assembled in parallel to form the typical collagen fibrils which become cross-linked and interact with other type of collagen molecules and proteoglycans (Yamanchi and Mechanic, 1988; Labat-Robert and Robert, 1988). The network of collagen fibrils formed provides the tensile forces needed to neutralize the swelling pressure created by absorption of water by the proteoglycan.

Collagens of types V, VI, IX, X, XI are each essential molecules of the cartilage matrix in which they are present (Poole, 1993). Types V and VI collagens, which are not unique to the articular cartilage, constitute a very small proportion of the total collagen (1% or less). Their functions in the articular cartilage are still unknown. Type IX collagen is a structuraly unique collagen because it is composed of three collagenous domains, named COL1, COL2, and COL3 that are interupted by four non-collagenous domains (NC1 to NC4) in which a CS/DS chain is attached to the amino terminal non-collagenous domain (NC3) of the α 2(IX) chain. This collagen molecule can be considered as a proteoglycan since it contains a GAG chain (McCormick et al., 1987; Huber et al., 1988). It is closely associated with the type II collagen fibrils. In fact studies by electron microscopy of rotary shadowed collagen fibrils have

demonstrated the periodic distribution of type IX collagen along the surface of collagen type II fibrils (Vaughan et al., 1987; Vaughan et al., 1988). This association is mediated by lysine-derived cross-links between the two collagens. The N and C terminal telopeptides of the collagen type II α1 chain are connected to the central collagenous domain (COL2) of the α2(IX) chain (Eyre et al, 1987; van der Rest and Mayne,1988). Because of its predominant location in the fine small fibrils in the pericellular matrix of chondrocytes, as detected by immunolocalization, this collagen is believed to be important for the lateral growth of collagen type II fibrils (Hartman et al., 1982; Wotton et al., 1988). Type X collagen is composed of three α1(X) chains and is found exclusively in the hypertrophic region of the growth plate. It is believed to play a role in the process leading to cartilage calcification (Poole and Pidoux, 1989a). Type XI collagen is associated with the fibril containing collagen types II and IX (Mendler et al., 1989) and is thought to be involved in fibril formation.

3.3.2 LARGE AGGREGATING PROTEOGLYCAN (AGGRECAN)

As its name indicates, this cartilage molecule consist of a protein to which glycosaminoglycans chains are bound (Figure 2). These account for more than 85 % of the total dry weight of the molecule which has a M_r of 1-3 x 10^6. The complete sequences of the core proteins of rat chondrosarcoma PG (Doege et al., 1987), human PG (Doege et al., 1991), and mouse PG (Glant et al., 1992) have been recently identified. Aggrecan, also called proteoglycan (PG), which is found in normal cartilage consis' · of a core protein of Mr 210 000 to which are attached many glycosaminoglycans and oligosaccharide chains. The oligosaccharides consist of about 40 O-linked and 7 N-linked chains

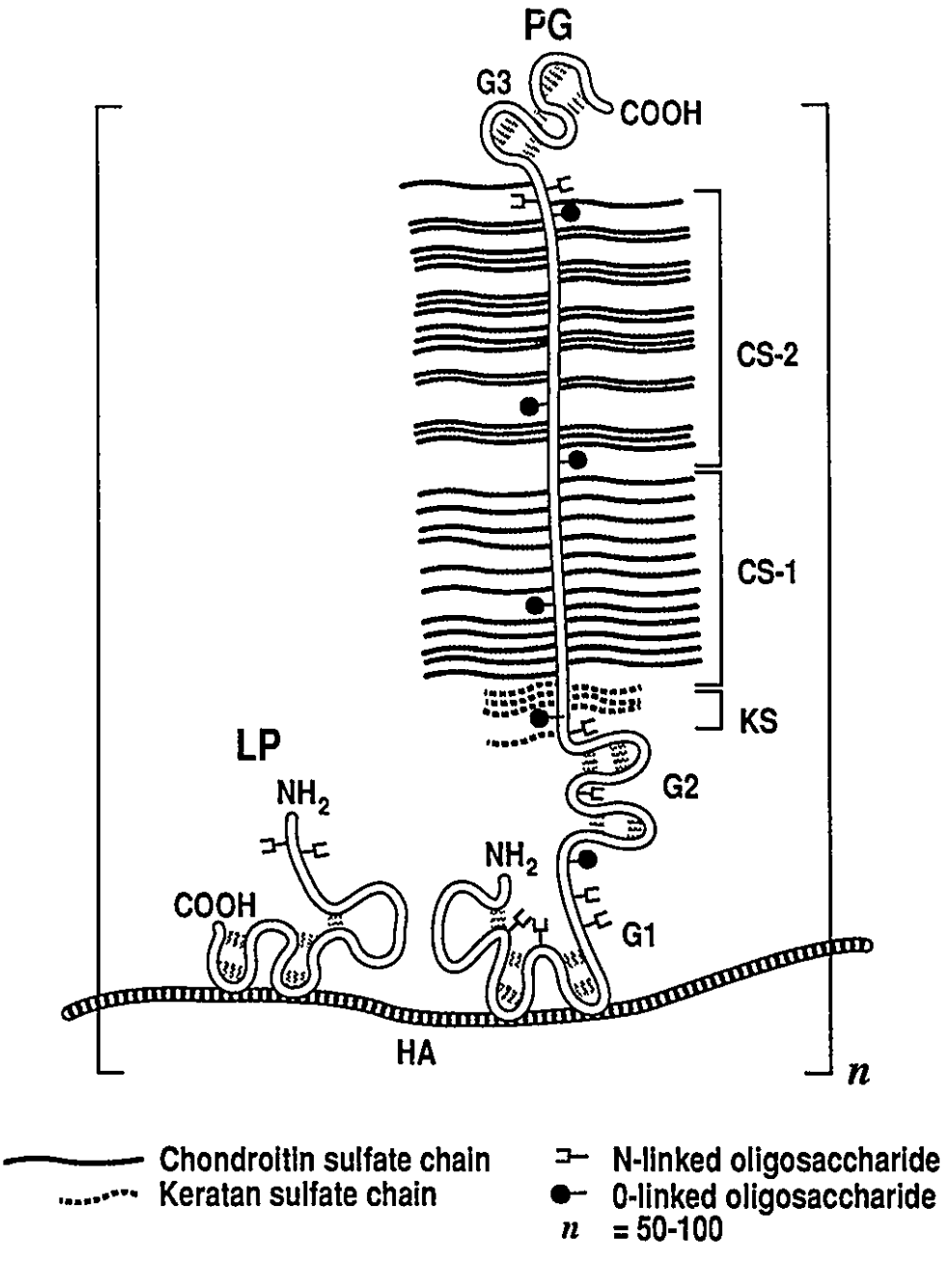

FIGURE 2. Schematic Representation of Aggrecan (PG) in Association with Link Protein (LP) and Hyaluronate (HA)

G1 : Globular domain 1; **G2 :** Globular domain 2; **KS :** Keratan sulfate region; **CS-1 :** Chondroitin sulfate region 1; **CS-2 :** Chondroitin sulfate region 2; **G3 :** Globular domain 3

which are distributed sparsely throughout the core protein but with a higher concentration of the N-linked chains at the N-terminal end of the molecule (Doege et al., 1987; Neame et al., 1987) and with the O-linked chains at the C-terminal end (Heinegård et al., 1985). There are as many as a 100 chondroitin sulfate chains (Mr of aproximately 20 000) and 30 keratan sulfate chains (Mr of about 8 000) per molecule with the majority distributed along the core protein (Figure 3 and Figure 4) in groups which form specific domains (Heinegård et al., 1985). The structure of this molecule is as follows (Figure 2). At the N-terminus is the G1 globular domain which is responsible for the specific non-covalent interaction with hyaluronate (Morgelin et al., 1988; Fosang and Hardingham, 1989). This G1 domain was previously known as the hyaluronic acid binding region (HABR). It can be subdivided into 3 regions delineated by three disulfide-bounded loops. The nearest loop to the N-terminus has an immunoglobulin fold-like sequence (Neame et al., 1986, Neame et al.,1987; Dudhia and Hardingham, 1989; Perkins et al., 1989). The link protein has an homologous structure to that of the G1 and these two Ig-folds (one on each molecule) are in fact responsible for the interaction between link protein and the PG subunit (Périn et al., 1987). This region is followed by two other disulfide-bonded loops of the G1 domain named B and B'. The amino acid sequence of these two loops exhibit homology with each other and with the corresponding regions in the G2 domain and link protein. These two loops are known to mediate the hyaluronate-binding interaction of PG and link protein (Figure 2) (Neame et al., 1986, Neame et al.,1987; Périn et al., 1987; Goetnick et al., 1987). This does not seem to be the case with the G2 domain even if it contains the same tandem repeats as the G1 domain (Doege et al., 1987; Neame et al.,1987; Fosang and Hardingham, 1989, Paulsson et al., 1987). The G1 and G2 domain are separated by a long segment of

approximately 134 amino acids called the interglobular domain where some oligosaccharides are found attached to the core protein as well KS chains in some PG like the bovine and pig PG. This region is susceptible to protease cleavage since trypsin will cleave in that region to liberate an intact G1 from PG aggregate. This susceptibility may have some relevance with observation of increase release of free G1 in the synovial fluid (Witter et al., 1987) and the presence of a stromelysin susceptible site (Fosang et al., 1991), stromelysin being an important active metaloproteinase in the cartilage (Wilhelm et al., 1987). The function of the G2 domain remains unclear.

At the C terminus of the G2 domain is found the KS rich domain. However this domain is absent from the rat chondrosarcoma PG (Doege et al., 1987; Stevens et al., 1984; Antonsson et al., 1989). The KS glycosaminoglycan chains consist of repeated disaccharides of galactose attached to an N-acetyl glucosamine by a β 1,4 linkage. The disaccharides are linked to each other by β 1,3 linkages (-1,3 gal-β-1,4-glcNAC-β-) (Figure 3). This non-sulfated carbohydrate chain is named a polylactosamine chain and is found on many glycoproteins (Wight et al., 1991). In the KS chain, either or both oligosaccharide are sulfated at the 6 position (Figure 3). Each chain is usually 20-40 disaccharides long (8-20 kD). An hexameric repeat consensus in the KS attachment domain has been observed in both human and bovine PG in the KS rich region: the serine that serves as the attachment site for the KS chains is preceeded by a proline which is itself preceeded by an aromatic amino acid or a serine, another proline, any amino acid, and a glutamic acid. While usually O-linked to serine or threonine, the KS chains can also be N-linked to asparagine as in proteoglycans of the cornea and in the proteoglycan fibromodulin (Plaas et al., 1990) (Figure 4).

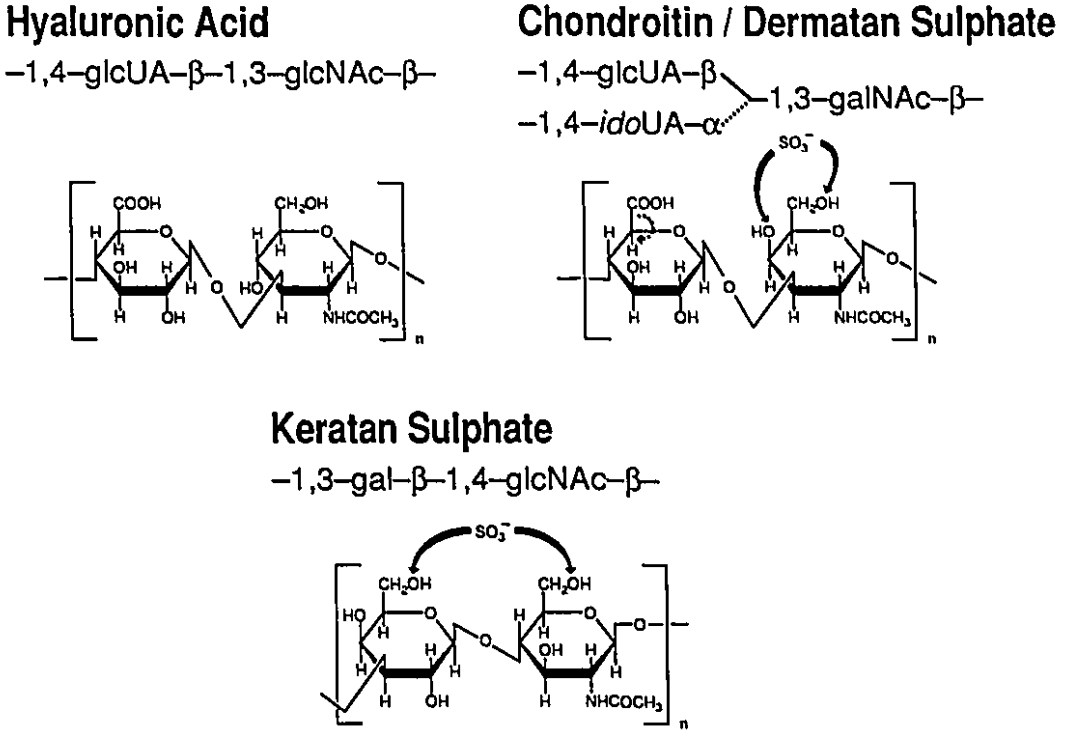

FIGURE 3. Representation in Developed Planar Configuration of the Repeating Disaccharide Unit of Different GAG

Hyaluronic acid and chondroitin sulfate (CS) have a similar uronic acid composition and similar linkage except that in the latter a galNAC replaces the glcNAC and this oligosaccharide can be sulfated (dark arrows). In dermatan sulphate (DS) the glucuronic acid is change for an iduronic acid which occur from epimerisation of the carboxyl group of the glcUA (dotted arrows). Keratan sulfate consist of polylactosamine chains which are sulphated at either one or both of the 6th carbons of each disaccharide. Heparan sulphate and heparin (not shown) consist in similar disaccharide composition as for the CS and DS GAG except the linkage is 1,4 instead of 1,3.

This KS rich region is followed by three CS domains (CS-1, CS-2, CS-3) which are highly substituted by CS chains (Doege et al., 1987). These CS chains consist of repeated disaccharides of glucuronic acid attached to an N-acetylgalactosamine in a β 1,3 linkage. These disaccharides are attached to each other by a β 1,4 linkage. Sulfation occurs usually at either 4 or 6 positions of the N-acetylgalactosamine (-1,4-glcUA-β-1,3-galNAc-β-) (Figure 3). Each chain can possess more than 50 repeated disaccharides (30kD). These are attached to serine via a xylose-gal-gal-GlcUA oligosaccharide sequence (Figure 4). The CS-1 and CS-2 domains show a high degree of homology in which contiguous sequences are arranged in consecutive 19-20 residue blocks with many serine-glycine pairs to which are attached the CS chains in an O-glycosidic linkage to the hydroxyl group of the serine (Doege et al., 1987; Doege et al., 1991).

At the carboxy terminus of the molecule is located the third globular domain G3. It contains a lectin-like sub-domain that shares homology with the hepatic lectin (Sai et al., 1986; Doege et al., 1987; Oldberg et all., 1987). This domain interacts with low affinity, but in a specific manner with several sugar ligands, principally galactose and fructose (Halberg et al., 1988). Therefore this region may interact with sugar residues present on other matrix glycoproteins. The lectin-like domain is followed by two alternatively spliced sub-domains, the epidermal growth factor (EGF)-like and the complement regulatory protein (CRP)-like sub-domains (Baldwin et al., 1989; Doege et al., 1991). For example the EGF sub-domain is not found in the RCSPG nor in chicken PG (Baldwin et al., 1989; Doege et al., 1991) but is present in the human. This EGF-like sub-domain has been estimated to be present in about one third to one half of the human aggrecan transcripts of fetal and juvenile cartilage mRNA (Doege et al., 1991). The CRP-like sub-

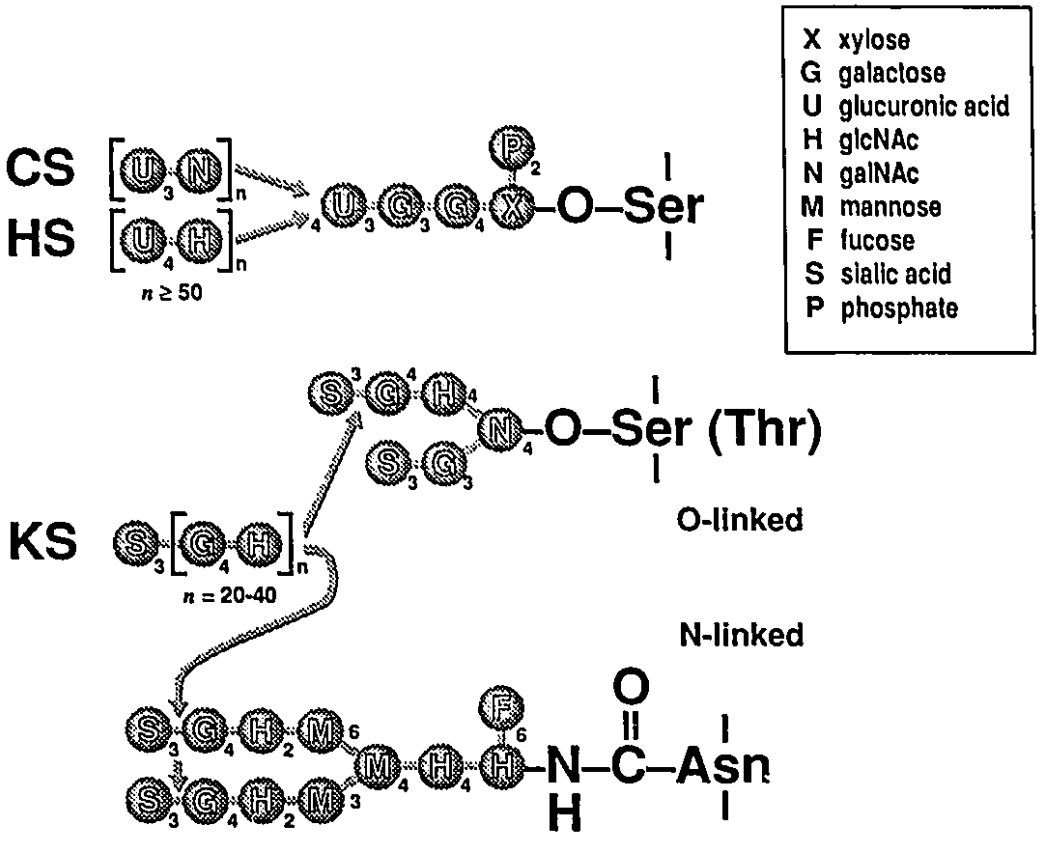

FIGURE 4. Schematic Representation of the Linkage Region of the Different Glycosaminoglycans (GAG) to the Core Protein

The disaccharide repeats of heparan sulfate (HS) and chondroitin sulfate (CS) GAG chains are linked to the oxygen of the hydroxyl group of serine or threonine through a quadrasaccharide arm. Keratan sulphate (KS) can be either O or N linked to serine/threonine, or asparagine through an arm of bi-antenary complex type of sugar. In both cases the disaccharides are attached to the last galactose of the arm. Numbers represent linkage carbon. (Adapted from Wight et al., 1991)

domain has, however been found in the human , rat (Doege and Yamada, 1988) and chicken (Tanaka et al., 1988). This sub-domain is apparently subject to alternative splicing since it was reported by one group (Baldwin et al., 1989) and not found in cDNA clones by others in the human (Doege et al., 1991).

It is important to mention that the G3 domain is retained in a proportion of about 40% of extractable PG. This may be due to its loss in the extracellular matrix by the action of proteolytic enzymes (Paulsson et al., 1987).

3.3.2.1 PG BINDS TO HYALURONATE AND LINK PROTEIN TO FORM A MACROMOLECULAR AGGREGATE.

Hyaluronate is composed of a linear polymer of dissacharide repeats like the CS chains found on PG except that the N-acetylgalactosamine is replaced by an N-acetylglucosamine (Figure 2). Its Mr is of the order of 1-4 X 10^6 Da. Unlike other glycosaminoglycan chains it is not sulfated and it is not attached to any protein component (Heinegård and Paulsson, 1984). Each of the HA binding sites for either PG or link protein extends over five repeated disaccharides (Hardingham and Muir, 1974; Hascall and Heinergård, 1974). An average of 25 disaccharide repeats of HA separate each PG-link complex (Faltz et al., 1979). The link protein (LP) is a glycoprotein of 48 kD that shares homology with the G1 domain of PG (Deák et al., 1986; Neame et al., 1986; Dudhia and Hardingham, 1989). Two other components of LP have been described with Mr of 44 and 41 kD which represent a single oligosaccharide form with the same core protein length (Baker and Caterson, 1979; Mort et al., 1985) and a shorter core protein lacking oligosaccharide due to proteolytic cleavage near the N-terminus (Le Glédic et al., 1983). This non-covalently bound ternary complex formed by PG, HA and LP is very stable (Hascall and

Heinergård, 1974). All these components, as for type II collagen, are synthesized by the chondrocyte.

3.3.3 OTHER PROTEOGLYCANS OF CARTILAGE

In addition to the aggregating PG there are three non-aggregating PG of low molecular weight core protein (approximately 37 kD for the core protein size). One is called decorin (DS-PGII) and another biglycan (DS-PGI). These contain at the N-terminus either one or two CS or DS chains respectively (Choi et al., 1989). The third member is fibromodulin (59 kD) that bears N-linked keratan sulfate chains in the central portion of the molecule (Plaas et al., 1990). These small PG's are members of a leucine rich family of PG having the common characteristic of a central leucine-rich segment formed by at least 10 repeating sequences of 24 amino acids in which leucine and cysteine residues are found in conserved positions (Heinegård and Oldberg., 1989; Fisher et al., 1989). Decorin and fibromodulin bind to collagen types I and II and limit fibril formation (Hedbom and Heinegård, 1989). It has been suggested that both molecules are involved in the regulation of collagen fibril formation and that decorin's primary function is in the organization of the extracellular matrix (Hedbom and Heinegård, 1989). Decorin binding to collagen is not mediated through the GAG chains (Vogel et al., 1984). Since these two molecules possess different GAG the collagen binding domain may reside in the homologous region of the core protein of both molecules (Hardingham and Fosang, 1992).

3.3.4 OTHER COMPONENTS OF THE CARTILAGE MATRIX

Type II collagen and aggrecan are the principal components of the matrix of articular cartilage. But in addition to these molecules there is a panel of other molecules for which the structures have only recently been characterized (Heinegård and Oldberg, 1989). In most cases their functions remain unknown. The proteins include a 21 kD protein, a 36 kD protein, a 58 kD protein, chondronectin and fibronectin (Paulsson and Heinegård, 1984; Heinegård and Oldberg, 1989).

Anchorin CII is a collagen binding protein of Mr 34kD. It is present both in the chondrocyte plasma membrane (Mollenhauer and von der Mark, 1983) and as a secreted protein although no signal peptide could be found in the cDNA clone (Fernandez et al., 1988). It has been proposed that it acts as a mechanoreceptor for chondrocytes to mediate cell-matrix interaction since it serves as site for anchorage of pericellular collagen type II fibrils to the chondrocytes.

Other large molecules have been described in hyaline cartilage. The cartilage matrix protein (148 kD) described in bovine tracheal cartilage but not in articular cartilage and intervertebral disc, is characterized by two large repeated domains separated by a region with high homology to EGF (Kiss et al., 1989). It is tightly associated with PG aggregates (Heinegård and Oldberg, 1989). Another molecule of high Mr (550 kD, being composed of five subunits of 116 kD); has been found in both tracheal and articular cartilage (Fife and Brandt, 1984; Heinegård and Oldberg, 1989). It is called cartilage oligomeric protein.

CHAPTER IV : ARTHRITIC DISEASES

4.1 COMMON ARTHROPATHIES

Arthropathies are characterized by the destruction of articular hyaline cartilages in joints. Several types of arthropathies exist. They are usually classified into two major groups: one involving primarily cartilage destruction and one involving the synovium. The former is represented by the most prevalent form of arthritic disease, osteoarthritis or degenerative joint disease. The disease is associated with aging and involves joints of the knee, hip, spine, the hands and feet. The highest prevalence is in the knee and hip joint. The disease is a progressive degeneration and loss of articular cartilage associated with changes in the subchondral bone and joint margins leading to ankylosis of bone.

The second group of the classified arthropathies involves primarily the synovium. These comprise joint disease with diverse etiologies. Examples of these are the induced synovitis where microorganisms such as bacteria induce the immunopathology. Lyme disease is an example where a spirochete is responsible for the induction of the disease (Steere, 1989). Other examples which fall in this group are the crystal induced arthritides such as gout and pseudogout.

The second group also includes arthropathies in which the synovial inflammation is of unknown etiology. These are split into two more groups: those thought to be autoimmune diseases which comprise systemic lupus erythematosus, rheumatoid arthritis and juvenile rheumatois arthritis ; and in the second group are found Reiter's syndrome, psoriasis, ankylosing spondylitis and scleroderma.

Of all these disease, osteoarthritis and rheumatoid arthritis are the most prominent joint diseases. But these two joint diseases are distinct because only RA is likely to be an autoimmune disease. RA may in fact be the most common autoimmune disease. Ankylosing spondylitis which is an inflammatory disease involving axial and appendicular joints may also be autoimmune by nature.

4.2 IMMUNOPATHOGENESIS OF RHEUMATOID ARTHRITIS

4.2.1 PATHOLOGY

The pathological features of RA vary with the development of the disease. At early stages the changes seen in the joint are related to the synovium, with the appearance of hyperplasia of the synovial membrane due to increased number of both type A and B synovial cells and the influx of lymphocytes, monocytes, plasma cells, and neutrophils (Harris, 1989). Changes also include increased vascularity. The inflammated synovium develops numerous villi that form a pannus which spreads to cover in part the articular cartilage. This pannus also destroys the underlying articular cartilage and subchondral bone. The earliest and most prominent bone erosions occur at the joint margins where articular cartilage ends and the joint capsule attaches to bone. Later bony ankylosis can unite the opposing joint surfaces (Harris, 1989). These changes result in severe pain of the inflammed joints. It has been observed that inflammation subsides when articular cartilage has been totally removed or destroyed (Poole, 1993), strongly suggesting that the elements driving the inflammation may be contained in the cartilage.

4.2.2 ETIOLOGY

Despite the many causative agents proposed to be responsible for the appearance of RA, the real cause(s) is still unknown. Research has been focused mainly on infectious agents as well as endogenous molecules of the cartilage which may be involved in the perpetuation of the disease, in part because of antigenic mimicry with microbial antigens (van Eden et al., 1987).

Several possible candidates have been proposed in the past as causative agents in RA. These include the Epstein-Barr virus, parvovirus, T-cell lymphotropic virus type 1, the rubella virus, the cytomegalovirus, the herpes virus, and mycobacteria (Harris, 1990). Among these, the Epstein-Barr virus and mycobacterias have attracted growing interest in recent years because of the following observations. Eighty percent of RA patients have circulating antibodies directed against antigens of Epstein-Barr virus (Alspaugh et al., 1981). However the possibility that the phenomenon observed for the Epstein-Barr virus in RA patients is a secondary phenomenon rather than a causative one comes from reports that the titers of antibodies to the virus antigens are not elevated in early RA (Silverman and Schumacher., 1981). The link between Epstein-Barr virus and RA has regained interest through the concept of molecular mimicry. The viral Epstein-Barr virus glycoprotein gp 110 and the "RA susceptibility sequences" of the β chain of HLA-Dw4, Dw14, and DR1 haplotypes of the class II molecules share homologous sequences (Roudier et al., 1988). Serologic studies have shown that serum antibodies of patients with previous Epstein-Barr virus infections recognize the same peptides from both gp 110 and HLA-Dw4 (Roudier et al., 1989).

The link between mycobacteria and RA was made because these bacteria express heat-shock proteins (hsp). These molecules have been shown to be the arthritogenic factors of adjuvant arthritis in the rat (van Eden et al., 1988).

In synovial fluids from patients with RA, αβ T cells as well as γδ T cells are present which proliferate in response to mycobacterial antigens (Hill-Gaston et al., 1989; Holoshitz et al., 1989). On the contrary it is also possible that the reactivity to hsp is a secondary phenomenon. T cell clones reactive to the mycobacterial 65 kD hsp were isolated from patients with RA but were not found to be cross-reactive to the eukaryotic hsp and therefore were not considered the cause of joint inflammation in RA (Hill-Gaston et al., 1990). However cross-reactions of T cells to endogenous hsp60 and mycobacterial hsp65 have been found in juvenile chronic arthritis in synovial fluid and peripheral blood. But no such proliferative response to hsp has been found in RA patients (De Graeff-Meeder et al., 1991).

It is generally agreed that cartilage molecules are involved in the perpetuation of the disease, but these are not necessarily believed to be instigators of RA (Harris, 1990). Recently, it has been proposed that the etiologic agent might act as a superantigen (Palliard et al., 1991; Howell et al., 1991). This was proposed from the finding that patients with RA have similar Vβ family T cell clonal deletions from the peripheral blood, which are characteristic of superantigen deletion (Herman et al., 1991). Moreover the same Vβ bearing T cell clones that have disappeared from the peripheral blood were found in the joints of RA patients (Palliard et al., 1991; Howell et al., 1991). Whether this has relevance to disease remains to be established.

4.2.3 IMMUNOGENETICS OF RHEUMATOID ARTHRITIS AND ANKYLOSING SPONDYLITIS

As with many autoimmune diseases the MHC locus is associated with disease. While in ankylosing spondylitis (AS) the association is made with

the MHC class I molecules, in RA it is with the MHC class II locus. AS is the autoimmune disease with the strongest correlation with the MHC since 90 % of these patients have the HLA-B27 haplotype (Brewerton et al., 1973; Schlosstein et al., 1973; Calin, 1989). In RA there is a significant association with the class II molecules HLA-DR4 and HLA-DR1 (Stastny, 1978;Harris, 1990). However these haplotypes do not constitute the only genetic components of susceptibility to the disease. Other undefined gene elements may also govern resistance or susceptibility. As discussed earlier under immunogenetic's in Chapter 2, there is a shared epitope among certain HLA haplotypes and Epstein Barr Virus molecule at amino acid positions 70-74 in the third hypervariable region of the β chains which is associated with increased susceptibility to RA and perhaps severity of the disease (Harris, 1990).

4.2.4 HOMING OF T CELLS TO THE JOINT

It is in the early stage of disease that lymphocytes home to the synovial membrane. This coincides with the neovascularization of the synovial membrane. Then lymphocytes migrate through endothelial walls and aggregate around the blood vessels below the synovial surface (Harris, 1990). The adhesion of the lymphocytes on the post-capillary venules is possible through molecules found on endothelial cell surfaces called addressins, for which lymphocytes possess receptors (Harris, 1990). Some cytokines enhance the adhesiveness of endothelial cells for lymphocytes. Among these are IL-1, TNF-α, and IFN-γ (Harris, 1990). It is interesting to note that the structure of the addressins found at the surfaces of the post-capillary venules in the rheumatoid synovial membrane is different from the addressins of the other

secondary lymphoid organs (Jalkanen et al., 1986). An essential observation, which may have a link with this difference in addressins, is that the helper-inducer T cells adhere better to endothelial adhesive proteins than do the other subsets (Pitzalis et al., 1988). This may relate to the finding that there are more T cells than B cells in the synovial membrane (van Boxel and Paget., 1975).

4.2.5 CYTOKINES IN RHEUMATOID ARTHRITIS

Much progress has been made in the last decade on the identification and the action of cytokines. The molecules are produced mostly by immune cells but can also be produced by other cell types like the fibroblast. The cytokines are hormone-like proteins that enable inter-cellular communication and modulate cellular activity which in turn modulates the local environment. The integrity of cartilage matrix and the turnover of its molecular constituents are maintained by the metabolic rates of chondrocytes. This equilibrium can be shifted toward net degradation over synthesis by the action of various cytokines in synovitis (Poole, 1993). The major immune cell type that produces cytokines during inflammation is the T cell, which produces cytokines such as IL-2, LT, and IFN-γ, while macrophages and fibroblasts synthesize primarily IL-1, IL-6 , GM-CSF, and TNF-α. However discrepancies concerning the presence of these cytokines in the joint are observed in RA inflammation. The IL-2, IFN-γ, and IL-4 cytokine levels are usually very low in the RA synovial fluid. In contrast, large amounts of the macrophage and fibroblast type of cytokines have been found in RA synovium (Firestein, 1991). This restricted secretion pattern could be determined by *in situ* hybridization of freshly isolated synovial tissue cells

(Firetsein et al., 1990). This technology allows quantitation of gene expression of cytokines by cells and perhaps better reflects the levels of cytokine activity in the tissue than evaluating cytokine levels in the serum or synovial fluid. This study suggested that synovial T cells do not produce significant amounts of their specific cytokines but high level of cytokines are produced by macrophages or type A synoviocytes (IL-1, IL-6, IL-8, TNF-α, M-CSF, GM-CSF, and TGF-β) and fibroblasts or type B synoviocytes (IL-6, GM-CSF, FGF) (Firestein, 1991). Many of these cytokines can have a direct effect on the chondrocyte balance of synthesis over degradation (Poole, 1993). TGFβ, IGF-1 and bFGF have been found to promote matrix synthesis while IL-1α,β, TNFα and the combination of IL-1 with bFGF promotes degradation of matrix (Poole, 1993).

In RA inflammation, IL-1 has been demonstrated to be the most important cytokine involved in cartilage destruction. IL-1 exists in two forms, IL-1α, and IL-1β, with the latter being the predominant form. They are derived from two distinct genes, have the same Mr in their active mature form (17.5 kD), bind to the same high affinity receptors on cell membranes and elicit similar biologic responses (Dinarello, 1989). IL-1 can act as a paracrine/autocrine factor, and could almost be considered as an hormone. Mainly secreted by macrophages, but also secreted by different cell types, it elicits a wide range of biological responses. It is considered as an important mediator of inflammation. *In vivo*, IL-1 elicits systemic inflammatory responses such as fever, the acute phase response of the liver, and local inflammatory reactions such as binding of neutrophils to endothelial cells, induces PGE_2 release by cells, increases production of proteases normally produced during cartilage and bone metabolism, increases growth of fibroblasts and activates B and T cells (Durum and Oppenheim, 1989). The

biologic effects of IL-1 in RA are similar to those of local inflammation produced by IL-1 with the addition of the production of collagenase by synovial fibroblasts (Dayer et al., 1986) and chondrocytes (Evêquoz et al., 1984). Chondrocytes have been shown to be a potential source of IL-6 (Guerne et al., 1990) and secrete IL-8 on stimulation with IL-1 (Lotz et al., 1992). These data confirm the observation made in another study of the level of the different cytokines found in the synovial fluid of RA patients by immunoassays (Firestein, 1991). All of these studies suggest that the synovium is the source of most articular cytokines which can act directly on the chondrocyte.

These observations are somewhat against a role for the T cell in perpetuation of the disease. Many T cells of the helper-inducer subset are found in the synovial membrane, yet they do not apparently synthesize the lymphokines that they usually secrete in inflammation. Questions are raised as to the possibility that some undefined cytokines are secreted in the joint which promote activation and proliferation of the T cells in the joint but at the same time other factors secreted by macrophages, suppress the expression of cytokines by the T cells (Harris, 1990).

TGF-β is found in large amount in synovial fluids and is a potent inhibitor of immune cell function (Lotz et al., 1990). This cytokine appears to be constitutively produced by synovial fluid macrophages and can inhibit the biological effects of IL-1 (Wahl et al., 1990).

IFN-γ appears to downregulate the stimulating effect of other factors such as TNF-α-mediated proliferation, collagenase production, and GM-CSF production (Alvaro-Gracia et al., 1990). The utilization of antagonistic cytokines is of therapeutic interest in RA. But the use of IFN-γ in clinical trials produced disappointing results. This might be due to the inability of IFN-γ to block all possible redundant or alternate cytokines pathways

(Firestein, 1991). Cytokine therapy seems to be more promising with cytokine inhibitors. The IL-1 receptor antagonist protein (Mr. 22 kD) is a molecule produced by cells of the macrophage lineage. It inhibits the action of IL-1 by binding to the IL-1 cell surface receptor (Arend and Dayer, 1990). The gene encoding this molecule has now been cloned (Eisenberg et al., 1990). This recombinant IL-1-receptor-antagonist protein was shown to inhibit IL-1-mediated activation of cultured synoviocytes (Arend et al., 1990). Another potent cytokine inhibitor has been described recently; the inhibitor of TNF-α (Mr. 33 kD) has been found in the urine of fibrile patients (Seckinger et al.,1988). This inhibitor forms a tight complex with TNF-α and perhaps represents a form of soluble high-affinity TNF-α receptor (Seckinger et al., 1990).

4.2.6 EFFECTOR MECHANISMS

While T cells are orchestrating the development of the autoimmune response by communicating with different cells through a cytokine network, some of these cells are actually the final actors in that process of destruction which will lead to net degradation of cartilage over synthesis. Stimulated by the proper cytokines, these cells will secrete enzymes and oxygen radicals that will degrade the matrix components. These cells are predominantly the macrophage, the neutrophil, the synoviocyte type A and B and the chondrocyte itself. Among the cytokines produced in RA synovium, IL-1 and TNF-α are important modulators of metalloproteinase production by synoviocytes. The high levels of these lymphokines may account for the increased levels of collagenase and stromelysin gene expression in RA synovium.

The mechanism of destruction of the articular cartilage in RA is intimately interelated to the effect of the cytokine on chondrocytes (Poole, 1993). Cytokines such as IL-1 with bFGF activate the cells to secrete proteinases and free oxidative radicals from oxygen metabolites, that in turn will act directly on the molecules composing the cartilage matrix to lead to net degradation of matrix (Poole, 1993).

It is principaly the polymorphonuclear leukocytes, but also the monocytes and macrophages that produces oxygen-derived free radicals. Upon activation (phagocytosis or opsonisation), these cells increase their oxygen consumption (respiratory burst) and convert oxygen to the superoxide anion ($\cdot O_2^-$) (Gallin, 1989). Superoxide anion is catalyticaly converted by the action of superoxide dismutase to hydrogen peroxide which in turn interacts with myeloperoxidase (stored in neutrophil azurophil granules) to produce hypochlorous acid, hypochlorite and chlorine (Gallin, 1989). The hydroxyl radical ($\cdot OH$) is produced by the interaction of H_2O_2 with ferrous (Fe^{2+}) and cuprous (Cu^{2+}) ions (Gallin, 1989). $\cdot OH$ is a powerfull one-electron oxidant that can attack a large variety of compounds to turn them into new radicals. These hydroxyl radicals can cleave macromolecules like hyaluronic acid and collagen (Goetzl and Goldstein, 1989) and are capable of modifying amino acid residues on the protein cores of proteoglycan subunits and LP and cleaving the protein core (Roberts et al., 1989). The fragmentation pattern of LP produced by $\cdot OH$ is similar to that seen in adult human cartilage in ageing (Mort et al., 1983; Roberts et al., 1989). Loss of interaction between PG subunits and hyaluronate can result from the action of $\cdot OH$ (Roberts et al., 1989). It is believed that these reactive oxygen metabolites ($\cdot OH$, OCl^-) could account in part for the age-related decrease in size of hyaluronate chains since there is no

evidence for the presence of glycosidases or hyaluronidases in the extracellular matrix (Roughley and White, 1980; Holmes et al., 1988).

The cartilage matrix molecules are also degraded by proteinases which can act inside the cells in the lysosomal compartment after phagocytosis, or can be secreted by the cells and act in the vicinity of the secreting cells. The proteinases involved in cartilage matrix degradation are derived principally from the chondrocytes but also from synoviocytes, neutrophils and macrophages (Harris, 1989). There are four classes of proteinases based on the amino acid involved in the catalytic site: the aspartate and cysteine proteinases, which act principally at acid pH and the serine proteinases and metalloproteinases which are active mainly at neutral pH. Most of the proteinase have now been cloned and sequenced. Not all of their mechanisms of action, biologic and pathologic roles are fully understood. A brief description of the known proteinases that are believed to be important in the pathology of cartilage destruction follows.

Cathepsin D is a major aspartate proteinase found in the lysosomes of most mammalian cells. It can be secreted in the extracellular matrix of rheumatoid synovial tissue explants (Poole et al., 1976; Poole and Mort, 1981). However it is believed that it would be active only in the pericellular environment where a sufficiently acid pH may be found (Barrett, 1978). This enzyme was found to degrade proteoglycan subunits into large proteoglycan fragments (Roughley and Barrett, 1977).

Among the cysteine proteinases of the lysosome, cathepsin B and L are the best known proteinases of this family. These enzymes are associated with inflammation. Cathepsin B was found present in the extracellular environment of human RA synovial tissue (Mort et al., 1984). Cathepsin L has broader specificity and is more potent than cathepsin B in its action.

Cathepsin B is known to be capable of cleaving connective tissue macromolecules such as PG (Morrisson et al., 1973; Roughley and Barrett, 1977), collagen (Burleigh et al., 1974), elastin (Masson et al., 1986), and fibronectin (Isemura et al.,1981). Both proteinases cleave at the N-terminal end of collagen, in the telopeptides that contain the covalent cross links within and between molecules (Kirschke et al., 1982). Both proteinases cleave elastin (Mason et al., 1986).

The serine proteinases are the largest class of mammalian proteinase. Many members of this family appear to be implicated in cartilage degradation. Plasmin and the plasminogen activators and the plasma kallikren are thought to play a role in the destructive process of cartilage erosion, by being involved in the enzymatic cascade that serves to activate procollagenase (Werb, 1989). Human articular chondrocytes are known to produce plasminogen activators. There are two major serine proteinases which are present in the azurophil granules of polymorphonuclear leukocytes: elastase and cathepsin G. These two enzymes may participate in cartilage destruction (Janoff et al., 1976; Sandy et al., 1981). While elastase specifically degrades the highly protease resistant elastin, it can also degrade in a more potent way than cathepsin G the proteoglycan subunit, LP and the telopeptides of collagen type II (Keiser et al., 1976; Starkey et al., 1977).

The metalloproteinases are dependant on Zn^{2+} and Ca^{2+} ions for activity. There are 6 well described metalloproteinases in human tissues: the specific collagenase, stromelysin, stromelysin-2, PUMP-1, and the 72 kD and 92 kD gelatinases. The cDNA sequencing of these enzyme has been performed. All of these enzymes share a high degree of homology. Collagenase will digest collagen of type I, II and III. The digestion occurs in the alpha I chain of the triple helix at one susceptible point (between residues 775 and 776) and

generates characteristic 3/4 and 1/4 fragments (Werb, 1989). This enzyme can also cleave collagen type VIII and type X but not the type IX and XI (Gadher et al., 1988). Collagenase is produce by a vaierty of cells including macrophages , fibroblasts, synovial cells, osteoblasts, chondrocytes and endothelial cells (Werb, 1989).

Stromelysin is the other major metalloproteinase which is secreted by fibroblasts and synovial cells. It possesses 55% sequence homology with collagenase (Whitham et al, 1986). Stromelysin has a wide spectrum of connective tissue and plasma protein substrates. It can degrade cartilage PG, fibronectin, laminin, elastin, gelatin, collagen type IV, type V, type VII, denatured type I, casein, IgG2a, and α1-proteinase inhibitor (Chin et al., 1985; Okada et al., 1986, Wilhelm et al., 1987). It was also found to digest collagen types IX and XI and cleave the telopeptides of type II collagen. It is also an activator of procollagenase (Murphy et al., 1987). Studies of *in situ* catabolism of PG revealed a major cleavage site within the interglobular domain (Sandy et al., 1987; Sandy et al., 1991), which could be accounted for by stromelysin activity (Nguyen et al., 1989).

Stromelysin-2 possesses 78% sequence homology to stromelysin. It is however only found at low level in comparison with stromelysin-1, in human rheumatoid synovial cells (Sirum and Brinckerhoff, 1989).

Collagenase and stromelysin are believe to be involved in the destruction of arthritic cartilage on the basis of their tissue distribution, their subtrate specificity and their localization in the joint. In fact, increased levels in collagen and proteoglycan breakdown has been observed in human osteoarthritic cartilage (Pelletier et al., 1983; Martel-Pelletier et al., 1984) and in synovial tissues (Martel-Pelletier et al., 1986). Collagenase has been detected at the erosive sites of RA joints (Wooley et al., 1977). Prostromelysin has been

also detected in large amounts in rheumatoid synovioblasts (Okada et al., 1989) and was shown to be secreted by human chondrocytes (Nguyen et al., 1989).

Pump-1 has 44 % and 49 % sequence homology to collagenase and stromelysin, respectivelly (Muller et al., 1988). It degrades casein, gelatins of collagen types I, III, IV and V, fibronectin and can activate procollagenase (Quantin et al., 1989).

The two forms of gelatinase (72 and 92 kD) seem to possess the same substrate specificity. They both digest gelatin and collagen types IV, V, VII and XI but they do not cleave fibrillar collagens, laminin and proteoglycan (Murphy et al., 1982; Collier et al., 1988; Wilhelm et al., 1989). The lower Mr form is the predominant species expressed in connective tissue cells while the higher Mr form is predominantly expressed in haemopoietic cells (Murphy et al., 1989).

All active proteinases of all classes can be inhibited by plasma α2-macroglobulin by irreversible trapping of enzyme (Barrett and Starkey, 1973). However this inhibitor may not be relevant in the articular cartilage since none is found in the matrix. A major relevant proteinase inhibitor which has been found in the joint is the tissue inhibitor of metalloproteinases (TIMP). TIMP binds non-covalently to collagenase, stromelysin and gelatinase and inhibits their activity (Cawston et al., 1981; Murphy et al., 1981; Dean and Woesner, 1984). TIMP-2 (Stetler-Stevenson et al., 1989) and other homologous members of the TIMP family have been described (Cawston et al., 1990). These inhibitors are needed for the balance in the turnover of matrix elements by metallo proteinases. This balance is perturb in favor of increased levels of proteinases compared to inhibitors and result in the net loss of cartilage matrix in RA.

4.3 ANIMAL MODELS OF RHEUMATOID ARTHRITIS

Many animal models of rheumatoid arthritis have been developed in the past. The following is a brief description of the most important models and specific features of these models that have brought about an improved understanding of the mechanism of this disease. Several experimental models of RA present clinical and histopathological features similar to those seen in human RA. These animal models can be induced by a variety of agents in the susceptible strains. These includes the microbial agents and their components (i.e. mycobacteria, and streptococcal cell walls), the administration of adjuvant with bacterial component (FCA, muramyl dipeptide), or mineral oil alone (pristane), the injection of cartilage components such as collagen type II and aggrecan, the injection into the joints of antigen (methylated-BSA) or spontaneous disease in certain strains of mice (review in Wooley, 1991). Though RA has been studied in different animal species, a large amount of the data have been obtained from studies done in rabbits, rats and mice. However, because of the well understood immunogenetics of rats and mice, these two species have proven the most useful in understanding the immune response that occurs in these animals.

Spontaneous arthritis have been observed in several strains of mice. In 12 month old BZH mice ($H\text{-}2^q$) strain, arthritis appeare in 28 % of mice with a predominance in males (Bouvet et al, 1990). Spontaneous arthritis was also observed in MRL/lpr mice and subcutaneous injection of IL-1β accelerated the onset of arthritis in these mice (Hom et al, 1990). More recently spontaneous arthritis was also observed in the CIA susceptible strain of mice DBA/1. With increasing age, arthritis developed in 80 % of the mice (Nordling et al., 1992). These observations strengthen the concept of the importance of genetic

predisposition and aging in favoring the development of this disease. These mouse models are perhaps the most relevant to the human disease since no intervention such as massive injection of antigen is needed, nor perturbation of the immune system by adjuvant.

An earlier model of RA consisted of injection of antigen such as methylated-BSA into the knee joints of rabbits or rats. This model provided mainly information about the histopathology in relation to the development of the disease (Klasen et al., 1990). Using this model it was found that intra-articular injection of IL-1 and methylated-BSA was sufficient to induce an acute erosive monoarticular arthritis in naive mice (Staite et al., 1990). However using beige mice in this model of arthritis it was found that the disease was more severe in those mice which are deficient in leukocyte proteases indicating the possibility of a secondary role for the mediators of inflammation (Schalkwijk et al., 1990). Other animal models, such as the streptococcal cell wall induced-arthritis in the rat, have also provided information about the T cell requirement for inflammation (Van den Broek et al., 1990). But they also provided information concerning the expression of important metalloproteinases in the synovium as detected by in situ hybridization, which was observed independant of the presence of T cells (Case et al., 1989).

Other models of arthritis have provided some insights into the mechanisms of the disease. Relevant animal models of rheumatoid arthritis are those which involve molecular constituents of the cartilage as autoantigens. These resemble the human disease in term of the establishment of an immune response to autoantigens. These models include the collagen type II-induced arthritis (CIA), the proteoglycan-induced arthritis (PGIA). The adjuvant and pristane-induced arthritis could also be included here because

they may involve cross-reactivity with autoantigen such as PG and hsp's through molecular mimicry (van Eden et al., 1985; van Eden et al., 1987; Thompson et al., 1990).

Adjuvant arthritis (AA) was first introduced by Pearson in 1956, by using FCA, which contains *Mycobacterium tuberculosis* (MT) cell wall fragments (Pearson, 1956). Much later muramyl dipeptide was found to be the arthritogenic component of adjuvant (Kohashi et al., 1977). Later it was found that mineral oil alone, such as pristane, without any bacterial components, could induced arthritis (Chang et al., 1980; Wooley et al., 1989).

Later cross-reactivity to *Mycobacterium tuberculosis* hsp65 and PG and its importance in adjuvant arthritis were demonstrated (van Eden et al., 1985). In the rat models, T cell lines derived from rats with FCA-induced arthritis were shown to transfer the disease or protect against the disease (van Eden et al., 1987). A particular arthritogenic T cell clone (A2b) could recognize a component of normal joint tissue. This T cell clone was in fact shown to proliferate in the presence of several cartilage PG preparations isolated from various species (van Eden et al., 1985). Furthermore, synovial fluid from the knee of a patient with RA or osteoarthritis was found to stimulate this T cell clone (van Eden et al., 1985). This specific T cell clone recognized the amino acid sequence 180-188 of the hsp65. But no particular similarity exist between the human and the mycobacterial hsp65 (van Eden et al., 1988). It was also reported that injection of PG synergized with CFA in the induction of adjuvant arthritis in rats (van Vollenhoven et al., 1988). However the proposed cross-reacting antigen is not well defined, since the PG preparations used for assessing T cell reactivities were not pure. Monoclonal antibodies that cross-react with both human and mycobacterial hsp65 were found to react with material from RA joints whereas normal joints did not (Karlsson-Parra,

et al., 1990). However the monoclonal antibody used then was found to be unreliable as a probe for hsp65 (Sharif et al., 1992). Using another specific monoclonal antibody to mammalian hsp65, it was found to be widely distributed in the synovium of both OA and RA indicating that hsp65 was not disease-specific (Sharif et al., 1992). Immunization with soluble *Mycobacterium tuberculosis* hsp65 cannot induce the disease but rather induces protection against adjuvant arthritis by preimmunisation of animals with the *Mycobacterium tuberculosis* hsp65 (van Eden et al., 1988; Billingham et al., 1990). This protective effect of *Mycobacterium tuberculosis* hsp65 is also observed in other experimental models of arthritis: streptoccocal cell wall induced arthritis (van den Broek et al., 1989), and in pristane induced arthritis (Thompson et al., 1990). These studies suggest that hsp's reactivity may not be the triggering event that leads to arthritis but rather a consequence of arthritis.

Relevance of this model to the human disease has been strengthened by several observations. Cross-reactivity between mycobacteria and cartilage components has been proposed for human RA. This link was suspected with the association of arthritis with tuberculosis where increased incidence of arthritis was observed in cancer patients treated with BCG immunotherapy (van Eden et al., 1987; Torisu et al., 1978). Furthermore anti-mycobacterial hsp65 immunity has been described in human RA (Pope et al., 1991; Res et al., 1988; Res et al., 1990). Also αβ T cells from synovia but not from peripheral blood, of inflammatory arthritis patients, responded to the mycobacterial hsp 65kD protein (Hill Gaston et al., 1989; Hill Gaston et al., 1990). In another report, αβ T lymphocytes of RA patients were shown to have increased reactivity to a fraction of mycobacteria and were also reactive with cartilage (Holoshitz et al., 1986). T-cell reactivity could also be directed to endogenous mammalian hsp. This was investigated by studying the reactivity of

peripheral blood mononuclear cells to human hsp60 in adult RA patient and JRA patients. Only the latter group recognized the human hsp60 kD and showed significant cross-reactivity with the homologous region of mycobacterial hsp65 (De Graeff-Meeder et al., 1991). Several T cell clones from the synovial fluids of RA patients that reacted with mycobacterial components, expressed the γδ TCR and one of the clones reacted to mycobacterial hsp65 (Holoshitz et al., 1989). Whether hsp's and γδ T cells play a role in RA remain to be proven.

Cartilage type II collagen induced arthritis (CIA) in rats and mice represent models of arthritis which have been the most widely studied. CIA was first observed in 1977 by Trentham and coworker (Trentham et al., 1977) in the rat. It was also demonstrated in mice by Courtenay et al., 1980. The arthritis was found to be transferable with spleen and lymph node cells from the type II collagen immunized rats (Trentham et al., 1978). This CIA has also been demonstrated in primates (Cathcart et al., 1986). The immunogenetics of the CIA model in the rat and the mouse have been extensively studied. In the rat, the susceptibility to CIA is linked to strains carrying the MHC haplotypes RTI^{l}, RTI^{a} and RTI^{u}. All of these strains have a high incidence of arthritis when immunized with heterologous type II collagen. In mice susceptibility to arthritis induced with heterologous type II collagen is associated with $H\text{-}2^{q}$ and the $H\text{-}2^{r}$ MHC class II haplotypes: it was mapped by using recombinant strains, to the I-A region (Wooley et al., 1981). A parrallel has been drawn between the human RA association with HLA-DR MHC class II molecules and the MHC molecules in both the rat and mouse models. This was demonstrated in the diabetes-resistant BB rats of which 100% of the animals get arthritis when injected with human type II collagen. There was complete sequence homology between position 69 and 79 of the third

hypervariable region of the RTI.D β chains in the rat strain, with the sequence for the human HLA.DRw10, and close homology with DR1-Dw1, DR4-Dw4, DR4-Dw14 and DR4-Dw15 (Watson et al., 1990), which all encode for the RA susceptible sequence (Todd et al., 1988). Examination of the sequence homology between the B.10.G ($H\text{-}2^q$) susceptible and resistant congenic B.10.P ($H\text{-}2^p$) mice has revealed no differences in the α chain sequences. Differences were only observed at position 85 to 89 in the β chain (Gustafsson et al., 1990). Similar sequence homology was observed in the same region of the $H\text{-}2^r$ haplotype of susceptible strains where only the difference of one amino acid substitution at position 86 on the β chain could explain the resistance of these mice to the arthritis induced by chick collagen (Gustafsson et al., 1990).

The Mls genes have also an important role in the susceptibility to CIA. The Mls product acts as a superantigen. During ontogeny of the T cells in the thymus, T cells that bear Vβ TCR which recognize the MLs antigen with MHC class II are deleted (Herman et al., 1991). Susceptibility to disease in appropriate MHC strains, may result if self-reactive T cells are not deleted during T cells ontogeny because of the absence of endogenous-superantigens, such as Mls antigens. Deletion of the Vβ6, Vβ7, Vβ8.1 and Vβ9 T cell clones occurs in the thymus when T cells recognize the MLs antigens in the context of MHC class II I-E molecules (Kappler et al., 1988). However in $H\text{-}2^q$ mice, I-E molecules are absent and therefore the Vβ8.1 and Vβ6 T cell clones are not deleted in these mice (Banerjee et al., 1988). The importance of Mls genes was also confirmed in experiments where $Mls1^a$ and $I\text{-}E^+$ F1 hybrid mice (which delete Vβ6, Vβ7, Vβ8.1 and Vβ9 T cell clones) were compared with $MLs1^b$ and $I\text{-}E^+$ F1 hybrids (which express Vβ6, Vβ7, Vβ8.1 and Vβ9 T cell clones), for the development of arthritis . The latter group developed arthritis like the susceptible parent strain (B.10.Q) while the former group of mice exhibited a

lower incidence and severity of disease (Andersson et al., 1991; Wooley. 1991; David, 1990).

The limited usage of TCR Vβ elements in mouse CIA was also investigated using SWR mice. The SWR mouse strain can not develop the disease although it possesses the right H-2^q haplotype for disease induction. However this strain has a large deletion of the TCR Vβ gene which includes the Vβ8 and Vβ6 TCR elements (Behlke et al., 1986). It also lacks the complement factor C5 (Watson and Townes 1985). The resistance of this strain of mouse to CIA might therefore be due to either the lack of proper TCR usage and/or C5 deficiency. To test if the resistance of SWR mice to CIA was due to the absence of T cells reactive to the arthritogenic determinants on collagen type II, crosses between the SWR (H-2^q) and B10 (H-2^b) mice were made and compared with crosses between SWR and C57L mice (H-2^b with mutant Vβ TCR genes like SWR) (Banerjee et al., 1988; 1989). In B10 crosses. the strains with the H-2^q haplotype develop disease whereas the C57L crosses were resistant. C5 deficiency was not found to be crucial for the development of the disease but only delayed its onset as shown by induction of arthritis in crosses between SWR and A/J mice (Banerjee et al., 1989).

Anti-CD4 pretreatments abolished the induction of the disease while anti-CD4 therapy did not change the course of the disease (Ranges et al., 1985; Goldschmidt et al., 1992). There was the possibility that CD4 cells only play a role in the initiation of the disease but not the subsequent arthritogenic events. But it was shown that in this case the resistance to anti-CD4 therapy was rather due to the fact that activated CD4 T cells were found to be refractory to the treatment since no complete depletion was achieved (Goldschmidt et al., 1992). In contrast to the anti-CD4 therapy, the use of an anti-TCR monoclonal antibody was able to achieve prevention of arthritis when the

treatment started before the onset of the disease but not after the disease was already established (Goldschmidt and Holmdahl, 1991; Yoshino et al., 1991). This suppression of arthritis was achieved without reduction of anti-type II collagen autoantibody levels (Goldschmidt and Holmdahl, 1991). This therefore suggested that T cells were directly involved in the initial events as well as in the maintenance of disease. However it was suggested in another study also using anti α/β TCR monoclonal antibody depletion treatment in both AA and CIA in the rat, that α/β T cells have a central role in AA but not in CIA (Yoshino and Cleland, 1992). This conclusion was based on the observation that antibody treatment, started when arthritis reached a peak, markedly depleted the α/β T cells in both models but only markedly suppressed AA but not CIA (Yoshino and Cleland, 1992).

The restricted employment of TCR in CIA of the DBA/1 (H-2^q) susceptible strain was also investigated by in vivo depletion of the T cells bearing the specific TCR family, by using cytotoxic monoclonal antibodies to these TCR (Goldschmidt et al., 1990; Chiocchia et al., 1991). The addition of mouse anti-rat kappa antibodies enhanced the depletion of these T cells (Vβ8.1 and Vβ6) in vivo (Goldschmidt et al., 1988) when the mice were thymectomized to prevent development of mature T cells. These animals were immunized with rat type II collagen and assessed for the development of CIA. No differences in incidence and severity were observed between these groups and control groups. The Vβ8.1 and Vβ6 populations were alowed to reconstitute T cells after termination of the therapy. Arthritis then developed. Thus this treatment simply resulted in delayed onset of disease. It was suggested that activated T cells were more resistant to monoclonal antibody therapy.

The proof of restricted T cell clonal autoreactivity in CIA remains to be confirmed but the idea of limited TCR restriction usage is still valid in autoimmunity as demonstrated in immunotherapy with anti-Vβ8 TCR monoclonal antibodies in the EAE mouse model (Acha-Orbea et al., 1988). In mice developing EAE it was found that T cells that were specific for the N-terminal MBP fragment p1-9 were all restricted to the H-2^u haplotype, and all used the Vβ8 TCR (Zamvil et al., 1988). This anti-Vβ8 antibody treatment was able to prevent disease in these mice (Acha-Orbea et al., 1988).

4.4 IMMUNITY TO AGGRECAN IN HUMANS AND ANIMALS

Cellular immunity to PG, demonstrated by proliferative and lymphokine assays, has been shown using peripheral blood of patients with RA. Humoral immunity to PG in RA patients was also shown in one study but remains unconfirmed (Glant et al., 1980). Cellular immunity has also been observed with an incidence ranging from 14 to 59 %, in peripheral blood cells of patients with JRA (Golds et al., 1983a). and less commonly in patients with osteoarthritis (Golds et al., 1983a; Golds et al., 1984). Cellular reactivity to PG has been shown also in the inflammed synovium of patients with R.A. (Golds et al., 1983b). There have also been reports of cellular immune responses to PG in patients with relapsing polychondritis (Herman, and Dennis., 1973; Rajapakse and Bywaters, 1974). Cellular immunity to PG is observed in patients with AS (Gold et al., 1983a) whereas cellular immunity to type II collagen could not be found in these patients. This immunity to PG has been confirmed with the isolation of PG-specific T-helper cells lines and clones derived from the peripheral blood of patients with AS (Mikecz et al.,

1988). These T cells were highly specific for human PG and did not react with bovine or rats PG.

Extraarticular manifestations of rheumatic disease, such as scleritis in RA and iritis and aortitis in AS, are often observed (Calin, 1989; Ferry, 1989). In all of these tissues, there are PG epitopes that cross-react with cartilage PG which are defined with antibodies (Poole et al., 1982; Cöster et al., 1986).

Immunity to PG is also observed in animals with arthritis. In rabbits with *Mycobacterium butyricum*-induced inflammatory arthritis, articular cartilage degradation is accompanied by humoral and cellular immunity to rabbit PG (Champion et al., 1981; Champion et al., 1983). This immunity to PG was also demonstrated in rabbits with experimental osteoarthritis (Champion and Poole, 1982). However the humoral immunity in these animals seem to be due to natural antibodies which cross-react with the unsaturated glucuronic acid residues of chondroitin sulfate chains digested with chondroitinase ABC (Poole et al., 1985). Injection of rabbit immunoglobulin produces arthritis in rabbits which is associated with the development of humoral immunity to cartilage PG (Yoo et al., 1987). Intravenous injection of cartilage PG into rabbits can cause depletion of the cartilage PG (Kresina et al., 1988).

Recently, immunity to PG in the mice has been shown to produce joint and spinal lesions resembling those found in RA and AS, respectively. These are produced by repeated injection of human fetal PG in BALB/c mice (Glant et al., 1987). Both humoral and cellular immunity to PG are involved. This model is described below.

4.5 AGGRECAN-INDUCED ARTHRITIS MODEL IN THE MOUSE

PG-induced arthritis in the BALB/c mouse is the most recently described animal model of arthritis. The disease is induced by immunizing BALB/c mice with PG of human fetal origin. It usually takes 4 i.p. injections of 50-100 μg of PG depleted of the CS chains. Incidence is maximal in female mice. The presence of FCA in the first injection increases the incidence and severity of the disease. It is however not necessary to use FCA since injection in IFA also causes the disease. The pathological features resemble those seen in the human RA and AS. The clinical signs consists of initial redness and swelling of the paws. Then, as more limb joints become involved, a progressive chronic polyarthritis with fibrous ankylosis of the affected joints follows. Eventually, as disease progresses, gross joint deformities and stiffness develop in involved joints. In the early phase of the disease, the involved joints shows synovial hyperplasia and infiltration of the synovium with mononuclear cells and polymorphonuclear leukocytes. Polymorphonuclear leukocytes, mononuclear cells and fluid accumulate in the joint space. The synovitis is associated with increase vascularization of perisynovial and periarticular tissues with mononuclear cells infiltration concentrated in the perivascular area. Cartilage and bone erosion under the villous pannus occurs at this stage. Rear and front paws are both involved. A unique feature of the PG-induced arthritis is the involvement of the lumbar spine and the proximal caudal intervertebral discs of the tail: inflammatory and degenerative changes are observed. The inflammation is usually characterized by peripheral erosion of the intervertebral disc involving mononuclear cell infiltration. This leads to resorption of the intervertebral disc (Glant et al., 1987).

The involvement of joints of both the appendicular and axial skeleton is thought to involve an autoimmune response to the cartilage mouse PG (MPG), which possesses cross-reactive arthritogenic T and B cells epitopes(s) with HFPG. In this model the use of HFPG may be important in order to break the immunotolerance toward MPG. It is also important to mention that the cartilage is an immunopriviledged tissue since it is not vasculirized and not easily accessible to the immune system. Not all sources of PG can induce the disease. The highest incidence and severity is obtained with PG from human fetal epiphyseal cartilage while adult PG is less efficient. This difference may be due to age-related differences in epitopes on PG (Glant et al., 1986a). It is known that differences exist between fetal and adult PG as earlier discussed (see Chapter 3). So with age, arthritogenic epitopes may gradually decrease. The disease is also better induced after depletion of the CS chains on the core protein. These GAG chains may somehow prevent accessibility of the core protein-associated arthritogenic epitopes to the immune cells in vivo.

It is known that differences in the potential of PG for induction of the disease exist between PG's isolated from differents species. Beside the human, PG's from pig and dog cartilage are also arthritogenic, while PG from cows, rabbits and lambs are not arthritogenic in BALB/c mice, suggesting again differences in the biochemical composition of the different PG's which produce differences in immune responses (Glant et al., 1991).

Genetic predisposition to disease induction is an important consideration in mice. The disease was initially found in BALB/c mice and it occurs preferentialy in females (Mikecz et al., 1987). This may be due to hormonal influences. Other strains of mice with the same or diferent different haplotypes have been immunized in the same manner (Mikecz et

al., 1987). Only BALB/c (H-2^d) mice and to a lesser extent the NZB (H-2^d) strains developed disease (Mikecz et al., 1987). This indicated that susceptibility to the disease is regulated by a fine genetic restriction. In later studies it was shown that B10.D2 (H-2^d) mice could also develop disease but to a lesser degree than the BALB/c mice (Banerjee et al., 1992b). The background of this mouse is well enough understood so that a careful study can be made of the influence of the MHC genes by using the mutiple congenic strains available. Arthritis only developes in B10.D2/n (C5+) mice and not in the B10.D2/o (C5-) mice (Banerjee et al.,1992b). These congenic strains only differ in that the latter strain is deficient in C5. This result suggests that C5 is important for the development of the disease. Since other strains of mice having the same haplotypes (H-2^d), did not develop the disease, such as the DBA2 strain, other genes than those of the MHC region may have importance in the susceptibility to the disease. Using the BALB background and mice which only differ between each other in the H-2 haplotype, it was shown that BALB.K (H-2^k) were also susceptible to the disease but not BALB.B (H-2^b), showing the influence of MHC genes in the susceptibility of this disease (Banerjee et al., 1992b). The fact that B10.D2 (H-2^d) mice get the disease is further evidence that MHC genes are essential. However one important known difference between the three BALB strains is that BALB/c and BALB.K express I-E molecules while BALB.B does not (Mathis et al., 1983). Hence I-E molecules may prove to be important in the presentation of the arthritogenic epitope in these mice. The resistance among mice carying the same MHC haplotype, including (BALB/c X DBA/2) F1 hybrid mice, was proposed to be due in part to the clonal deletion of specific Vβ TCR bearing T cells by the endogenous superantigen Mls-1a (Banerjee et al., 1992b).

Both cellular and humoral immune responses to mouse PG accompany the development of PG-induced arthritis in BALB/c mice (Mikecz et al, 1987). The disease can be adoptively transfered by spleen and lymph node cells from arthritic mice to naive irradiated mice (Mikecz et al., 1990). The onset of the disease is more rapid with the transfer of the autoimmune cells (secondary transfer) than with the disease induced by PG-immunization. This onset is even faster when cells from spleen and lymph nodes of mice with a secondary arthritis are passively transfered into a third group of naive irradiated mice (tertiary transfer) (Mikecz et al., 1990). The presence of anti-proteoglycan antibodies and IL-2 during transfer enhanced the disease activation. It was shown by deletion of T cells that these cells played a pivotal role in the adoptively transferred disease. Transfer of disease requires both $CD4^+$ and $CD8^+$ T cells as well as B cells and macrophages (Mikecz et al., 1990). This was shown by *in vitro* complement sufficient antibody depletion of each cell type from the spleen cells of donor mice with primary arthritis. The requirement for Ia^+ cells was shown. Humoral immunity is also essential but not sufficient by itself to produce the disease. Antibodies that are cross-reactive to both human and mouse PG have been detected in the inflamed periarticular tissues. These antibodies were found to be cytotoxic to mouse chondrocytes (Mikecz et al., 1987). Some of these autoantibodies are capable of eliciting a loss of PG from articular cartilage *in vivo* (Dayer et al., 1990).

In vivo depletion studies with cytotoxic monoclonal antibodies specific to the $CD4^+$ and the $CD8^+$ T cell subsets have shown that mice depleted of $CD4^+$ cells resulted in inhibition of disease development and a decrease in anti-PG antibody response, while $CD8^+$ depletion enhanced the severity of the disease (Banerjee et al., 1992a). This study demonstrated the importance of

the $CD4^+$ T cells in the induction of the disease and suggested an immunoregulatory role for the $CD8^+$ T cell subsets.

PART II EXPERIMENTAL WORK

AIMS OF THE THESIS

The objectives of the present study were determined by the results of earlier experiments dealing with PG induced arthritis and ankylosing spondylitis in the BALB/c mouse. This initial study was performed by Katalin Mikecz and Tibor Glant in Robin Pooles, laboratory. They showed that T cell immunity plays a pivotal role in the onset and the course of this experimental disease. The involvement of specific PG-reactive T cell mediated immunity in this disease remains to be established.

On the basis of information obtained in these earlier studies of this model of autoimmunity, the specific objectives of this thesis were:

1) Isolation of PG-reactive T cells from arthritic mice and the characterization of their reactivities.

2) Establishment of PG-reactive T cell lines and T cell hybridoma clones.

3) Characterization of representative T cell lines and T cell hybridoma clones for their PG-reactivities and phenotypic profiles.

4) Determination of the influence of carbohydrate structure on the T cell reactivity.

5) Determination of T cell epitope(s) recognized by some of these T cells

6) Determination of the potential of these T cells to produce pathology .

The ultimate goal is to define arthritogenic T cell epitope(s) on the PG molecule.

CHAPTER V : MATERIALS AND METHODS

5.1 MATERIALS AND REAGENTS

The following reagents were used: guanidine hydrochloride (GuHCl) and iodoacetamide (BDH Chemicals, Toronto, ON); cesium chloride (CsCl) (Terochem Chemicals, Edmonton, AT); casein, diisopropyl fluorophosphate (DFP), PMSF, pepstatin A, EDTA, endoproteinase-Lys-C, endoproteinase-Asp-N, cyanogen bromide (CNBr), O-iodobenzoic acid, hyaluronic acid, 2-mercaptoethanol, LPS (E. Coli 055:B5) (Sigma Chemical Co, St-Louis,MO); chymotrypsin, endoproteinase-Arg-C and Glu-C (V8 proteinase) immobilized enzymes (MoBiTec, BIO/CAN, Mississauga, ON); TPCK-trypsin (Fluka Biochemical, Ronkonkona, NY); N-glycanase and O-glycanase (Genzyme, Boston, MA); neuraminidase (Calbiochem Corporation, San Diego, CA); chondroitinase ABC (protease free), hyaluronidase, keratanase I (Pseudomonas sp), keratanase II and keratan sulfate (bovine corneal) (Seikagaku America, Rockville, MD); CFA and IFA (Difco Laboratories, Detroit, MI); ^{3}H-thymidine (6.7 Ci/mmol) (ICN Biomedical Canada Ltd., Montréal, Qué.); Lympholyte M (Cedarlane Laboratories, Hornby, ON); Aurodye, sodium iodide (^{125}I) and iodinated protein A (Amersham, Oakville, ON); paraformaldehyde, and SDS (BDH, Montréal Qué.); CAPS (cyclohexylaminopropane sulfonic acid); (United States Biochemical Corporation, Cleveland, Ohio); polyvinyl difluoride (PVDF) membrane, nitrocellulose membrane, acrylamide, bis-acrylamide, and TEMED (Bio-Rad Richmond, MD); Sephadex G-25, Sephacryl S-200, Sepharose CL-2B and Sepharose CL-4B (Pharmacia Kirkland, Qué.); Spectra/por dialysis membrane (Spectrum Medical Industries inc., Los Angeles,CA).

Culture media and fetal calf serum were obtained from Flow Laboratories (McLean,VA). Normal mouse serum was obtained from

Bioproducts (Indianapolis,IN). Culture supplements were from GIBCO Laboratories (Burlington,ON).

5.2 MICE

Female 8-12 week old BALB/cAnNCrlBR, DBA/2NCrlBR, AKR/NCrlBR, and C3H/HeNCrlBR mice were obtained from Charles River, Canada (St-Constant, Québec). Female B10.D2/nSn, B10.BR/SgSn, B10.RIII(71NS)/Sn, DBA/1LaCJ, and C57BL/6J mice (all 8-12 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). Female B10.RDD mice were obtained from Dr. Chella David, Mayo Clinic, Rochester, MN.

5.3 ANTIBODIES

5.3.1 SOURCES OF ANTIBODIES

Monoclonal rat IgG_1 anti-mouse IL-2 antibody (CRIL-2) was obtained from Collaborative Research, Bedford, MA. The following monoclonal antibody secreting hybridomas were obtained from ATCC (Rockville, MD): hamster IgG anti mouse αβ TCR (H57-597); rat IgG_1 anti-mouse IL-4 (11B11); rat IgG2a anti-IL-2 (S4B6): rat IgG2b anti-CD4 (GK1.5), anti-CD8 (2.43), anti-I-A^d+I-E^d (M5/114.15.2) and anti-HLA-B6 (SFR8.B6) antibodies; and mouse IgG2a anti-I-A^d (MKD6), and anti-I-E^d(14-4-4S). SFR8.B6 was used as a control antibody for the rat IgG2b isotype since it does not react with mouse lymphocytes. Ascities of mouse IgG1 anti-HABR (1C6) (Stevens et al., 1984) was from Developmental Studies Hybridoma Bank (Univ. Iowa, Iowa City, IA, USA). Rat and mouse anti-Vβ8 (KJ16,F23.1, and F23.2), rat IgG2a anti-Vβ2 (B20.6), anti-Vβ4 (RT4-10), rat IgG2b anti-Vβ6 (44-22-1), rat IgM anti-Vβ14 (14.2) and mouse IgG anti-Vβ9 (MR10-2) TCR antibodies were kindly donated

by Dr. Chella David (Mayo Clinic, Rochester, MN). FITC conjugated $F(ab')_2$ fragments of mouse anti-rat IgG, and FITC conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Fc specific) were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Mouse IgG2a myeloma protein used as an isotype control was obtained from Sigma Chemicals (St-Louis, MO). Anti- KS antibody AN9P1 (IgG2a) (Poole et al., 1989) was available in this laboratory and 5D4 (IgG1) (Caterson et al., 1983) from ICN (Montréal, Qué.).

5.3.2 PREPARATION OF ANTIBODIES

The following antibodies 1C6, 5D4 and AN9P1, used as ascites, were clarified by centrifugation on a benchtop Eppendorff centrifuge (12 000 g, 15 mins) and diluted with PBS-3%BSA. The antibodies obtained by secreting hybridomas were cultured in T75 (75 mm^2) flasks (Falcon Laboratory,) until confluency. The cells were sedimented and the antibodies contained in the medium were immunoaffinity purified. The rat antibodies were affinity purified on a goat anti-rat Ig agarose column (Sigma Chemicals, St-Louis, MO) and the mouse antibodies were purified on a protein-A Sepharose column (Pharmacia Chemicals, Uppsala, Sweden).

5.4 PREPARATION OF ANTIGENS

5.4.1 SOURCES OF THE PROTEOGLYCAN AGGRECAN

PG preparations were isolated as previously described (Roughley and White, 1980) from condylar cartilage of 12-20 week old human fetuses (for HFPG), cartilage from the femoral condyle of a 35 year old man (for HAPG), condylar cartilage from the metacarpal-phalangeal joint and nasal cartilage of 24-32 week old bovine fetuses (for BFPG), and the Swarm rat chondrosarcoma

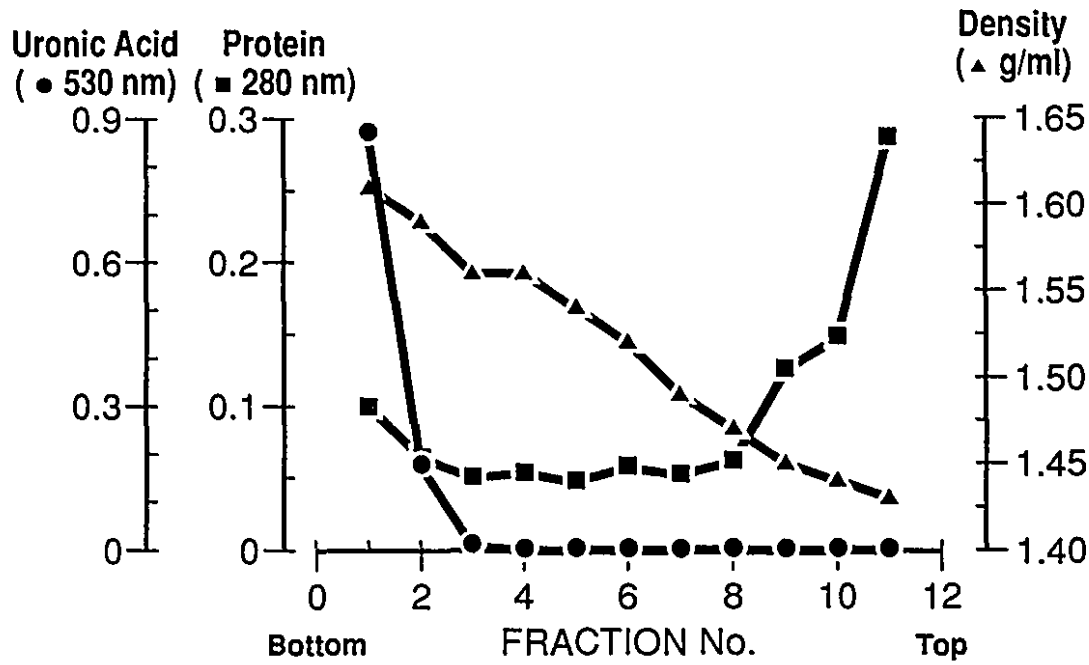

FIGURE 5. Purification of PG Monomer from a Fetal Cartilage Extract by Ultracentrifugation on a CsCl Density Gradient Under Dissociative Conditions

Three parameters were monitored for each fraction: protein, uronic acid and the density.

(RCSPG). In the case of the RCSPG the tumor cells were passaged in Lewis rats (Charles River, St.-Constant, Qué.) by s.c. injection in the dorsal region of 10-50 x 10^6 cells in saline. PG was extracted from the resulting tumor after it was excized on reaching a size of about 3 cm diameter in about 2-3 weeks.

5.4.2 EXTRACTION AND PURIFICATION OF AGGRECAN

5.4.2.1 CENTRIFUGATION UNDER DISSOCIATIVE CONDITIONS

Ten grams of 1-2 mm^3 pieces of freshly chopped cartilages were extracted in 100 ml of 100mM Tris/acetate buffer, pH 6.8 with 4 M GuHCl for 48 h at 4 ^oC in the presence of proteinase inhibitors (PMSF and iodoacetamide at 1mM, pepstatin A at 1 µg/ml and 10mM EDTA). The PG extracts were filtered through glass wool and ultracentrifuged at 100 000g for 48 h on a CsCl density gradient (starting density of 1.5 g/ml) in Beckman 25 x 89 mm Quick-sealed polyallomer tubes (Beckman Inst. Co., Palo Alto, CA) with a total volume of 36 ml using a Beckman TI-70 rotor head and a Beckman L8-70M Ultracentrifuge. The high buoyant density proteoglycan fraction D1 (consisting of the bottom 2-3 fractions out of 12 fractions with densities of more than 1.6 g/ml) was isolated. The presence of the native PG monomer was confirmed by the analysis of each fraction for uronic acid content , and protein content (Figure 5). The density of each fraction was determined by weighing 1 ml volumes. The D1 fractions were pooled and extensively dialyzed over 96 h at 4 ^oC against deionized water (9 changes with the third being in potassium acetate 100 mM, pH 6.0): they were then lyophilized. Native PG preparations were dissolved in sterile PBS, sterilized by UV irradiation in a laminar flow hood (1-2 hrs) or filtered with a 0.22 µm nylon membrane filter and assayed for protein concentration. All concentrations of

antigens were expressed as nM protein. This takes into account the molecular weight difference between PG and smaller molecules such as bovine link protein (LP) and the G1 domain. The molecular sizes of the protein cores of these three molecules were assigned as follows: PG 200 kD, LP 48 kD, G1 60 kD. The *in vitro* concentrations of these molecules in assays of comparative T cell reactivity were thus expressed on an equivalent molar basis of protein content.

5.4.2.2 EXTRACTION AND PURIFICATION OF MOUSE PROTEOGLYCAN

Mouse proteoglycan was extracted in the same manner except for the following modifications to minimize proteolytic degradation (Rostand et al., 1982). Articular epiphyseal cartilages dissected immediately after death from 2 day old BALB/c mice were frozen on dry ice and then stored at -70 ^{o}C. Cartilages were frozen-sectioned at 20 μm with a Tissue-Tek II cryostat (Miles Co, Elkhart, IN) and extracted for 8 hrs as above in a salt/ice bath at -10 ^{o}C (Rostand et al., 1982).

5.4.2.3 CENTRIFUGATION UNDER ASSOCIATIVE CONDITIONS.

PG's were extracted as above in 4 M GuHCL in the presence of enzyme inhibitors for 48 h and then isolated as aggregates under associative conditions (Roughley et al., 1982). The extract was filtered through glass wool and exogenous HA added (0.5 mg HA/g of cartilage), stirred slowly at room temperature for 1 h and dialysed against 50 volume of 100mM potassium acetate (pH 6.0) for 20 h at 4 ^{o}C. Cesium chloride was added to a starting density of 1.65 g/ml: centrifugation was as above. After 48 h the tubes were fractionated into 12 fractions and assayed for protein, uronic acid and density as above. These parameters gave profiles similar to the dissociative gradient. The bottom fraction (A1) contained the PG monomer bond to link protein

and HA. After dialysis and lyophilisation the preparations were dissolved in PBS, filter sterilized, and protein concentration estimated as above.

5.4.3 GENERAL CHEMICAL ASSAYS

5.4.3.1 URONIC ACID

This was determined by the carbazole assay (Bitter and Muir, 1962). Samples (500µl) were mixed with 3 ml of sulfuric acid and digested at 100 °C for 10 mins, cooled in an ice bath and 100 µl of carbazole reagent were added, mixed and incubated at 100 °C for 15 mins. Tubes were cooled down and read at 530 nm.

5.4.3.2 PROTEIN

Protein content was determined by measuring absorption in a 1 cm light path cuvette at 280 nm, or by the Lowry reaction (Lowry et al., 1951) or Bradford assay (Bio-Rad protein determination method) (Bradford, 1976).

5.4.4 GLYCOSAMINOGLYCAN DIGESTION

5.4.4.1 CHONDROITINASE ABC

CS chains were removed from aggrecan by digestion with chondroitinase ABC (0.01 unit/mg PG) (Glant et al., 1986b). Lyophilized PG monomer or bovine G1 were dissolved at 10mg (dry weight)/ml and 250 µg/ml respectively in 200 mM tris-acetate buffer, pH 7.3, containing the proteinase inhibitors iodoacetamide, pepstatin A, PMSF or DFP and EDTA (all at final concentrations of 1mM except DFP at 2 mM), to which was added chondroitinase ABC (protease free from *Proteus vulgaris*). After incubation at 37 °C for 24 h the enzyme was inactivated by boiling for 5 mins. The free oligosaccharides were removed either by dialysis or by chromatography on

Sephacryl S-200 in 4 M GuHCl (Glant et al., 1987) followed by extensive dialysis against a 100mM potassium acetate (pH 6.0) followed by water. The CS-depleted PG preparations were usually concentrated, sterile filtered, protein content determined and stored aliquoted at -20 °C. Otherwise they were lyophilized and stored dessicated at 4 °C. Where necessary these preparations were dissolved in PBS, filter sterilized (0.22 μm) and protein contents were determined before use.

5.4.4.2 KERATANASES

PG and bovine G1 were dissolved at 10mg/ml and 250 μg/ml respectively in 0.2 M Tris-HCl buffer, pH 7.4, with 80mM NaCl, containing the following protease inhibitors: 1mM EDTA, 1mM iodoacetamide, 5 μg/ml of pepstatin A and 2 mM DFP or 1 mM PMSF. The mixture was digested with keratanase I (*Pseudomonas sp.*) (1 unit/mg/ml) or where indicated, with keratanase II (*Bacillus sp.*) at 37 °C for 20 h . The reaction was terminated by boiling the mixture for 5 mins. The digested samples were dialyzed against water, lyophilized on a Speed vac model SVC-100H (Savant Instrument Inc., Farmingdale, NY) and redissolved in sterile PBS at desired concentrations.

5.4.4.3 HYALURONIDASE

This enzyme digests both condroitin sulfate and hyaluronic acid (HA). Digestion of PG leaves a saturated oligosaccharide at the non-reducing end of the chondroitin sulfate stub compared to an unsaturated non-reduced product with chondroitinase ABC. The digestion with hyaluronidase was performed by dissolving 10 mg (dry weight)/ml PG in 100mM sodium acetate, pH 5.0, 150 mM NaCl in the presence of proteinase inhibitors as above at 1 mM and 240 U/ml of bovine testicular hyaluronidase. After incubation at 37 °C for 24 h,

the digestion was stopped by boiling for 5 mins. The digest was dialysed against water as for the other glycosidase digestion protocols and stored either lyophilized or sterile in PBS at -20 °C. In most instances, the inactivated enzyme remained in the antigen preparations. Controls for T cell assays included wells with inactivated enzyme and no antigen. In some earlier experiments with HFPG preparations, the enzymes were removed by Sephacryl S-200 gel filtration column in the presence of 4 M GuHCl.

5.4.5 G1 DOMAIN OF BOVINE PG

The purified bovine G1 domain was a generous gift from Dr. L. C. Rosenberg (Orthopaedic Research Laboratory, Montefiore Hospital and Medical Center, Bronx, N.Y.). It was prepared from PG aggregate (PG fraction A1A1) as previously described for calf articular PG (Tang et al., 1979). The PG fraction A1A1 from an associative density gradient was dialysed directly without lyophilization against 0.15 M NaCl, 50 mM Tris, pH 7.5, and diluted to 500 ml with the same buffer. Sodium azide (0.02%) was then added. TPCK-trypsin (5mg in 5ml of 0.15 M NaCl, 30 mM $CaCl_2$, 50 mM Tris, pH 7.5) was added and the A1A1 was digested for 6 h at 25 °C. An additional 5 mg of TPCK-trypsin was added and the A1A1 was digested for another 16 h at 25 °C. TLCK (29.5 mg) was added to terminate the trypsin digestion. The solution was chilled to 0 °C and adjusted to pH 7. EDTA (5mM) and CsCl (3.5M) were added, while the pH was maintained at pH 7. Equilibrium density gradient centrifugation was carried out at 5 °C for 60 h at 100,000 g. The gradient was divided into six equal fractions. Aliquots of each fraction were taken for SDS-PAGE and analyzed for protein, uronate and hexose. Of the six fractions, the two fractions of highest buoyant density (fractions 1 and 2, at the bottom of the gradient) contained chondroitin sulfate-peptides but no LP, nor G1. The

fractions of intermediate buoyant density (fractions 3, 4 and 5, from the middle of the gradient) contained LP, G1 and HA. The fraction of lowest buoyant density (fraction 6, from the top of the gradient) contained low molecular weight peptides, and was not studied further. The fractions of intermediate buoyant density from the middle of the gradient were used for the preparation of G1.

The complex consisting of G1 and LP non-covalently bound to HA was isolated by gel chromatography under associative conditions on Sepharose CL-4B in 0.15 M NaCl, 5 mM EDTA, 50 mM Tris, pH 7 as follows. The pooled fractions from the middle of the CsCl density gradient were concentrated on an Amicon YM-2 filter and dialyzed against 0.15 M NaCl, 5 mM EDTA, 50 mM Tris, pH 7. The solution was divided into equal aliquots, each of which was applied to a Sepharose CL-4B column (3.7 x 228 cm 2500 ml) equilibrated with the same solvent. Fractions were eluted at $4^{o}C$ at a flow rate of 55 ml/h, monitored for protein , uronate and hexose, and analyzed by SDS-PAGE. Fractions containing the LP-G1-HA complex, which eluted in the void volume, were pooled for further use. Fractions containing the complex were concentrated and dialysed against 4 M GuHCl, 0.15 M sodium acetate, 5 mM EDTA, pH 6.3. The solution was applied to a Sephacryl S-300 column (3.7 x 228 cm 2500 ml) equilibrated in the same solvent. Fractions collected at a flow rate of 30 ml/h were monitored for protein, uronate and hexose, and by SDS-PAGE. Fractions were recycled on Sephacryl S-300 in 4M GuHCl. Relatively monodisperse G1 containing fragments separated from LP and HA, were recovered: their molecular weights were determined by SDS-PAGE. The G1 containing fragment used in these studies had an apparent Mr of 71.5 kD on SDS-PAGE. This fraction was dialyzed extensively against water, filtered and used after further dilution in culture medium.

5.4.6 LINK PROTEIN

LP was also generously donated by Dr. L. C. Rosenberg. It was extracted from fetal bovine epiphyseal cartilage and purified as described earlier (Tang et al., 1979). The purification procedure consisted of the following: formation of an aggregate from cartilage extract by dialysis against 20 volume of 0.15 M sodium acetate for 16 h in the presence of proteinase inhibitors, and ultracentrifugation of an associative density gradient (starting density 1.5 g/ml). This was followed by dissociative density gradient centrifugation of fraction A1 (starting density 1.5 g/ml). A second dissociative density gradient centrifugation of fractions D5 and D6 (starting density 1.35 g/ml) was used. Chromatography of the second D5 and D6 fractions on Sephacryl S-200 in 4 M GuHCl was folowed by extensive dialysis against 100 mM Tris/acetate, pH 7.2.

5.4.7 HEAT SHOCK PROTEIN

The recombinant 65 kD mycobacterial heat shock protein (HSP) was generously donated by Dr. Ruurd van der Zee, National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands.

5.4.8 REDUCTION AND ALKYLATION OF PG AND G1

PG (1mg/ml) or G1 (200 μg/ml) was dissolved in 4M GuHCl, 0.1 M Tris-HCl, 1mM EDTA pH 8.5. DTT was added to a final concentration of 10 mM and the mixture incubated at 60 °C for 2 h. The mixture was rapidly cooled, and iodoacetamide was added to a final concentration of 40 mM. It was incubated for 2 h at 21 °C in the dark before being extensively dialysed against 25 mM Tris-acetate, pH 8.2 at 4 °C before use.

5.4.9 CHEMICAL CLEAVAGE OF THE CORE PROTEINS OF PG AND THE G1 GLOBULAR DOMAIN

5.4.9.1 CNBr CLEAVAGE

Lyophilised molecules were dissolved in 70 % formic acid at a concentration of 200-250 µg/ml of protein to which was added 12 mg/ml final concentration of CNBr from a stock solution of CNBr of 480 mg/ml dissolved in acetonitrile (Dodge and Poole, 1989). The tubes were flushed with nitrogen, sealed and incubated at room temperature for 24 h in the dark. The digestion was terminated by diluting the mixture with ten volumes of water. The mixture was dialyzed against water, lyophilised and stored at -20 °C.

5.4.9.2 O-IODOBENZOIC ACID

The CNBr digested bovine G1 (20 µg) was electrophoresed on a 12.5 % SDS-PAGE BIO-RAD minigel under reducing conditions and transfered onto a PVDF membrane. The membrane was stained with aurodye and the stained bands cut into 1mm^2 fragments. These were added to Eppendorf tubes and covered with 50 µl of 80 % acetic acid containing 3 mg/ml of o-iodobenzoic acid, based on the digestion protocol of Mahoney et al. (1981). The mixture was incubated in the dark at room temperature for 24 h and the reaction terminated by adding ten volumes of water. The membrane was then dried in the Speed vac and rinsed with water. This rinsing and drying cycle was repeated three times. The bands treated this way were either used directly in an antigen presentation assay or eluted with SDS-PAGE sample buffer for 90 minutes to be re-electrophoresed and re-blotted. This treatment will cleave peptide bonds at the C-terminal end of tryptophane when used in

combination with the electrophoreticaly purified CNBr fragments, it allows further shortening of peptides containing epitopes recognized by T cells.

5.4.10 ENZYMATIC CLEAVAGE OF THE CORE PROTEINS OF PG AND G1

5.4.10.1 TRYPSIN

The G1 was also digested with 5 µg/ml of TPCK-trypsin (cleaves at the C-terminal ends of arginine and lysine residues) per 250 µg/ml of G1 in 100mM Tris-HCl, 150 mM NaCl, pH 7.4 for 18-20 h at 37 °C.

5.4.10.2 CHYMOTRYPSIN

Immobilized chymotrypsin was used to cleave the G1 molecule at specific aromatic amino acid cleavage sites according to the manufacturer's protocol. One hundred µg of G1 in a 100 µl of 50mM Tris/HCl, pH 7.5 was added to the immobilized enzyme column and incubated for 20 h at room temperature. The cleaved G1 was eluted with the same buffer by centrifugation of column into Eppendorf tubes at 4000 g for 5-10 sec..

5.4.10.3 Glu-C

Immobilized endoproteinase Glu-C (V8 protease) was used to cleave at the C-terminal end of glutamate and aspartate residues under conditions recommended by the manufacturer. These consisted of incubating 10-20 µg of G1 in 100 µl of 50 mM ammonium bicarbonate, pH 7.8 for 3 h at room temperature. The sample was eluted by centrifugation of column into an Eppendorf tube at 4000 g.

5.4.10.4 Arg-C

Immobilized endoproteinase Arg-C was used to cleave at the C-terminal end of arginine under conditions recommended by the

manufacturer. These consisted of incubating the column with 10-20 µg of G1 in 100 µl of 50 mM phosphate buffer pH 8.0, with 1.3 mM glycine for 6-8 h at room temperature. The sample were eluted by centrifugation of columns into Eppendorf tube at 4000 g.

5.4.10.5 Lys-C

The lyophilized G1 was dissolved in 100 mM of ammonium bicarbonate, pH 8.1 and incubated with 2% (w/w) of enzyme at 27°C for 4 h.

5.4.10.6 Asp-N

The G1 was mixed with 5% (w/w) of enzyme in 50 mM phosphate buffer, pH 8.0 and incubated for 4 h at 37 °C.

In all of these incubations the reaction was terminated by boiling for 5 min. and dialyzed against PBS before testing.

5.5 SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The antigen preparations were electrophoresed (constant voltage: 200 V, variable current) for 45 mins in 12.5% polyacrylamide gel or, where indicated, in a continuous 5-15 % polyacrylamide gradient mini-gel (BIO-RAD, Richmond, CA) under non-reducing or reducing condition as described previously (Laemli, 1970).

5.6 IMMUNOBLOTTING

Following electrophoresis, gel contents were electrotransferred (Towbin et al., 1979) (constant current; 300 mA, and 60 V) in CAPS buffer with 10% methanol onto a PVDF membrane for 75 minutes according to the manufacturer's recommendations. The membrane was immunoblotted with one of the following monoclonal mouse antibodies: 1C6 for the G1 domain,

and AN9P1 or 5D4 for KS. Briefly, the PVDF membrane was blocked with 3% BSA-PBS for 1 h and cut into slices in the direction of electrophoresis. Each slice of the PVDF sheet was incubated with one of the different antibodies (diluted 1:1000 from ascites with PBS-3%BSA) for 1 h at room temperature with shaking followed by 3 washes in PBS-0.1% Tween 20 for 5 mins. The second antibody, a rabbit anti-mouse IgG, (diluted 1:1000 with PBS-3%BSA) was then added to the membranes and incubated at room temperature for 1 h followed by 3 washes in PBS-Tween. The specific antibody binding was detected by incubation with iodinated protein A (5×10^4 CPM/ml), washed and autoradiography was performed using Kodak X-Omat-ray film (Eastman Kodak Co., Rochester, NY).

5.7 SEQUENCE ANALYSIS

The purified bovine G1 fragments generated by CNBr digestion, by endo Asp-N or by trypsin digestion, were resolved by SDS-PAGE mini-gel (12.5% gels, 1mm x 10 x7.5 cm) and electroblotted onto PVDF membranes, as described by Matsudaira (1987). The membranes were stained with Coomassie brillant blue R-250 in 50% methanol for about 15 mins and destained in 50% methanol, 10% acetic acid until the bands could be visualized. The bands were cut and loaded on an Applied Biosystem model 470A sequencer attached to a PTH analyser, model 120A. The PTH-amino acid derivatives were separated by reverse phase HPLC using a C18 column (220 x 2.1 mm).

5.8 IDENTIFICATION OF T CELL EPITOPE MOTIFS

The synthesis of peptides containing putative T cell epitopes was based in part on identification of sequence homology between the known sequences of the bovine (Neame et al., 1987) and human G1 domains (Doege et al., 1991)

of PG and the known human LP sequence (Dudhia and Hardingham, 1990). An alignement and homology prediction program, McCAW (Schuler et al., 1991) running on DOS was used to help determine blocks of homology between the sequences. This was particularly helpful in determining the most probable T cell epitopes for the T cells cross-reactive with both G1 and LP.

Prediction of T cell epitopes was also based on a computer program called TSITES which groups the combination of four models that predict patterns in the amino acid sequence of peptides which are likely to be either T cell epitopes or MHC class II binding peptides. Two out of the four models represent score assignment for the most probable MHC class II amino acid sequence motif for IA^d or IE^d. This is based on known sequences of IA^d and IE^d binding motifs (Sette et al., 1989). The prediction of the IA^d motif is based on the OVA(327-332) sequence (VHAAHA) and scores are given for the substitution of the amino acid at each position of the hexamer. From this, a calculation is made and predicted values are obtained. The IE^d motif is defined by a "basic, basic, non-charged, basic" motif (Sette et al., 1989).

The third model predicts T cell epitopes by an algorithm based on the amphipathic helix model in which the amino acid residues of the epitope are postulated to form a helix with one face (toward the TCR) being mainly polar and the other face (toward the MHC) being predominantly apolar (Margalit et al., 1987). This algorithm could predict 75% of the known T cell epitopes in earlier studies (Margalit et al., 1987). The fourth model uses the Rothbard motif which consists of the pattern of a positive residue followed by three hydrophobic amino acids to predict T cell epitopes (Rothbard et al., 1988).

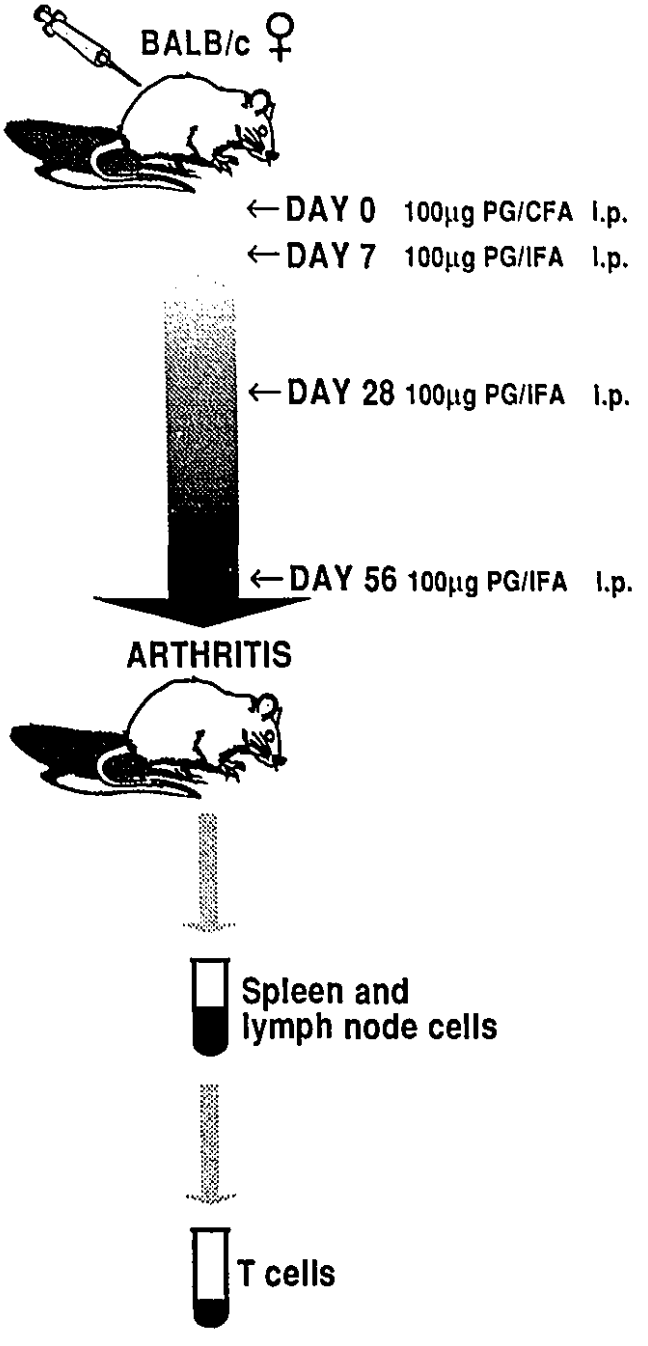

FIGURE 6. Immunization Protocol for the Induction of Arthritis in Female BALB/c Mice with HFPG for the Isolation of PG-Reactive T Cells

5.9 SYNTHETIC PEPTIDES

Peptides to be tested for T cell epitopes were synthesized with an Applied Biosystem model 431A peptide synthesizer using FMOC chemistry. Synthetic peptides were purified on reverse phase HPLC (C18 column, 220x 2.1 mm).

5.10 INDUCTION OF ARTHRITIS BY IMMUNIZATION WITH PG

Arthritis was induced in 8 week old or in retired breeder female BALB/c mice (5-6 months) as earlier described (Glant et al., 1987). Briefly, mice were immunized i.p. with a 100 µg (protein) of HFPG CS-depleted in 100 µl of CFA, followed by i.p. injection of 100 µg (protein) of CS-depleted HFPG in IFA at 1, 4, and 8 wk after the first injection (Figure 6). If necessary, a fifth injection was given at 12 wk. On average clinical signs of arthritis usually appeared by about 80 days after the first injection in 80 % of the mice.

5.11 GENERATION OF PG-REACTIVE T CELL LINES AND CLONES

PG-reactive T cell lines were obtained from spleens and lymph-nodes (inguinal and periaortic) of mice within 7 days of onset of arthritis as described earlier in other studies (Kimoto et al., 1980; Hom et al., 1986) (Figure 7). Briefly, freshly isolated splenocytes and lymph-node cells were cultured *in vitro* in both 6 well plates (9.62 cm^2, Limbro, Flow Lab., McLean, VA) and T25 flasks (25 cm^2, Falcon, Becton Dickinson Labware, Lincoln Park,NJ) with HFPG at 50µg/ml in DMEM with the following additives : 10mM HEPES, 100 U/ml penicillin, 100µg/ml streptomycin, 0.10 mM non-essential amino acids, 1.0 mM sodium pyruvate, 50 µM 2-mercaptoethanol and 1% of heat inactivated normal mouse serum (BALB/c) for 3 days. Viable cells were isolated over a Lympholyte M gradient and cultured for 4 days in

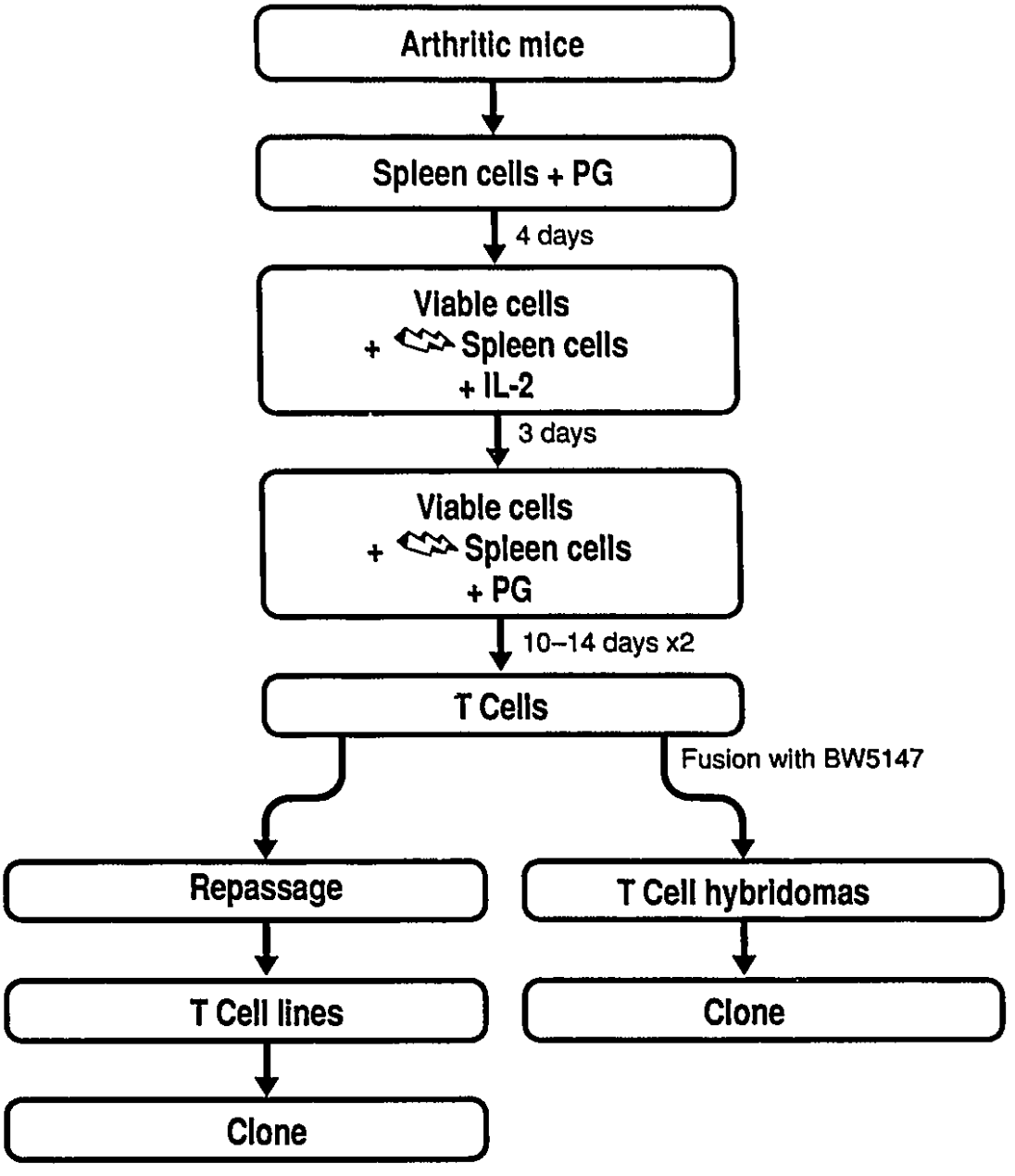

FIGURE 7. Flow Chart for the Generation of PG-Reactive T Cells

The lymphocytes from the arthritic mice were from both spleen and lymph nodes. Viable cells were isolated by centrifugation on Lymphocyte M. The "flashes" represent the irradiation of the spleen cells which serve as a source of APC. The source of IL-2 is TCGF from supernatant of rat spleen cells stimulated with 5µg/ml ConA for 48 hours.

the presence of irradiated (3300 rad) syngeneic spleen cells in complete medium wihout antigens but with 10% FCS and with 10% TCGF. Conditioned medium of rat spleen cells stimulated with 4 μg/ml Con-A for 48 h in complete medium, was used as a source of IL-2 (TCGF). This TCGF was made ConA-free by passing the medium through an α-methyl-mannoside column (5 ml gel) (Sigma, St-Louis, MO). The viable cells were reisolated and cocultured with antigen-presenting cells [irradiated (3300 R) syngeneic spleen cells] and CS-depleted HFPG at 50μg/ml in DMEM with supplements and 5 % heat inactivated fetal calf serum for 10-14 days. At the end of the culture period, viable T cells were recultured in the preceeding fashion at least 3 times. Thereby PG-reactive T cell lines were obtained. These T cell lines were propagated in the same manner with intermittent supplementation with 10% TCGF to enhance cell proliferation. Ovalbumin T cell lines were also obtained in the same way, from ovalbumin immunized mice and by using ovalbumin as the antigen instead of HFPG for the *in vitro* stimulation. This line was used as control. Attempts to clone the T cell lines JY.A and JY.D at 0.3 cell/well were unsuccessful. However, cells have been grown at dilutions up to 2 cells/well with growth in less than 30% of the wells. They have stable phenotypes and specificities for over a year. The identifications of the T cell lines as T cell clones are pending TCR analyses.

5.12 GENERATION OF T CELL HYBRIDOMAS

T cells at the third passage were harvested and viable cells were fused with BW5147 (H-2^k) thymoma cells (kindly provided by Dr. P. Marrack, University of Colorado Health Sciences Center, Denver, CO) as described (Kappler et al., 1981). The cells were seeded into 96 well flat bottomed plates

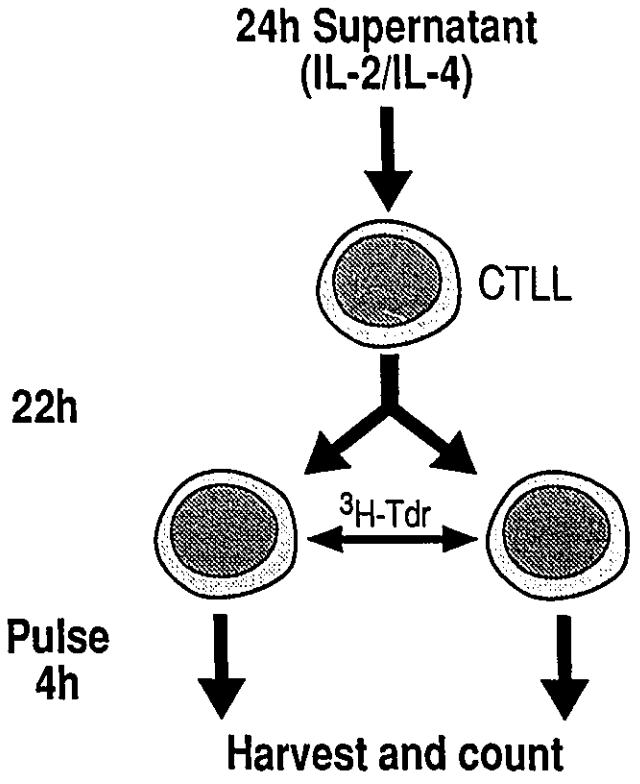

FIGURE 8. Schematic Representation of CTLL Proliferation Assay for the Detection of Lymphokines Secreted by Activated T Cells

at 3 x 10^6 cells/ml or 3 x10^5 cells per well in 100 µl of medium. Hybridomas were selected in HAT complete medium and screened for reactivity against HFPG and HAPG as described below. Wells containing responsive cells were cloned twice by limiting dilution (0.3 cells/well).

5.13 T CELL REACTIVITY ASSAY

The T cell lines and hybridomas were tested for their reactivities against antigens by incubating 2 X 10^4 cells per well with 5 X 10^5 freshly isolated irradiated syngeneic spleen cells in triplicate with various antigens in 96 well microtiter plates at 37^{o}C for 24 h. On some occasions the APC were derived from non-irradiated spleen cells of both naive and arthritic mice or mice which had been depleted of CD8 cells in vivo (Baneerjee et al., 1992a). APC have also been been derived from lipopolysaccharide (LPS) stimulated spleen cells. This was done by incubating 0.5-1.0 x 10^6 cells/ml in complete medium with 10% FCS in T75 flask (80 cm^2, Nunclon, Gibco BRL, Burlington, ON), with 25 µg/ml of LPS (Sigma, St.-Louis, MO). The principal behind this assay is described in Chapter 1 and depicted in Figure 1. All antigen concentrations were expressed as nM of protein contents. The supernatants (100 µl per well) were harvested 24 h later and assayed for IL-2/IL-4 with the CTLL-2 (ATCC, Rockville, MD) proliferation assay (Figure 8). Some CTLL proliferation assays involved measurement of endogenous N-acetyl-β-D-hexosaminidase activity by colorimetric assay (Landegren, 1984). The majority of CTLL cells proliferation assays involved ^{3}H-Tdr incorporation (Mosmann et al., 1986). Briefly, 4 X 10^3cells/well in 96 wells flat-bottomed plate were pulsed with 1 µCi/well of ^{3}H-Tdr (specific activity: 6.7 Ci/mmol) for 5 h at 37 ^{o}C. The cells were harvested by a Skatron cell harvester onto a glass fiber filter mat and the

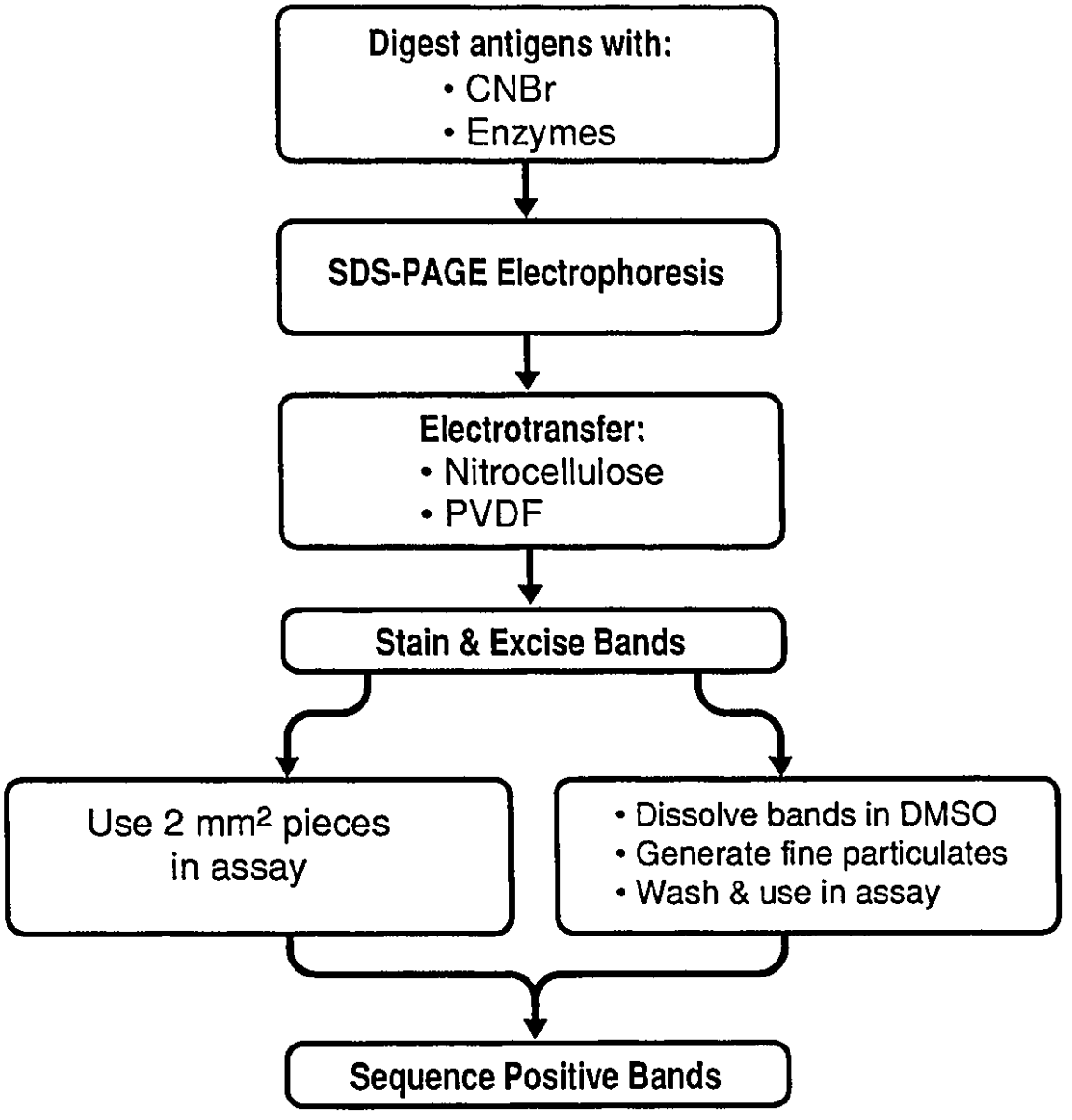

FIGURE 9. Flow Chart for the Utilization of Membrane-bound Peptides as Antigens for T Cells *in vitro*

discs distributed into liquid scintillation vial to which Ready value liquid scintillent cocktail (Beckman Inst. Inc., Fulleton, CA) were added. The vials were then counted in a liquid scintillation analyzer, model 1900 CA Tri-Carb (Packard Inst. Co., Meriden, CT). Both assays were comparable in determining the secretion of IL-2/IL-4 by the T cells. Most results are expressed as either Δ CPM (mean cpm with antigen - mean cpm with medium alone) of CTLL cells or as a Stimulation Index (mean cpm with antigen / mean cpm with medium alone) of CTLL cells. The standard errors of at least triplicate determinations were ordinarily less than 15 % of the mean.

5.14 MEMBRANE -BOUND ANTIGEN ASSAY

To further delineate the T cell reactive peptides of antigens, G1 or PG were digested with CNBr or endoproteinase and electrophoresed on a SDS-PAGE mini-gel with subsequent electrotransfer onto nitrocellulose or PVDF membranes, as previously described (Figure 9). The membranes were blocked with PBS-0.3% Tween-20 for 30 mins at 37 ^{o}C with shaking. Membrane were wash 3 times in PBS-0.3% Tween-20 (5 mins each wash), rinsed in water and stained with aurodye for 2 to 18 h. The visualized bands were cut into small pieces of 2 mm^2, sterilized under U.V. light for 30-60 mins and added to the wells of 96 wells plates for T cell reactivity assay.

5.15 LYMPHOKINE DETERMINATION (IL-2/IL-4)

The CTLL cells were shown to proliferate in the presence of both recIL-2 and recIL-4 (Figure 10) and therefore the CTLL assay could not distinguish between the two different subset of T cells. To discriminate between the two types of lymphokine secretion pattern, two fold dilutions of affinity purified

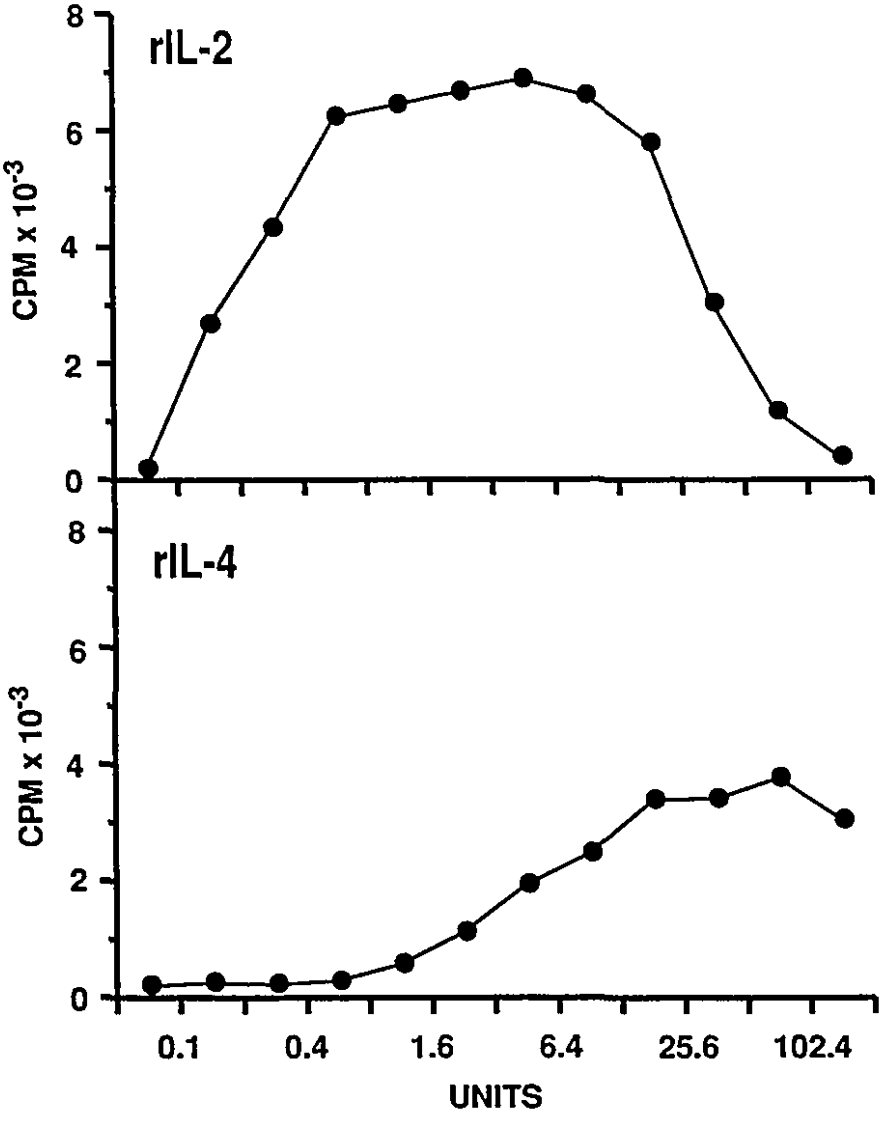

FIGURE 10. Standard Curve of CTLL Proliferation Produced by Recombinant IL-2 and IL-4

The CTLL cells (4 x 10^3 cells/well) were incubated for 24 h with the different cytokines in two fold dilutions and pulsed with ^{3}H-Tdr for the last 4h.

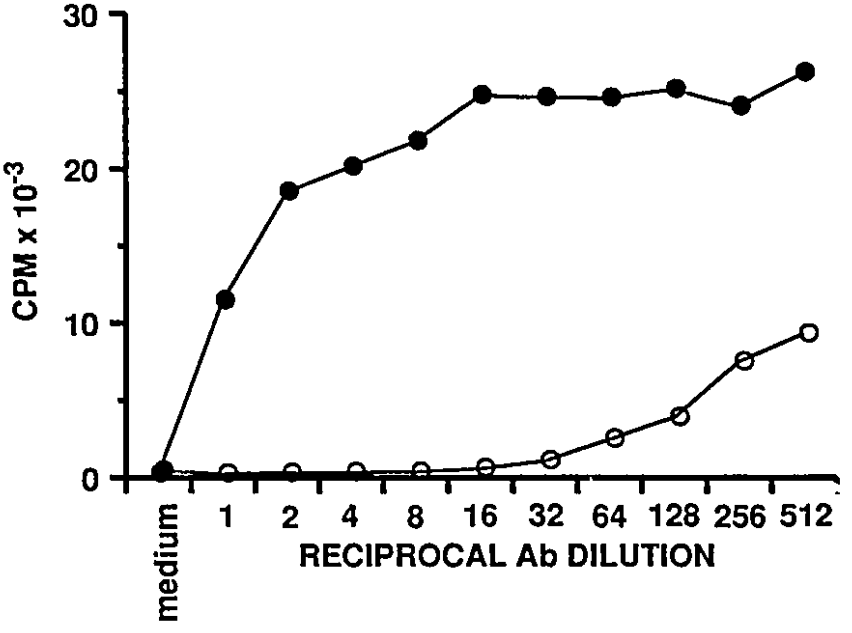

FIGURE 11. Titration Curve of Ab Neutralization of Recombinant IL-2 and IL-4 in CTLL Proliferation Assay

Ab were used in two fold dilutions. Starting concentration of anti-IL-2: (5 μg/ml) and anti-IL-4 : (2 μg/ml). IL-2 (●) (25 U/ml), IL-4 (O) (250 U/ml).

anti-IL-2 and anti-IL-4 (1-10 μg/ml) antibodies were added to the wells of the 96 wells plate at the begining of the CTLL assays to determine the identities of the released lymphokines (IL-2 and/or IL-4) (Figure 11). These antibodies neutralized the effect of the respective specific lymphokines. Figure 11 shows data for 5 μg/ml of anti-IL-2 and 2 μg/ml of anti-IL-4.

5.16 MHC RESTRICTION ANALYSES

Determination of MHC restriction was performed using affinity purified M5/114.15.2, 14-4-4S, and MKD6 antibodies (at 10μg/ml) in the antigen specificity assays with appropriate isotype matched controls. This concentration of antibodies was found to be optimum in preliminary dose-response analyses *in vitro*. Spleens from mouse strains with different haplotypes were also used as antigen presenting cells in the assays to confirm the nature of the MHC restriction.

5.17 FLOW CYTOMETRY

The antibodies used for phenotyping were GK1.5, 2.43, H57-597, B20.6, RT4-10, 44-22-1, KJ16, F23.1, F23.2, MR10-2, and 14.2. The T cells were phenotyped by flow cytometry as described earlier (Banerjee et al., 1989). Briefly, cells were incubated with purified primary antibodies for 30 min. at 4 ^{o}C, washed twice in washing buffer (PBS with 0.3 % BSA and 0.05 % sodium azide) and reincubated with FITC labeled $F(ab')_2$ mouse anti-rat IgG, and FITC labeled $F(ab')_2$ goat anti-mouse IgG (Fc specific) or biotin-Streptavidin-conjugated affiniPure goat anti-hamster IgG (H+L) for an additional 30 min. at 4 ^{o}C. The cells were washed, fixed in 1% formaldehyde (from paraformaldehyde) in PBS for 10 min., washed again and analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View,CA).

5.18 CASEIN ASSAY FOR PROTEOLYTIC ACTIVITIES OF GLYCOSIDASE PREPARATIONS

Radiolabelled casein was prepared according to the method of Cawston and Barrett, (1979). Two hundred mg of 3% TCA insoluble casein is dissolved in 40 ml of 0.1 M Na-borate, pH 9.0, at 4 °C. The solution is acetylated by adding ^{3}H-acetic anhydride (25mCi; 8.7 Ci/mmol) in 1.0 ml dry dioxane. The reaction was continued for 60 min. at 0 °C with rapid stirring, followed by exhaustive dialysis against distilled water at 4 °C. The ^{3}H-casein was diluted with cold casein to a specific activity of 2.6 X 10^{6} CPM/mg. The tritiated acetate derivatizes the ε amino group of the side chain of lysine and the amino terminus of casein.

The assay (Campbell et al., 1986) consisted of mixing 2.6 x 10^{5} cpm of labelled casein with the glycosidase preparation (1 U for keratanase and 0.1 U for chondroitinase) for 24 h at 37 °C in 250 µl of the digestion buffer. The reaction was terminated by adding a 100 µl of cold casein (5 mg/ml) and 150 µl of 10% (w/v) TCA for a total volume of 500 µl. The mixture was centrifuged at 4 °C for 15 min. at 16,000 g and TCA soluble radioactivity was determined by scintillation counting. Background and maximum release controls consisted of no addition of glycosidase to the buffer containing radiolabelled ^{3}H-casein and the addition of trypsin (100ng/250 µl), respectively .

5.19 RADIOIODINATION OF PURIFIED G1

The purified bovine G1 (200 µg) was labelled with Na^{125}I (Amersham, Oakville, ON) by the chloramine T method according to Oike et al., (1980). The radiolabeled G1 was separated from free iodine by chromatography on a Sephadex G-25. The radiolabelled G1 was treated with keratanase or ABC

chondroitinase or trypsin as described above. The preparations were then used for either gel electrophoresis or were precipitated with TCA as above in the casein assay and radioactivity in the supernatants determined in a LKB 1270 Rackgamma II gamma counter (LKB-Wallack, Turku, Finland).

5.20 DELAYED TYPE HYPERSENSITIVITY (DTH)

The potential for DTH by the T cell lines and the T cell hybridomas was assessed by classical methods (Bianchi et al., 1981). One to 2 x 10^4 cells in 20 µl of PBS with 10 µg of antigen were injected s.c. with a tuberculin syringe in the skin of the ear of naive BALB/c mice with a 26 gauge needle. The other ear serving as a negative control recieved only T cells. Some mice were injected with antigen alone.

5.21 PASSIVE TRANSFER OF T CELLS

PG-reactive T cells lines and hybridomas were injected by different routes into naive recipients which were either non-irradiated or irradiated (500 rad) . Five. 10, 50 and 100 x 10^6 T hybridoma cells in 200 µl PBS, were injected by both i.v. and i.p. routes with a second injection after 7 days in some cases. The T cell lines were injected in the same manner but with reduced cell numbers at 1 x10^4, 10 x 10^4, and 100 x 10^4 cells per mouse. The T cell lines were also injected intra-articular by using a 26 gauge needle and injecting 1-2 x 10^4 cells in 20 µl of PBS.

5.22 HISTOLOGY

Ten days after the injection of the T cell lines or T cell hybridomas i.a., the mice were sacrificed and the limb dissected with skin peeled off, muscle and most of the soft tissues removed from around the capsule. The knee

joint was fixed in 10 % buffered formalin (Fisher Scientific Co., Fairlawn, NJ) for 24 h and decalcified in a solution of 10 % formic acid for one day at 21 ^{o}C. The knee were embeded in paraffin and cut into 5 μm sections and stained with hematoxylin and conterstained with eosin. Some sections were stain with toluidine blue (0.25 %) which stains for PG. The decalcification, embedding and staining (except the toluidine blue staining) were done by personel of the histology laboratory of the Montreal General hospital or the Institute of Neurology, McGill University.

RESULTS

CHAPTER VI CHARACTERIZATION OF PG-REACTIVE T CELLS

6.1 INTRODUCTION

Proteoglycan-induced arthritis in mice reproduces some of the features of rheumatoid arthritis and spondylitis in humans (Glant et al., 1987, Mikecz et al., 1987). Injection of BALB/c mice with the large aggregating cartilage proteoglycan (PG) (aggrecan) of fetal human origin in CFA followed by repeated boosts of the same antigen in IFA results in histologically and clinically recognizable changes associated with polyarthritis and spondylitis (Glant et al., 1987, Mikecz et al., 1987). The disease is accompanied by the development of cellular and humoral immune responses to human and mouse PG (Mikecz et al., 1987, Mikecz et al., 1990). This animal model is of special interest since patients with rheumatoid arthritis and ankylosing spondylitis exhibit cellular immunity to human PG (Glant et al 1980, Golds et al., 1983a; Mikecz et al., 1988).

PG depleted of chondroitin sulfate (CS) chains has been shown to be more efficient than the native molecule at inducing this experimental disease (Glant et al., 1987). In addition, CS-depleted fetal human PG (HFPG) induces disease with a higher frequency than CS-depleted adult human PG (HAPG). These observations suggest that the arthritogenicity of PG is influenced by the presence of glycosaminoglycan chains and structural changes associated with age. Previous studies in this laboratory have indicated the importance of T cells in the arthritis induced by PG immunization since the disease can be adoptively transferred from arthritic mice into naive irradiated syngeneic mice using spleen and lymph nodes cells: T cells are essential for this transfer (Mikecz et al., 1990). The role of PG-reactive cells in the adoptive transfer was shown by the ability to transfer the disease with cultured lymphocytes after stimulation in vitro with HFPG and mouse PG (MPG) (Mikecz et al., 1990). The disease could be prevented by *in*

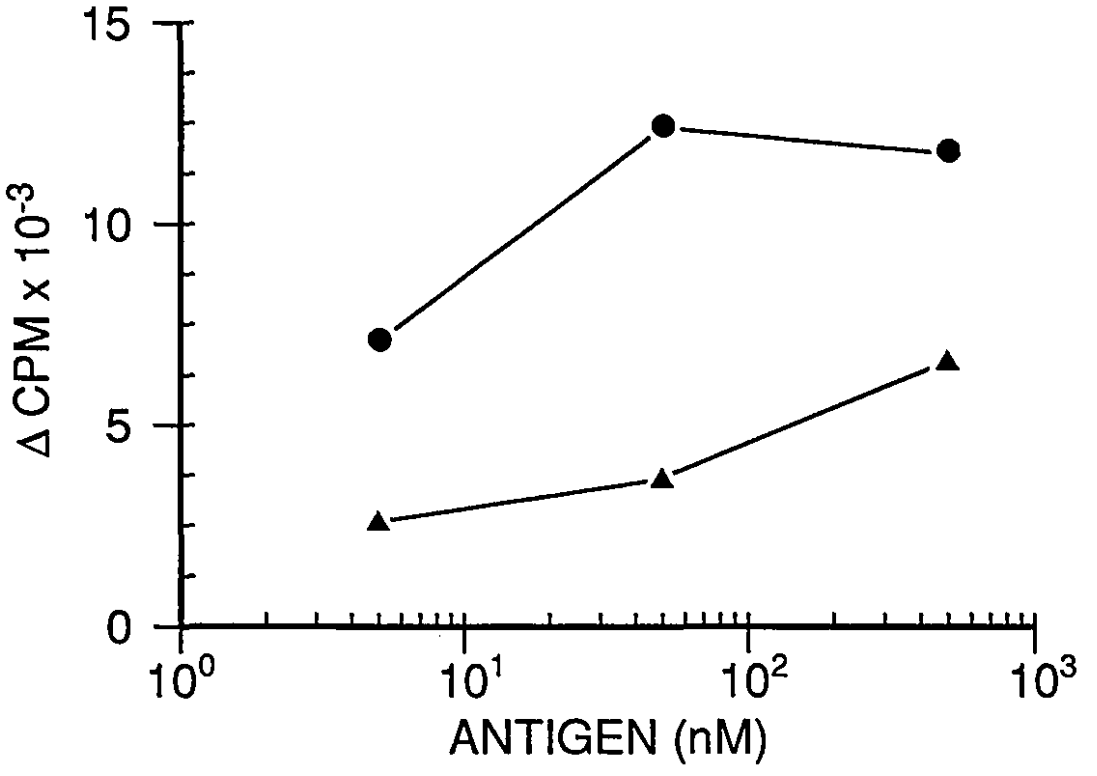

FIGURE 12. Reactivities of Splenic Lymphocytes from a PG-induced Arthritic Mouse to HFPG and HAPG at Various Concentrations

2 X 10^5 cells/well were incubated with the antigens and 24 h later supernatants were removed for assay with CTLL-2 cells as described in the text. Similar results were obtained with five other arthritic mice. PPD gave a Δ CPM of 10,900 at 25 μg/ml. HFPG (●), HAPG(▲)

vivo anti-CD4 antibody treatment but not by anti-CD8 (Banerjee et al., 1992a). Anti-PG antibodies alone are not sufficient for transfer of this disease (Mikecz et al., 1990, Dayer et al., 1990), although they can cause limited synovitis and damage to cartilage (Dayer et al., 1990).

In order to determine the nature of the T cell epitopes on PG which are recognized in immunized mice and which may be of pathogenic importance in this animal model, T cell lines and hybridomas reactive to PG have been derived from mice with PG-induced arthritis and their immune reactivities have been analyzed. In the present chapter the data show that the N-terminal globular domain G1 of the PG molecule, which constitutes the hyaluronate binding region (Doege et al., 1991), contains T cell epitopes, and that HFPG and HAPG possess different T cell epitopes.

6.2 RESULTS

6.2.1 PG REACTIVITY OF SPLEEN CELLS FROM ACUTELY ARTHRITIC MICE

Freshly isolated splenic T cells from arthritic mice immunized with HFPG were reactive to HFPG, and HAPG in a dose-dependent manner (Figure 12), besides being reactive to PPD. The T cells showed greater reactivity to HFPG than to HAPG (Δ CPM 12,500 for HFPG, and 4,000 for HAPG at 50 nM).

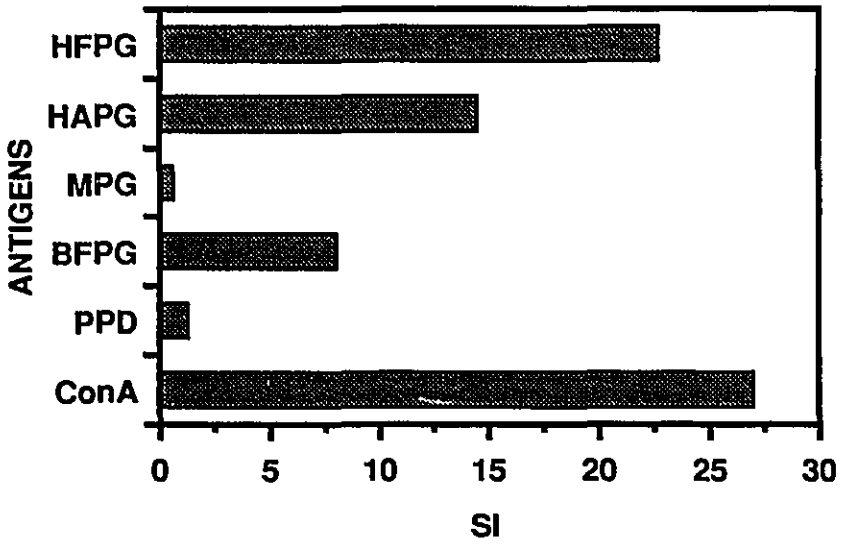

FIGURE 13. Reactivities of a Primary Culture of Enriched T Cells Against PG Derived from Different Species

Spleen cells from arthritic mice were cultured for 3 days with HFPG followed by expansion of viable cells in 10 %TCGF with fresh irradiated feeder cells for 4 days. Viable cells were recultured with irradiated feeder cell only. Seven days later the viable cells were harvested and used in the assay for reactivity against HFPG, HAPG, MPG, and BFPG at 100 nM. Reactivity to ConA (10μg/ml) and PPD (25μg/ml) are also shown.

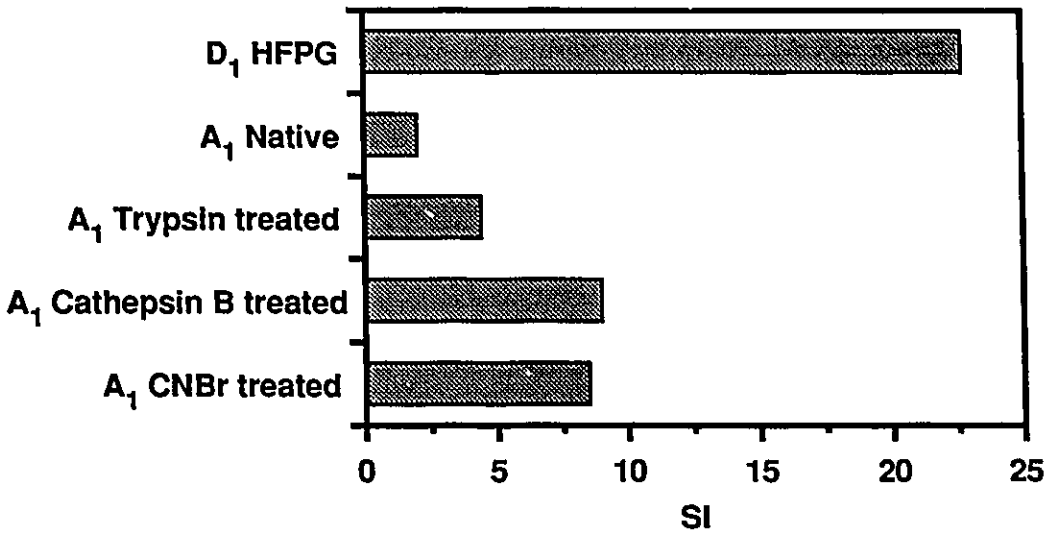

FIGURE 14. Comparison of the Reactivity of Primary Splenic T Cells to PG Aggregate (A1) Before and After Cleavage with Protease or CNBr

T cells were enriched as described in figure 13 and assayed against native HFPG aggregate with or without treatment with trypsin, cathepsin B and CNBr. All antigenic preparations were used at 100 nM.

6.2.2 EARLY STAGE T CELL LINE

Single cell suspensions of lymph node and spleen cells from two arthritic mice were used to establish T cell lines. These cells were tested for antigenic reactivity before the 2nd round of stimulation which was 2 weeks after the initiation of the bulk culture. The cell population of these cultures consisted of enriched T lymphocytes.

As shown in Figure 13, these cells were tested against a panel of PG derived from the cartilage of different species. The highest response was against HFPG (SI= 22) followed by HAPG (SI= 14.5) and BFPG (SI =8). However no reactivity was detected against MPG. PPD showed no response while maximal response was given by the reactivity to ConA (SI= 27). A similar pattern of reactivity was also obtained if the cells were derived from draining lymph nodes instead of the spleen but the differences in reactivity seen with HFPG and HAPG were less (SI = 28.5 for HFPG; SI = 24 for HAPG). PG human fetal aggregate (A1) was also tested for reactivity with these cells in the same experiment and elicited lower responses (SI = 2) than the monomer (SI = 22.7) when using similar protein concentration (20 μg/ml) (Figure 14). However greater reactivity was observed when the aggregate was digested with trypsin (SI = 4.5), cathepsin B (SI = 9) or CNBr (SI = 8.5) (Figure 14) but the reactivity never reached the level obtained with the monomer. Similar results were obtained in another experiment using cells derived from lymph nodes where the monomer gave an SI of 28.5 and the aggregate an SI of 4. The tryptic digest of A1 gave an SI of 8.6 and A1 digested with cathepsin B gave an SI of 20 while the CNBr digest of A1 gave an SI of 12.6.

TABLE 1. Reactivity of Four T Cell Lines to D1 and D1D1 Preparations of PG

	T Cell Lines Reactivities Against*			
T cell lines	HFPG D1	HAPG D1	HFPG D1D1	HAPG D1D1
JY.A	3834	-2000	9778	-557
JY.D	9413	34573	23630	31351
JY.G	10436	1172	731	0
JY.H	10663	109	1364	203

*Reactivities are expressed as Δ CPM. Antigens were used at optimal concentration equivalent to 100nM of PG.

TABLE 2. Reactivities of the T Cell Lines to the Different Fractions (D1,D2,D3) of CsCl Density Gradient from Human Fetal and Mouse Cartilage Extracts*

T cell lines	Fractions of PG Density Gradient					
	HFPG D1D1	HFPG D1D2	HFPG D1D3	MPG D1	MPG D2	MPG D3
JY.A	10963	5669	24141	-1044	-2876	-2946
JY.D	30518	4268	4610	238	195	3673
JY.G	662	4061	37949	1292	-16	-54
JY.H	238	2203	35186	-93	-232	-205

The reactivity of T cell lines JY.G and JY.H is recovered in the D1D3 fractions.
*Reactivities are expressed as ΔCPM. Antigens were used at optimal concentration (20 μg/ml).

6.2.3 T CELL LINES

Many T cell lines were generated from arthritic mice. In fact as explained below, many unsuccessful attempts were made to generate a MPG-reactive T cell line. Among these T cell lines, 4 of them (JY.A, JY.D, JY.G and JY.H) were partially characterized for their PG-reactivities. Two of these 4 lines (JY.A and JY.D) were more fully characterized in terms of reactivities and phenotype. The reactivities of the 4 lines were characterized by two distinct patterns, one pattern being the recognition of both HFPG (D1) and HAPG (D1) as represented by the line JY.D. The second pattern corresponded to the recognition of only HFPG (D1) as shown by the three lines JY.A, JY.G and JY.H (Table 1). When retested with another HFPG preparation that was further purified on a second density gradient to give a D1D1 PG preparation, the reactivities of JY.G and JY.H were lost but reactivity of JY.A was retained (Table 1). It is likely that the epitope for which JY.G and JY.H T cell lines were reactive to the D1 preparation was a contaminant molecule which was removed by further purification. Alternatively there is the possibility that loss of reactivity by the T cells is due to the release of small labile part of the molecule in the lower density fraction of the gradient. This explanation is unlikely because of the extreme care taking during the purification procedure. The recovery of the reactivity in the lower density fractions of the second gradient does not necessarily mean it is due to a cleavage product of PG. However this possibility should be considered with MPG where no cross-reactivity is seen with any of the T cells. It is possible that this reactivity could be recovered in the other fractions of the density gradient. HFPG preparations D1D1, D1D2 and D1D3 representing high, medium and low density fractions, as well MPG preparations D1, D2 and D3 covering the entire gradient were

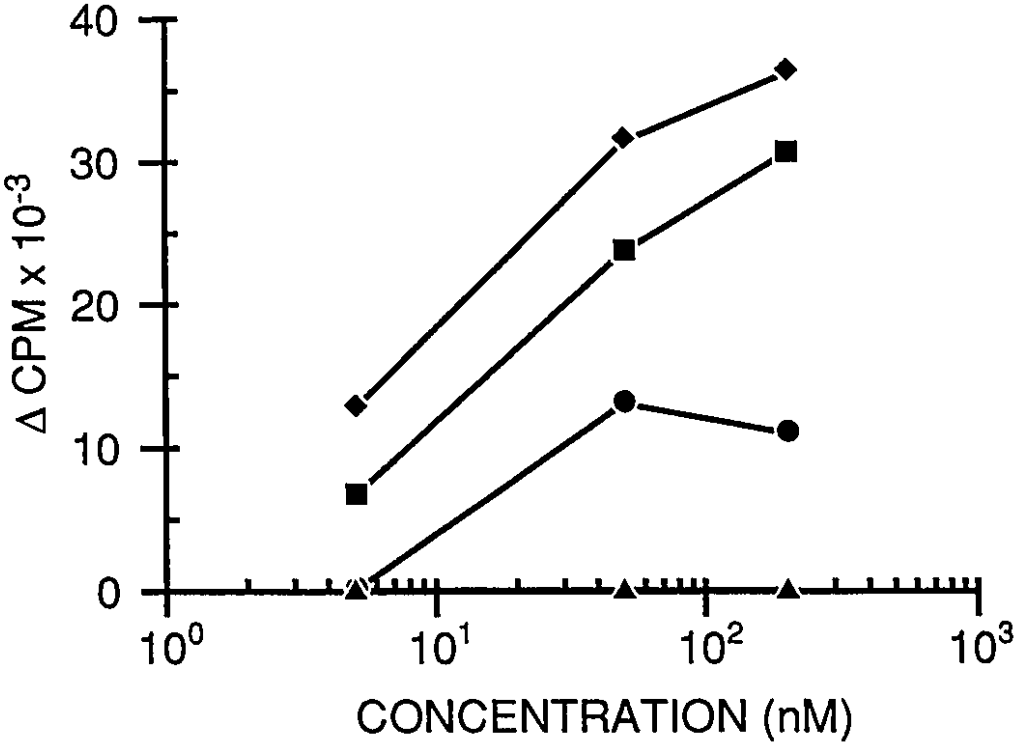

FIGURE 15. Proteoglycan Reactivities of the T Cell Lines JY.A and JY.D to HFPG and HAPG at Various Concentrations

Antigens measured as nM of protein under conditions described in the text. JY.A to HFPG: (●); to HAPG: (▲). JY.D to HFPG: (■); to HAPG: (◆).

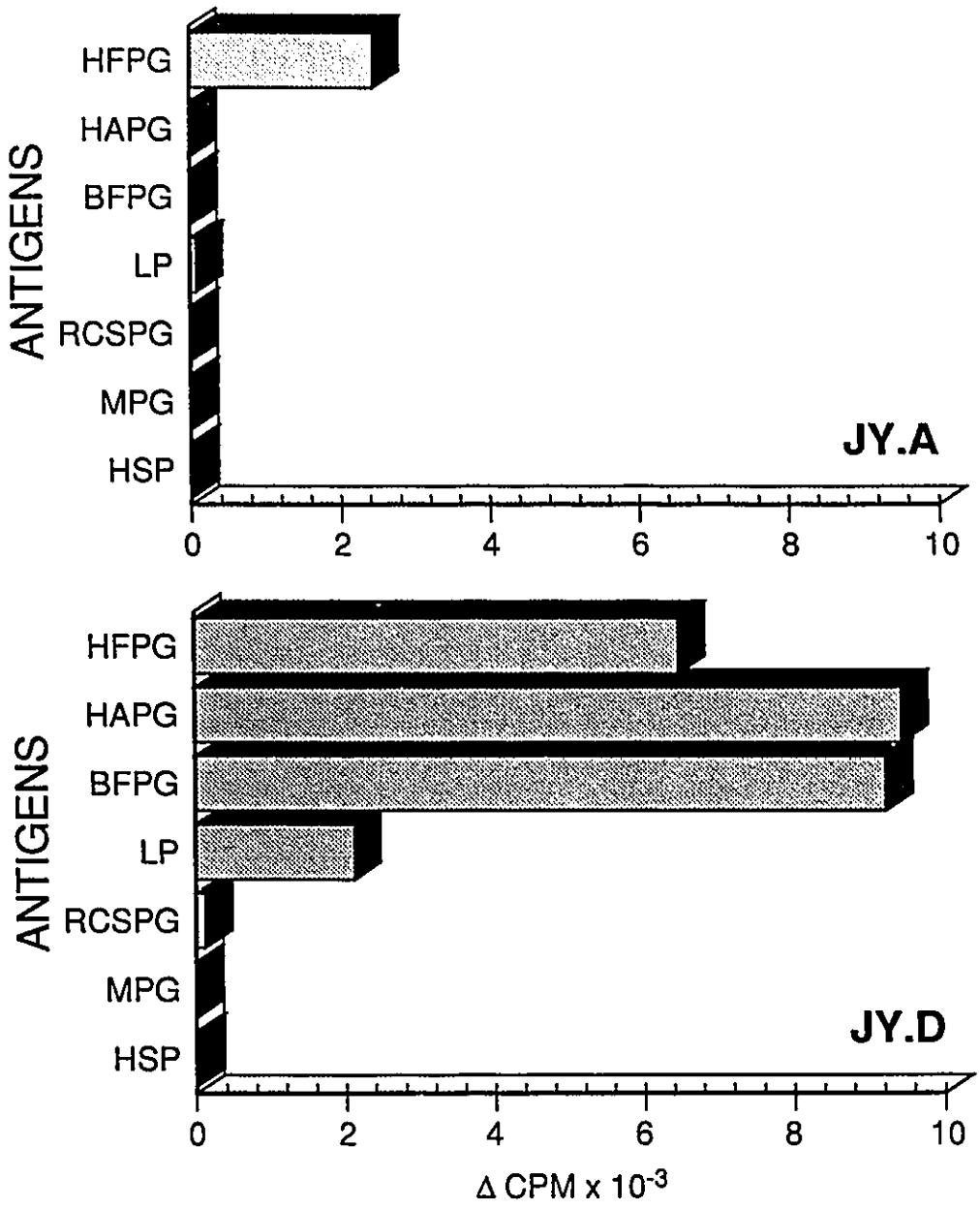

FIGURE 16. Antigenic Reactivities of the T Cell Lines JY.A and JY.D Against PG Derived from Different Species, Bovine LP and Recombinant Mycobacterial 65 kD HSP

The final antigen concentrations were (100nM) for all preparations except 50 nM for HSP. All the PGs were CS-depleted.

used to test this possibility. As seen in Table 2 reactivity to the HFPG preparation by JY.G and JY.H were found to be greatest with the D1D3 fraction of HFPG and not to the D1D1 preparation. JY.A was also reactive to the D1D3 fraction though still reactive to the D1D1 fraction. However JY.D only responded strongly to the D1D1 fraction. Reactivity to fractions of MPG was not detected with any of the T cells (Table 2). The reactivity of JY.D to MPG D3 and JY.G to MPG D1 are very low and not significant.

Since JY.A showed reactivity to the purified HFPG D1D1, this cell line was used in further studies as representative of the second pattern of reactivity. A dose-response curve of the age-related PG specificities of the two T cell lines JY.A and JY.D established the range of suboptimal dose and demonstrated that both T cell lines recognized different epitopes on PG depending on the age of the cartilage from which the PG preparation was derived(Figure 15).

Figure 16 shows the results at the optimum antigenic concentrations using CS-depleted PG preparations. Similar results were obtained in other experiments with native PG preparations. T cell line JY.A responded to HFPG but was unreactive to HAPG , BFPG, and LP (Figures 15 and 16). T cell line JY.D recognized HFPG, HAPG, BFPG,and LP (Figures 15 and 16). Neither of the T cell lines reacted to preparations of rat PG (RCSPG) nor mouse PG (MPG),at doses ranging from 1-40 μg/ml. Neither of the T cells reacted with 65 kD HSP at doses from 1 to 100 μg/ml (Figure 16 and data not shown).

6.2.4 AUTOREACTIVE T CELL LINES

In establishing antigen-specific T cell lines, many attempts led to the outgrowth of autoreactive cell lines characterized by high responsiveness to

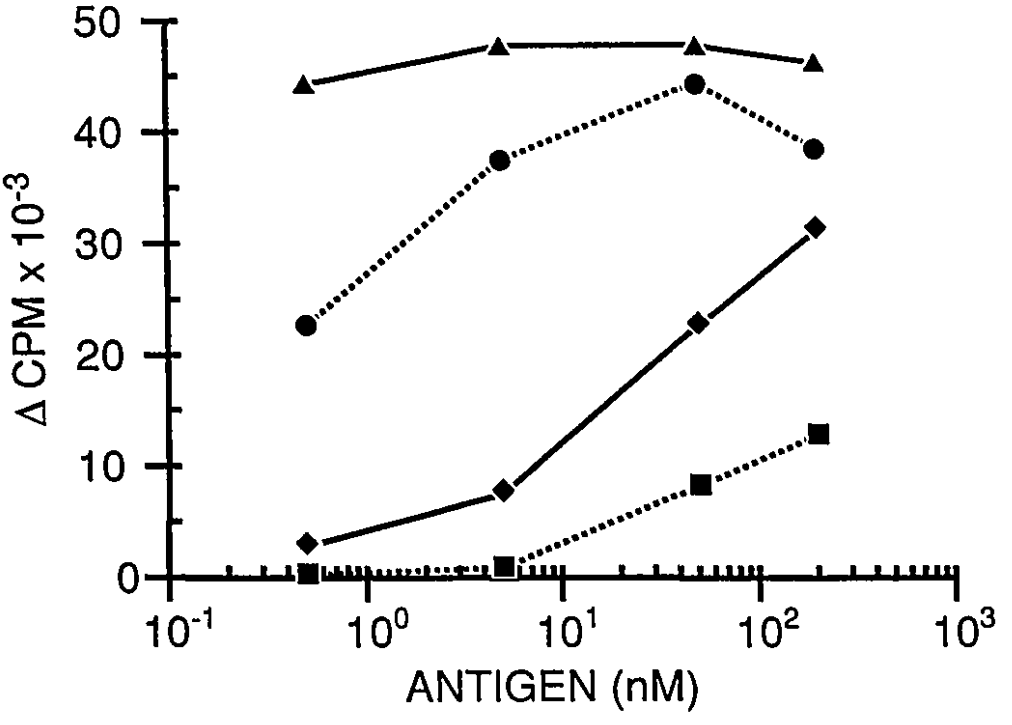

FIGURE 17. Influence of Age of the Cartilage Source on PG Reactivities of the T Cell Hybridoma TH5 and TH14

TH5 (HFPG [●], HAPG [▲]), and TH14 (HFPG [■], HAPG [◆])

autologous antigen-presenting cells even without addition of antigen. The T cell lines gave a high background in proliferation assays. These cells are characterized in culture by a very rapid growth. To minimize their appearance it was essential to use medium composed of 0.5 to 1 % normal mouse serum instead of FCS at the beginning of the culture. Also their rapid growth could be advantageously employed to eradicate these cells by diverse methodology, such as the ethidium-bromide incorporation in combination with UV irradiation or the "hot pulse" technique. In the latter, the rapidly growing cells take up high specific activity (20 μCi/mmole) tritiated thymidine for a period of 20 h. The thymidine that remains is chased with cold thymidine for 3-4 days. The rapidly growing cells that have incorporated high concentration of ^{3}H-Tdr are killed due to the intense radioactivity of the incorporated radionucleide (Cantor and Jandinski, 1974). This technique was used with good results for killing autoreactive T cell lines but without any success when generating PG-specific T cell lines. This is probably due to toxicity problems. In one example of the response of a T cell line established against RCSPG that contains autoreactive cells, the average medium response was 40,431 CPM for the culture with autoreactive cells compared to 907 CPM after the hot pulse. After treatment the responses to ConA and RCSPG diminished considerably but did not result in a better response to the specific antigen; RCSPG: 120, 676 CPM (SI = 2.6) before and 2329 CPM (SI = 3.0) after treatment.

6.2.5 T CELL HYBRIDOMAS

Multiple fusions were performed between PG-reactive enriched T cells lines and the Bw5147 thymoma. Many T cell hybridomas reactive to HFPG

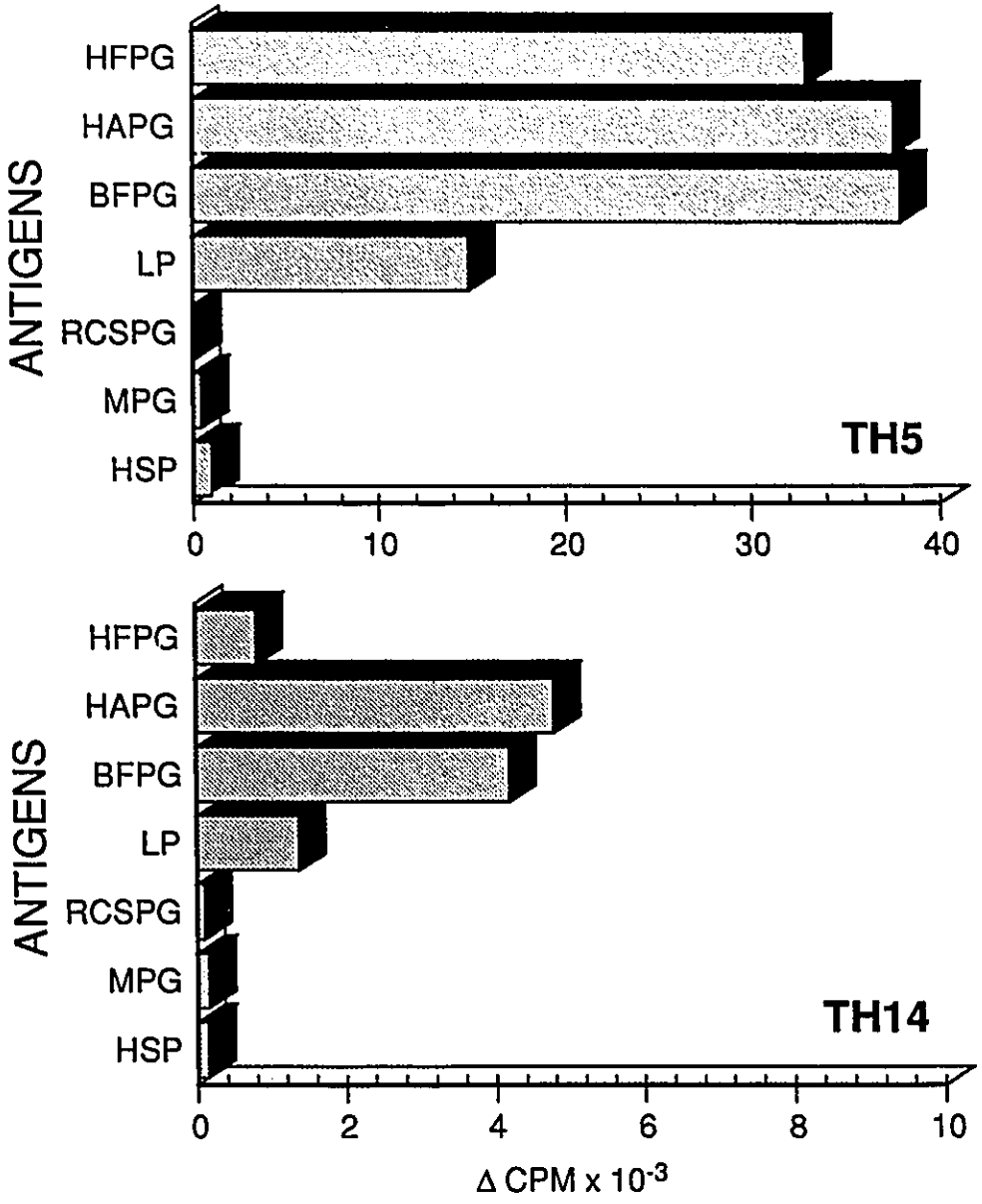

FIGURE 18. Antigenic Reactivities of the T Cell Hybridomas TH5 and TH14 to Different Antigen Preparations

The final antigen concentrations per well were 125 nM for all preparations.

were obtained. T cell hybridomas from two different fusions were further characterized. The growing cells were screened against HFPG, HAPG and MPG. The cells from the best growing wells were selected for cloning. All the T cell hybridoma clones showed a similar pattern of reactivity : no reactivity to PPD, RCSPG and MPG; reactivity to HFPG, HAPG (in both native or chondroitinase digested forms) and to BFPG chondroitinase digested (data not shown). Because of their similar reactivities, more fusions were performed to produces different patterns of reactivity as observed for the T cell lines.

In a second fusion, T cells after the third round of antigenic stimulation with HFPG *in vitro* were used. Among the T cell hybridoma clones produced, 4 were selected because they grew faster. Two patterns of reactivity were observed. One involved recognition of both HFPG and HAPG by the hybridomas TH1,TH5,TH6. The second pattern was characterized by the recognition of only HAPG by hybridoma TH14. But later in a complete dose-response analysis of the hybridomas against both HFPG and HAPG, TH14 was also found to be reactive to HFPG, although to a lesser degree. Only two of these hybridomas were further studied.

Specificities of the T cell hybridomas TH5.B.JY (TH5) and TH14.B.JY (TH14) are shown in Figures 17 and 18. Both T cell hybridomas were responsive to HFPG, HAPG (Figures 17), and to BFPG and LP. However, they were unresponsive to RCSPG and MPG (Figure 18) at doses ranging from 1-40 μg/ml. These T cell hybridomas, especially TH14, recognized HAPG better than HFPG (Figure 17). The two hybridomas however showed quantitative differences in their individual responses to all the PG preparations tested with TH5 producing larger responses to the antigens than TH14 at all concentrations tested (Figures 17 and 18). Neither hybridomas responded to HSP at concentrations from 5 to 80 μg/ml (Figure 18 and data not shown).

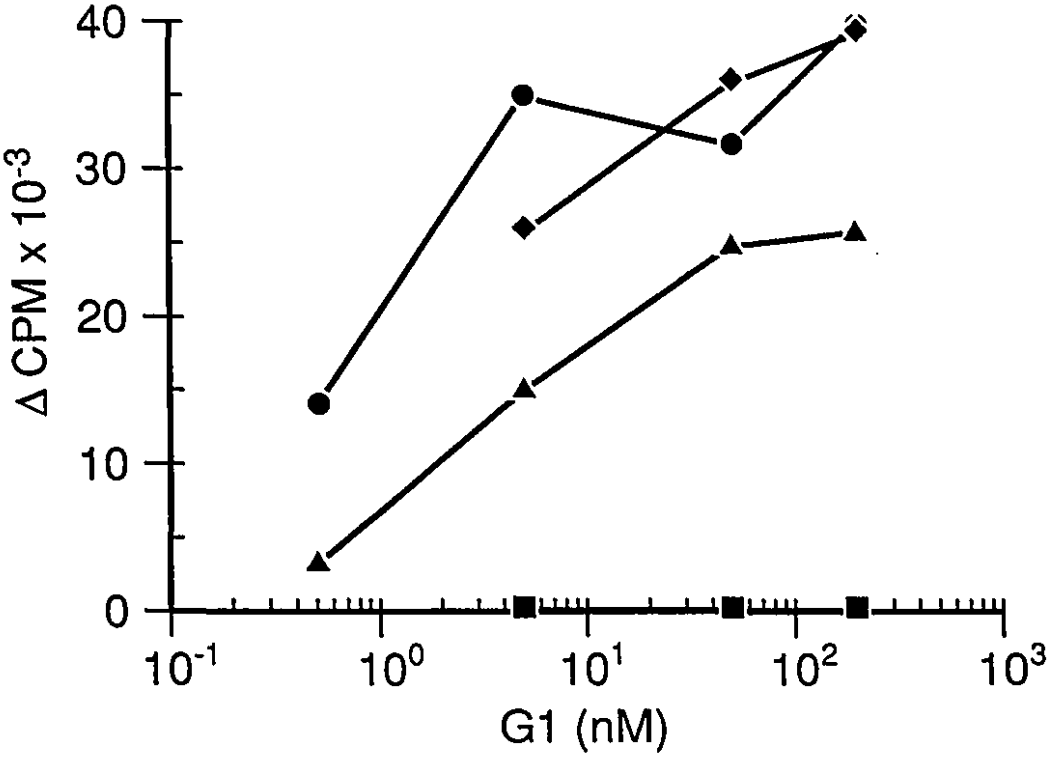

FIGURE 19. Reactivities of the T Cell Lines JY.A (■) and JY.D (◆) and the T Cell Hybridomas TH5 (●) and TH14 (▲) to the G1 Domain of PG

Reactivities at 0.5 nM were not measured for the T cell lines JY.A and JY.D.

6.2.6 REACTIVITY TO THE G1 DOMAIN

The reactivities of the T cell line JY.D and T cell hybridomas TH5 and TH14 to LP suggested that the epitope(s) on PG recognized by these T cells may reside in the G1 domain of PG, since this bears significant homology to LP (Neame et al., 1987). This was confirmed by the demonstration of the reactivities of these cells to the purified G1 domain of PG (Figure 19). In constrast the T cell line JY.A, which did not react with LP, did not respond to G1 (Figure 19).

Because three out of four T cells described here were reactive to the G1 domain it is possible that the G1 domain contains an immunodominant epitope(s) and that there is a strong selection of T cell reactivity to epitopes on the G1 domain. The dominance of the G1 T cell epitopes could be analysed by at least two methods. One is by T cell precursor frequency analysis where spleen cells are plated at a low cell number in 96 wells plates from which the precursor frequency is determined based on statistical analyis of the number of positive (G1 reactive) wells (Taswell, 1981). The other method, though not as elaborate and not quantitative as the first one, can give a relative value of the dominance of the G1 epitope(s). It consists of immunizing the mice with a single injection of HFPG in CFA in the foot pad and analysing the draining lymph nodes after 7 days for their reactivity to a panel of antigen at various doses that includes HFPG, BFPG and the bovine G1 domain. The slope of the curve or the percentage value of the T cell reactivities at any comparable dose expressed on molar basis for the G1 domain compared to the whole PG molecule provides an indication of the immunodominance of the G1 domain epitopes in the PG molecule.

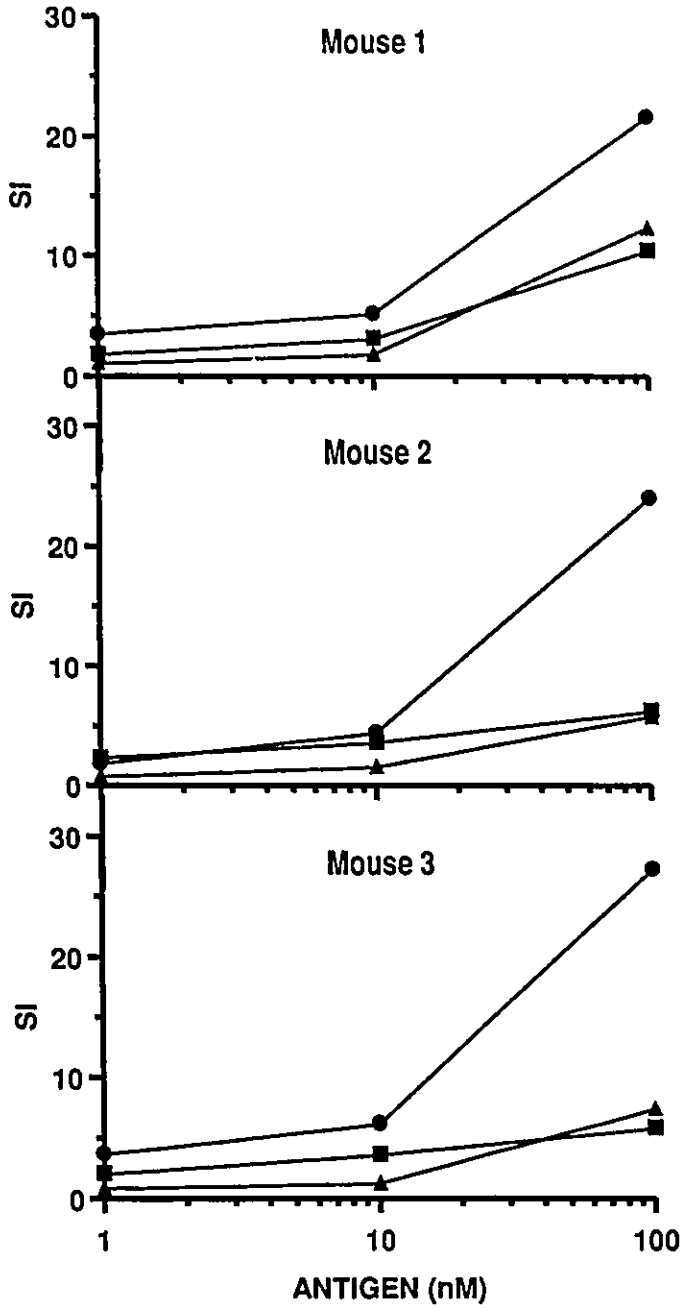

FIGURE 20. The G1 Domain Possesses One or More Dominant T Cell Epitope(s)
Three mice received a single injection of HFPG and draining lymph nodes were tested for T cell reactivities against HFPG (●), BFPG (■), and G1 (▲).

The lymph nodes of three mice immunized as mentioned above with 50 μg of HFPG were analysed in this way. These studies revealed that the G1 domain does contain dominant T cell epitopes as the same proportion of T cells reacted and at the same magnitude as for the BFPG (Figure 20). This may represent almost the total cross reactive BFPG T cell epitopes to the HAPG which in turn may represent the proportion of T cell reactive to the similar epitope on HFPG because it contains, with G2, the highest homologous region of interspecies PG. In comparison with the HFPG response, the reactivity to bovine G1 domain is from a quarter to half of that to HFPG (Figure 20). Since HFPG was used as the immunogen, the highest response to HFPG could be due to the T cell reactivities to part of the molecule outside the G1 domain and that are not cross-reactive to HAPG and BFPG because of lack of homology and/or glycosylation differences between these molecules. These experiments show that the G1 domain of PG contains immunodominant T cell epitopes. What these experiments do not tell us is how much of the non-homologous region of G1 contain T cell epitopes and this can only be determined by comparing the purified human G1 to the HFPG and the purified bovine G1 domain.

6.2.7 MOUSE PG REACTIVITY

No T cell has shown reactivity to MPG in any of the assays . In the spleen and lymph node populations directly tested from arthritic animals the frequency of MPG reactive T cell clone may be so low that they are not detectable in the assay. In the case of established T cell lines the selection phenomenon is biased towards production of T cells that recognize immunodominant T cell epitopes on HFPG which are not cross-reactive with

TABLE 3. Reactivities of the T Cell Hybridomas to HFPG and MPG Presented By Different Spleen APC

T Cell Hybrido-mas	Conditions	Source of Spleen APC*			
		Normal Mice	Arthritic Mice	Arthritic Mice† CD8-depleted	Arthritic Mice§ Thy-1-depleted
TH5	Medium	2948±673	29411±3701	15167±820	902±902
	HFPG	24618±547	38203±1823	38297±1871	27919±736
	MPG	5275±1140	27553±1145	15088±312	814±487
TH14	Medium	478±43	22322±2389	17067±1531	516±238
	HFPG	24170±902	33801±550	43648±3447	19236±2543
	MPG	1797±373	30393±1614	22365±1704	487±157
APC (alone)	Medium	3434±1155	1205±457	16373±3731	536±80
	HFPG	1049±169	6138±272	31425±1523	385±120
	MPG	1535±96	1427±484	8808±674	773±121

Values are in CPM with standard error of mean.

*Non-irradiated APC

†Arthritic mice treated *in vivo* with anti-CD8 antibody (2.43) (Banerjee et al., 1992). Flow cytometric analysis showed 2.5% CD8 spleen cells compared to 6% in normal spleen cells.

§Arthritic mice treated *in vitro* with anti-Thy-1 antibody (HO 13.4). Flow cytometric analysis showed 9.7% of total CD4 and CD8 spleen cells compared to 29% in normal spleen cells.

Addition of arthritic serum to both antigens (HFPG and MPG) with either normal or arthritic mouse APC did not change the level of reactivity of the T cell hybridomas to these antigens. Using thymus as the source of APC also did not change the T cell hybridomas reactivities to both antigens. For TH5 using thymus gave Δ CPM of 12,040 for HFPG and 808 for MPG compared to 11,711 and 654 for HFPG and MPG respectively with splenic APC.

TABLE 4. Reactivities of the T Cell Hybridomas to HFPG and MPG Presented By Different LPS Activated Splenic APC

T Cell Hybridomas	Conditions	Source of Spleen LPS Blast APC*		
		Normal Mice	Arthritic Mice	Arthritic Mice CD8-depleted
TH5	Medium	2585±685	304±64	2244±456
	HFPG	23701±2248	30047±1575	7555±968
	MPG	2610±455	375±31	3289±636
TH14	Medium	4011±496	500±217	1433±64
	HFPG	6111±1195	17400±3384	3907±579
	MPG	4058±1484	383±71	2107±176
APC (alone)	Medium	3607±1509	242±28	1980±681
	HFPG	1911±479	13994±341	2810±559
	MPG	4373±1864	305±76	1952±717

*All the spleen cells were LPS (25 μg) treated for 48 h before use.

MPG. This lack of response could also be due to high tolerance to autologous PG. Because none of the T cell lines and the T cell hybridomas generated in this manner have shown any responses to the MPG, several attempts were made to generate T cell lines reactive to MPG. *In vitro* stimulation with MPG and/or RCSPG of spleen and lymph node cells from acute arthritic mice was tried in order to increase the frequency of MPG T cells. However this was unsuccessful in generating MPG-reactive T cell lines. In another experiment mice were injected (200 µl), subcutaneously on the dorsal region of the rear paws with 100 µg of RCSPG or MPG, or a mixture of both, in CFA in native or CS-depleted forms. Eleven days later the draining lymph nodes (brachial and popliteal) were used to generate T cell lines. The cells were stimulated with either MPG, or RCSPG or HFPG. After four passages the lines were assayed for their antigenic reactivities. Though HFPG reactive T cell lines were obtained, no MPG reactive T cell line was found. A RCSPG reactive T cell line was obtained from RCSPG immunized mice. This reactivity to RCSPG was low with SI values of 2 to 3. However these cell lines often had high background counts due to the proliferation of autoreactive cells. A third possibility for the lack of reactivity may be due to the degradation of the MPG. Special care was taken in preparing the MPG preparation to avoid this. Moreover, this was ruled out by gel analysis of the preparation which did not reveal any significant degradation of the MPG.

Another possibility relates to the APC. This is an essential cell in the generation of T cell immunity. It is known that only APC can present antigen and deliver proper co-stimulatory signals to activate T cells. The source of APC may be important since APC without co-stimulatory signals can lead to anergy (Schwartz, 1990). Different APC may have varying abilities to process antigen and therefore to generate immunogenic or non-immunogenic

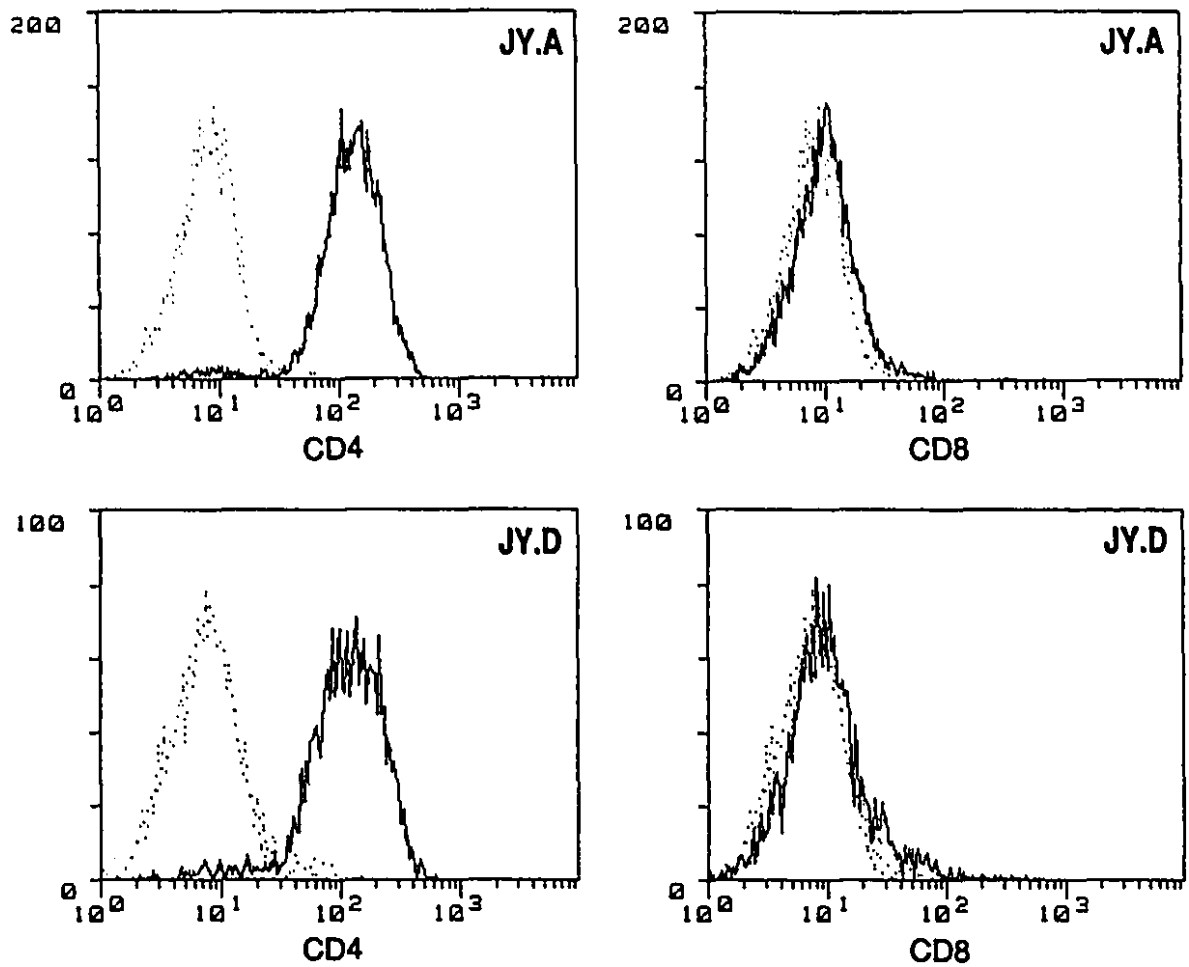

FIGURE 21. Flow Cytometric Analysis Histograms of CD4 and CD8 Expressions of T Cell Lines JY.A and JY.D

Each panel represents an overlay histogram of CD4 or CD8 expression (solid lines) over background (broken lines) (MAR-FITC control antibody).

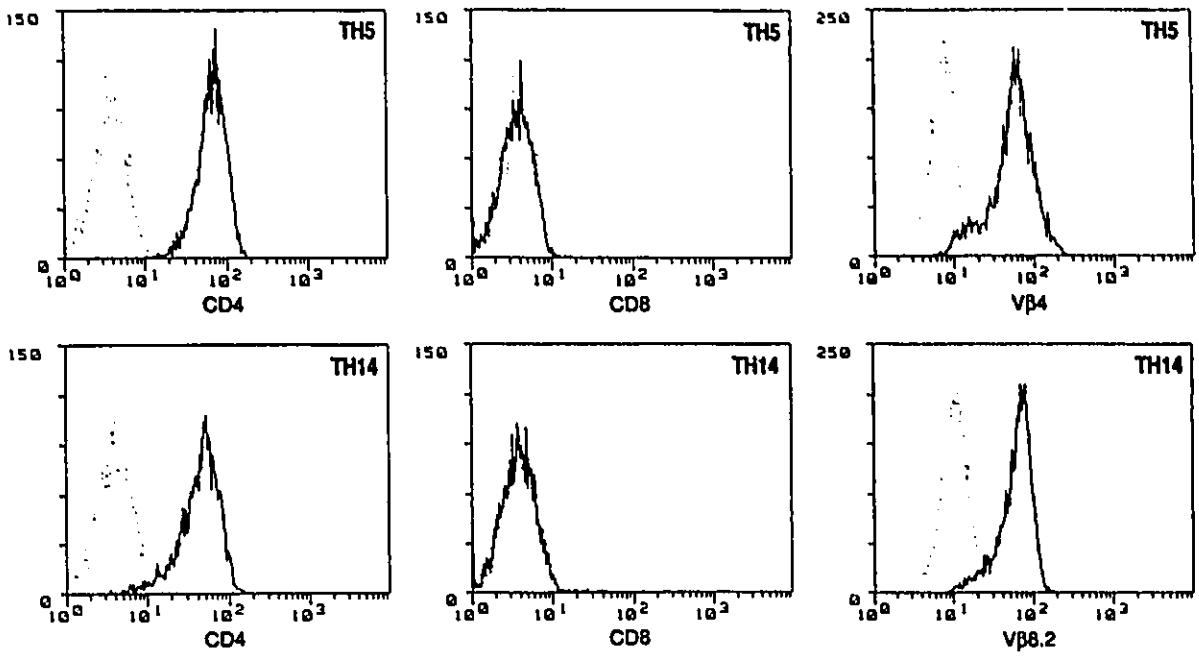

FIGURE 22. Flow Cytometric Analysis Histograms of CD4, CD8 and V_β TCR Expressions of T Cell Hybridomas TH5 and TH14.

Overlay histograms of first antibody staining (solid lines) over background (broken lines) from second antibody conjugated with FITC (MAR-FITC).

peptides (Sedgwick et al., 1989; Davidson and Watts, 1989; Michalek et al., 1989). Activated B cells are known to be very potent antigen-specific APC. To rule out the possibility that MPG was not presented by the proper APC, different tissue sources of APC and different protocols for generating activated B cells as APC were used with the T cell hybridomas TH5 and TH14 in the hope of observing a response to MPG. Thymic APC were tried without success. With irradiated spleen cells, the macrophages are believe to be the major source of APC since irradiation destroys the antigenic presentation ability of the radiosensitive B cells (Ashwell et al., 1984). However neither irradiated nor non-irradiated spleen cells supported responses to MPG. Activated B cells might be the most efficient APC (Chesnut and Grey, 1981; Krieger et al., 1985) and those that are MPG-reactive may best present MPG to T cell hybridomas . When these experiments were conducted, the PG specificity of the T cell hybridomas was known to be contained in the conserved region of G1 (the B loop) and it was assumed that the MPG sequence should be similar to those described for the RCSPG (Doege et al., 1987), HFPG (Doege et al., 1990) and BFPG (Neame et al., 1987). Very recently the MPG sequence has been revealed and was found to be indeed similar to the other species of PG in that G1 region (Glant et al., 1992). Spleen cells from acute arthritic mice, which have high levels of anti-MPG Ab, were compared with normal spleen cells and spleen cells from arthritic *in vivo* depleted CD8 and Thy-1 depleted mice (to remove T suppressor interferences) (Table 3), LPS treated spleen cells from normal, arthritic and arthritic CD8-depleted mice (Table 4), as a source of APC in order to search for a T cell response to MPG. However none of these cells supported reactivity to MPG at the level of Tcell hybridomas TH5 and TH14, nor did APC themselves exhibit such reactivity

TABLE 5. Comparison of the Responses of the T Cell Hybridomas TH5 and TH14 to HFPG at Various Protein Concentrations Using Antigen Presenting Cells (APC) from BALB/c and B10.RDD Mice

T CELL HYBRIDOMA	SOURCE OF APC	H-2				CONCENTRATIONS (μg/ml)†				
		K	I-A	I-E	D	Medium	0.1	1	10	40
TH5	BALB/C	d	d	d	d	7069 ± 762	15153 ± 249	37857 ± 2045	44770 ± 3090	41605 ± 2151
	B10.RDD	d	d	d/b*	b	4383 ± 653	15783 ± 1877	31214 ± 2919	37376 ± 1831	40949 ± 2492
TH14	BALB/C	d	d	d	d	325 ± 16	583 ± 37	1170 ± 90	8563 ± 1268	13103 ± 845
	B10.RDD	d	d	d/b*	b	1593 ± 877	198 ± 16	601 ± 207	7520 ± 1167	13209 ± 1085

*I-E is not expressed. B10.RDD spleen cells did not stain with 14-4-4S (data not shown)
†Results are expressed as mean cpm ± SEM

although reactivity to the control HFPG preparation was always detected in the assays (Table 3 and 4).

6.2.8 PHENOTYPES

6.2.8.1 CD4 VS CD8

The T cell lines JY.A, JY.D and the two T cell hybridoma clones (TH5 and TH14) were shown to be $CD4^+ CD8^-$ by flow cytometry (Figure 21, and Figure 22, and Table 6).

6.2.8.2 MHC CLASS II RESTRICTION

The responses of the PG-reactive T cells to antigen were MHC class II restricted since M5/114.15.2, which is reactive to both $I\text{-}A^d$ and $I\text{-}E^d$ (Bhattacharya et al., 1981), totally inhibited the antigen-specific responses of all of the T cells (Figure 23). To identify the MHC class II molecules involved ($I\text{-}A^d$ or $I\text{-}E^d$), blocking experiments were performed with an anti-$I\text{-}A^d$ antibody (MKD6) and an anti-$I\text{-}E^d$ antibody (14-4-4S). MKD6 blocked the JY.A, JY.D, TH5 and TH14 responses to PG antigens to various degrees whereas an isotype matched antibody had no effect, suggesting that the epitope(s) was being presented by $I\text{-}A^d$. 14-4-4S completely blocked JY.A responses to PG suggesting that here the presentation of epitope on PG involved $I\text{-}E^d$ (Figure 23). Additional experiments were done to confirm that the T cell hybridomas, which were cloned, were indeed $I\text{-}A^d$ restricted. Spleen cells from B10.RDD mice ($I\text{-}A^{d+}$,$I\text{-}E^{d/b-}$) were used as the source of antigen presenting cells (APC) besides BALB/c ($I\text{-}A^{d+}$,$I\text{-}E^{d+}$) spleen cells. It was shown that the APC from B10.RDD could present the PG antigens to

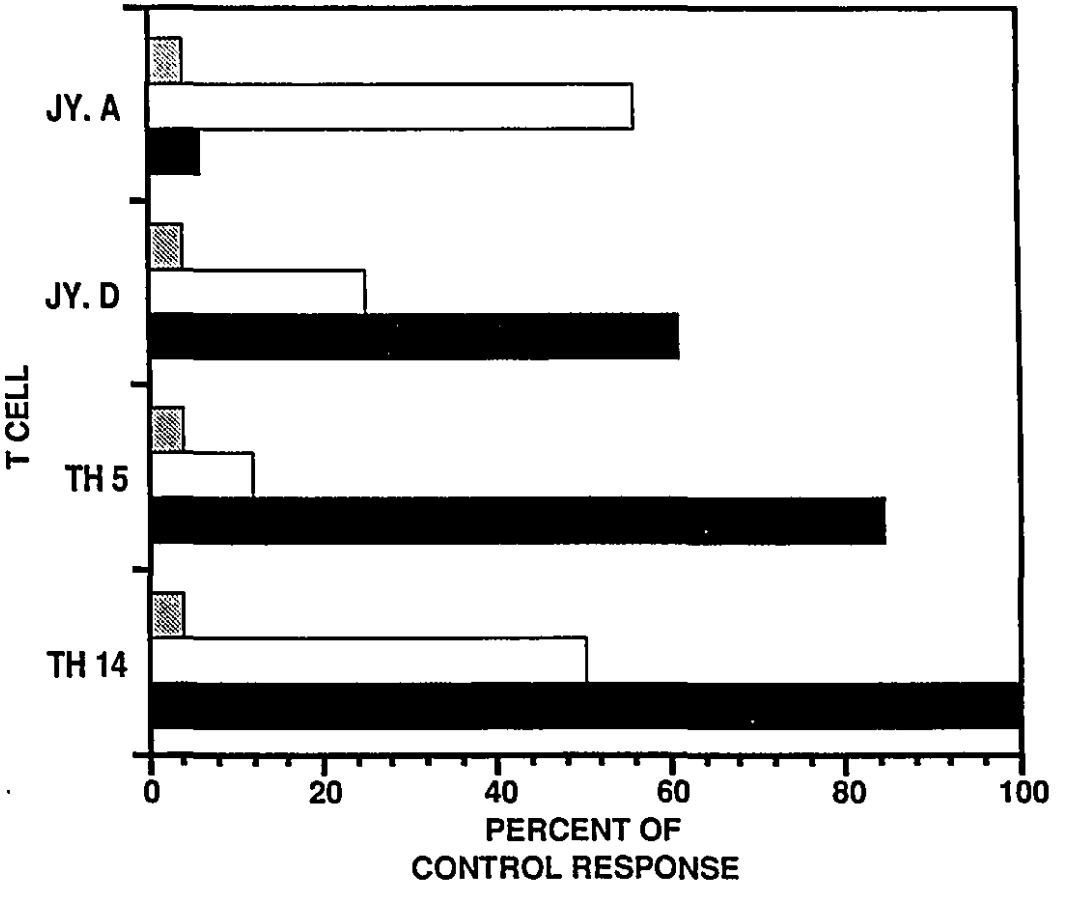

FIGURE 23. Influence of Addition of Anti-MHC Class II Antibodies on the Reactivities of the T Cell Lines and the T Cell Hybridomas to PG Antigens

The antigens used were HFPG [20μg(protein)/ml] for T cell lines JY.A, JY.D and T cell hybridoma TH5 and HAPG [20μg(protein)/ml] for T cell hybridoma TH14. Anti-I-Ad+I-Ed (M5/114.15.2) (shaded bar) anti- I-A^d (MKD6) (open bar); anti-I-E^d (14-4-4S) (solid bar). Both antigens used were CS-depleted The control responses (100%) represent the maximal responses of the T cell lines and hybridomas in the presence of their respective antigens and an irrelevent antibody [SFR8 (anti-HLA-BW6) for M5/114, and IgG_{2a} myeloma protein for MKD6 and 14-4-4S].

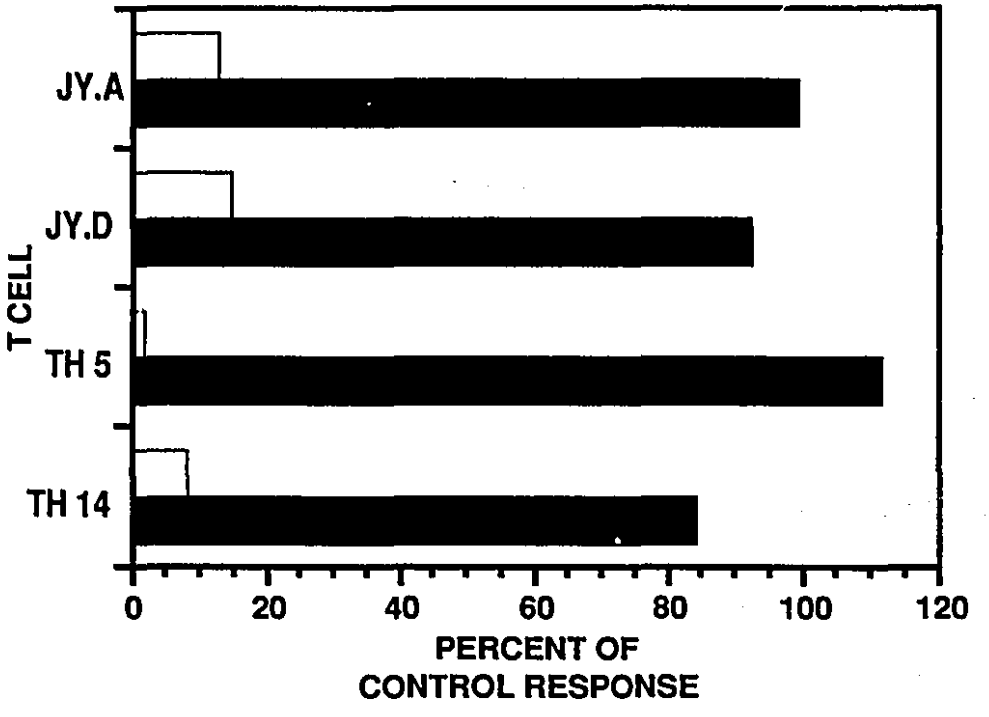

FIGURE 24. Determination of the Lymphokine (IL-2/IL-4) Secreted by the T Cell Lines and Hybridomas Stimulated with PG

The antigens used were HFPG [20μg(protein)/ml] for T cell lines JY.A, JY.D and T hybridoma TH5, and HAPG [20μg(protein)/ml)] for T hybridoma TH14. Anti-IL-2 (CR-IL-2)(□) ; anti-IL-4 (11B11)(■). Both antigens were CS-depleted. Control responses (100%) represent the maximal responses of the T cell lines and T cell hybridomas with their respective PG antigens in the absence of any antibody.

both T cell hybridomas, and the degrees of the responses were similar to those elicited with BALB/c APC at all antigen concentrations tested (Table 5).

6.2.8.3 TH1 VS TH2

Addition of anti-IL-2 antibody (5μg/ml) to the culture media from the T cell hybridomas and the T cell lines stimulated with antigen and feeder cells inhibited the proliferation of CTLL-2 cells, whereas the anti-IL-4 antibody (5 μg/ml) had no effect (Figure 24). CTLL cells did respond to recombinant IL-4 (Genzyme) and this response was blocked with 11B11 as previously shown in the Materials and Methods section (Figure 10 and Figure 11). These data indicated that the two T cell lines and the two hybridomas have a lymphokine secretion pattern ($IL\text{-}2^{+}$,$IL\text{-}4^{-}$) similar to the TH1 subsets of T helper cells (Mosmann et al., 1986) (Table 6).

6.2.8.4 TCR Vβ FAMILIES

A preliminary screening of the Vβ TCR usage by the T cells using two anti-Vβ TCR antibodies showed that TH14 was $V\beta8.2^{+}$ ($F23.1^{+}$,$KJ16^{+}$,$F23.2^{+}$), while TH5 was found to be $V\beta4^{+}$. T cell lines JY.A and JY.D were $V\beta8^{-}$,$V\beta6^{-}$ (Figure 22, Table 6), but they were not tested with the other anti-Vβ TCR families since the T cells were not cloned.

TABLE 6. Summary of The T Cell Phenotypes

T Cells	CD4	CD8	LYMPHOKINE (IL-2/IL-4)	DOMINANT MHC II RESTRICTION	Vβ T CELL RECEPTOR
· JY.A	+	−	IL-2	I-E^d	n.t.
· JY.D	+	−	IL-2	I-A^d	n.t.
· TH5	+	−	IL-2	I-A^d	$V\beta4^+$
· TH14	+	−	IL-2	I-A^d	$V\beta8.2^+$

n.t.: not tested

6.3 DISCUSSION

The reactivity profiles of spleen-derived T cells with regard to the age of the cartilage from which the PG was obtained showed that the T cell response was stronger against HFPG (which is the immunogen) than HAPG at equivalent molar concentration (Figure 12) indicating differences in recognition of the two molecule by the T cells. Qualitative differences between the two molecules may explain the differences in reactivities between HFPG and HAPG. These differences will be discussed later.

Early T cell lines were also used to study the age-related T cell responses to PG. These were the same as for the splenocytes. The T cells showed reduced reactivity to HFPG aggregate (A1) compared to PG monomer (Figure 14). It is possible that a large aggregate of HFPG, LP and HA of over five million kD is not efficiently endocytosed by APC and/or that some epitopes are protected from antigen processing and therefore not readily available for immune recognition. The proteolyticaly or chemically predigested aggregate increased the reactivity of the T cells perhaps by presenting the "pre-processed" antigen more efficiently by the APC. The G1, as well the LP have been shown to be protected from proteolytic digestion when complex with HA (Heinergård and Hascall, 1974; Faltz et al., 1979).

Two patterns of antigenic reactivities were recognized at the definite T cell line/hybridoma levels. The first pattern was characterized by reactivities to HFPG, HAPG, BFPG, the bovine G1 domain and LP (T cell line JY.D and T cell hybridomas TH5 and TH14). The G1 domain is known to have structural similarities with LP (Neame et al., 1987). There are two regions containing tandem repeats of sequences in each molecule, which are

involved in binding with HA (Goetinck et al., 1987; Doege et al., 1991). The B and B' regions in G1 are homologous with the corresponding regions in LP. The homology between the G1 domain of RCSPG and the corresponding regions of rat LP is 41 % (Neame et al., 1987, Doege et al., 1987) and between the G1 domain of human PG (Doege et al., 1991) and the whole human LP (Dudhia and Hardingham, 1990) the homology is 32 %. Since there is significant homology between bovine and human G1 domains (Doege et al., 1991; Neame et al., 1987; Sandy et al., 1990) and partial homology between bovine LP (Neame et al., 1987) and bovine G1 (Sandy et al., 1990), it is presumed that the epitope(s) on human PG and bovine G1 recognized by these PG-reactive T cells resides in the regions of the G1 domain homologous with LP. However, the reactivities to bovine LP by the three bovine G1-reactive T cells are much lower than to the PG and G1 molecules themselves (Figure 16, and Figure 18). This is suggestive of the fact that the T cell epitope(s) is partially but not totally contained in the G1-LP homologous regions or that LP is processed differently to PG and G1 and is less efficiently presented to the T cell receptor.

The data show that the G1 domain on PG bears immunodominant epitope(s). This may be of some pathological significance in arthritis. An earlier study in this laboratory had shown that one of the fragments that is most commonly present in the synovial fluid of arthritic patients is the G1 region of PG (Witter et al., 1987). Almost 40% of the total G1 in the synovial fluid in one of the patients was found to be functionally capable of forming HA aggregates. This suggests that both denatured/degraded as well as native G1 can be released from articular cartilage by enzymatic cleavage and/or damage due to oxygen radicals in various arthritides (Witter et al., 1987).

These G1 fragments could potentially elicit T cell responses in the joints and thereby perpetuate inflammation in human arthritides as well as in animal models. The presence of G1 in synovial fluids of RA patients, especially in the more severe and chronic forms of arthritis, has recently been confirmed (Saxne and Heinegard, 1992).

The second pattern of PG-reactive T cell responses (T cell line JY.A) was characterized by reactivity against HFPG, but not to HAPG nor to LP and the other above antigens . The possible basis of this difference in reactivity is described below.

A third pattern of reactivity was observed with two other cell lines which were not the object of further characterization. These T cell lines (JY.G and JY.H) may or may not recognize epitope(s) on fragments released from PG during further purification since they responded to the D1 preparation but not to the D1D1 preparation but responded well to the D1D3 preparation. In contrast, JY.A did respond to both preparations. These two T cell lines did not recognized the purified bovine G1. It is likely that the T cell lines JY.G and JY.H recognize a contaminant(s) in the PG preparation but JY.A recognized a PG epitope present also in the D1D3 fraction probably as a degradation product.

This studies clearly showed that different T cell epitopes on human PG are recognized by the two T cell lines JY.A and JY.D, and that fetal and adult human PG express different T cell epitopes. Age-related differences in cellular and humoral reactivities to PG have earlier been described in rabbits (Champion et al., 1982) and mice (Glant et al., 1986) using different human PG preparations. The sequencing of the cDNA of PG (aggrecan) isolated from fetal and juvenile human articular cartilages has shown that the G3 region

may contain alternatively spliced forms containing epidermal growth factor-like regions and/or a complement regulatory-protein like region in addition to a lectin-like region (Doege et al., 1991, Baldwin et al., 1989). The actual proportion of PG molecules at the protein level with or without the above alternatively spliced segments is not yet known either in fetal or adult cartilage (Doege et al., 1991). However, a high proportion (70 %) of mature cartilage PG monomers has been shown to lack the G3 domain by rotary shadowing in one study (Paulsson et al., 1987). Differences in the amount of G3 domain itself or in the regions in the G3 domain present in fetal vs adult PG may explain the difference in reactivity of the T cell JY.A. It may also explain the greater arthritogenicity of fetal PG over adult PG.

The differences in reactivities to HFPG and HAPG may also be related to the known differences in the contents of CS and keratan sulfate (KS) chains in the PG molecules (Roughley and White., 1980; Glant et al., 1986). HAPG is known to have more KS chains and fewer CS chains on the core protein than HFPG (Roughley and White., 1980). It is possible that glycosaminoglycan chains attached to the core protein of PG could interfere with the antigen processing of PG by antigen-presenting cells, as it has been shown with exogenous carbohydrates in a previous study on antigen presentation (Leyva-Cobian and Unanue, 1988). This may equally explain why the development of arthritis is observed less often after hyperimmunization with native human PG than with the human PG treated with chondroitinase, which may make the arthritogenic epitopes more accessible for recognition by T cells *in vivo* .

HAPG possess more KS chains on its core protein than HFPG but is yet better or as good at eliciting a response by the G1-domain reactive T cells lines

and hybridomas. This may be explained by the fact that the increase in KS substitution of HAPG in the KS rich region enhances antigenic recognition at the level of APC uptake and processing. KS may enhance uptake into APC and protect epitopes against degradation, although there is no evidence for this. However in HFPG there may be KS substitution closer to the G1 domain as is known for the pig and bovine G1 (Fosang and Hardingham, 1991; P. Neame and L. C. Rosenberg personal communication) compared to HAPG. This remains to be established. The increased responses of the G1 domain-reactive T cells to HAPG as compared with HFPG could also be explained by the shorter sizes of the core proteins of the majority of HAPG molecules as compared to HFPG (Webber et al., 1987). Hence a higher molar concentration of the former would be present when compared on a weight basis. The difference in sensitivities between the two hybridomas to various PG preparations could be due to differences in T cell receptor densities or the generation of intracellular second signal messages in these cells. These hybridomas are not derived from sister clones as evidenced by the fact that they have different Vβ TCR as shown by anti-Vβ4 (TH5) and anti-Vβ8 (TH14) TCR antibody staining (Figure 22).

The lack of reactivity of the G1 reactive T cells to MPG could be due to many possibilities. The first to consider is the degradation of MPG during purification leading to loss of the T cell epitopes. However, since extreme care was taken during MPG purification (see Materials and Methods) and SDS-PAGE analysis did not reveal any degradation of MPG, this was probably not the reason. If the G1 domain of MPG was cleaved, in part or in total, the immunoreactive fragments could possibly have been recovered in the upper fractions of the CsCl density gradient (D2 and D3). These fractions were tested

with the hybridomas and were not found to be antigenic. The lack of reactivity to MPG of the G1 reactive T cells could be due to sequence differences between human and mouse PG in the G1 region, as is seen between the sequences of bovine (Sandy et al., 1990, and P. Neame, unpublished data), human (Doege et al., 1991) and rat PG (Doege et al., 1987) in this region. However the MPG sequence was revealed recently (Glant et al. 1992) and showed similarity to the other PG in the conserve B and B' region of the G1 domain but differences as with the other species, in the N-terminal Ig fold region.

A recent study in another autoimmune disease model has shown that a pathogenic T cell clone shows lack of reactivity to the self antigen (rat retinal S protein) when tested *in vitro* in experimental autoimmune uveoretinitis in rats (EAU) (Fling et al., 1991). In addition, pathogenic T cells in EAU may be reactive with cryptic peptides of the self-antigen without showing any reactivity to the native molecule *in vitro*, possibly due to differences in processing by APC *in vitro* and *in vivo* (Lipham et al., 1991). Therefore non-reactivity to self-antigens, in this case MPG, *in vitro* does not necessarily signify that the T cells may not be pathogenic *in vivo*. Because of the possibility that normal irradiated spleen cells may lack the suitable APC and that activated B cells are known to be a very efficient APC (Chesnut and Grey, 1981; Krieger et al., 1985), a series of experiments were designed to use B cells as APC (Gontijo and Möller, 1991) with the hope of getting a response to MPG. However the T cell hybridomas never responded to MPG in any of the condition assayed. Differences in antigen processing of APC is still a possibility since purified dendritic cells have not been tested as APC. It is possible that the only apropriate APC which can present autologous MPG

may reside in the joint itself. However two attempts were made to derived MPG reactive T cell lines from the joint of arthritic mice using T cell cloning techniques described above but these were unsuccessful.

Cartilage PG and LP have been proposed to be crossreactive to the mycobacterial 65 kD HSP in the rat adjuvant arthritis model (van Eden et al., 1985; van Eden et al., 1988). Neither of the T cell hybridomas which recognize the G1 domain recognized the 65 kD HSP suggesting that the crossreactive epitope(s) is not recognized by these T cells.

The two $CD4^+$ PG-reactive T cell lines and two $CD4^+$ PG reactive T cell hybridomas from mice with arthritis induced by HFPG were extensively characterized for their phenotypes. These T cells apparently belong to the TH1 subset since they secrete IL-2 but not IL-4 (Figure 24). The T cell lines and hybridomas are MHC class II restricted as shown by complete inhibition with the anti-Ia antibody M5/114. The two hybridomas and the T cell line JY.D are predominantly I-A^d restricted while JY.A is predominantly I-E^d restricted (see summary Table 6 and Figure 23). The partial inhibition of the responses by the MKD6 (anti-I-A^d) antibody for JY.A, and MKD6 and 14-4-4S (anti- I-E^d) antibodies for JY.D may be explained by the presentation by mixed heterodimers as described below, or by heterogeneity in T cell clones in the T cell lines. The incomplete blockage of responses for TH14 by MKD6 could be explained by the presentation of the epitopes by MHC class II heterodimers ($A_\alpha{}^d E_\beta{}^d$) besides the more prevalent $A_\alpha{}^d A_\beta{}^d$ homodimers (Bhattacharya et al., 1981). Since MKD6 recognizes $A_\beta{}^d$ and 14-4-4 recognizes $E_\alpha{}^d$ (Bhattacharya et al., 1981; Buerstedde et al., 1988; Ruberti et al., 1991), these antibodies would not block presentation by $A_\alpha{}^d E_\beta{}^d$ heterodimers that may also present T cell epitopes to TH14. In contrast, the epitope recognized by

TH5 only in the context of $A_{\alpha}^{d}A_{\beta}^{d}$ since MKD6 totally blocked the response but 14-4-4S had no significant effect.

CHAPTER VII: CHARACTERIZATION OF THE T CELL EPITOPES: NATURE OF THE T CELL EPITOPE(S) AND IDENTIFICATION OF THE SEQUENCE STRUCTURE.

7.1 INTRODUCTION

As described above, the epitopes recognized by the two T cell hybridomas (TH5 and TH14) on PG reside in the G1 domain. It is also known that the epitopes on human PG recognized by these T cell hybridomas are also present on LP. This chapter describes studies designed to determine the location of the epitope recognized by TH5 and shows that TH5 and TH14 probably recognize different epitopes in this region. In addition, the presence of keratan sulfate on G1 was confirmed in the present study.

7.2 RESULTS

7.2.1 BIOCHEMICAL ANALYSIS FOR THE PRESENCE OF OLIGOSACCHARIDES AND GLYCOSAMINOGLYCANS ON THE PURIFIED BOVINE G1 DOMAIN

Radiolabelled ^{125}I-G1 was digested with several glycosidases and electrophoresed on 12.5 % SDS-PAGE along with undigested ^{125}I-G1, (Figure 25 and Figure 26). Faster migration of this protein band is indicative of the cleavage and partial or complete removal of specific carbohydrate chains by a glycosidase. Treatment of the purified bovine G1 with keratanase I decreased the Mr of G1 compared to the native molecule while treatment with chondroitinase ABC did not, suggesting that the G1 contained a KS-chain(s) but not CS-chain(s) (Figure 25). The G1 was treated with other glycosidases. As compared to keratanase I (*Pseudomonas sp.*) which hydrolyzes 1,4 β-galactosidic linkages where non-sulfated galactose residues participate, keratanase II hydrolyzes the 1,3-β-glucosaminidic linkages to galactose in KS.

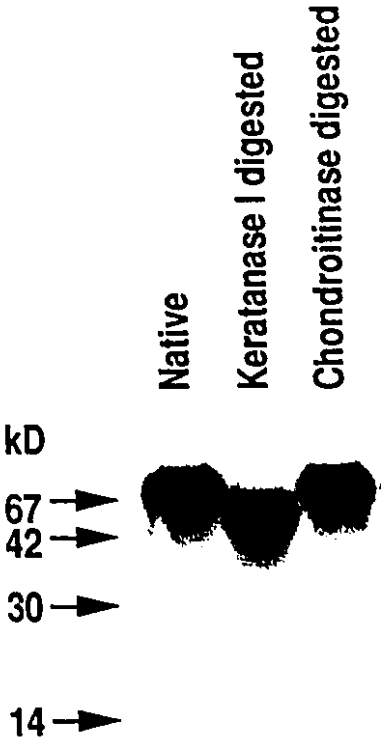

FIGURE 25. SDS-PAGE of Radiolabeled ^{125}I-G1 .I. Influence of Keratanase, and Chondroitinase Digestion

Native G1 or after treatment with keratanase I or chondroitinase ABC, were electrophoresed (10,000 CPM/lane) under reducing condition on a 12.5 % polyacrylamide gel. The gel was dried and autoradiographed.

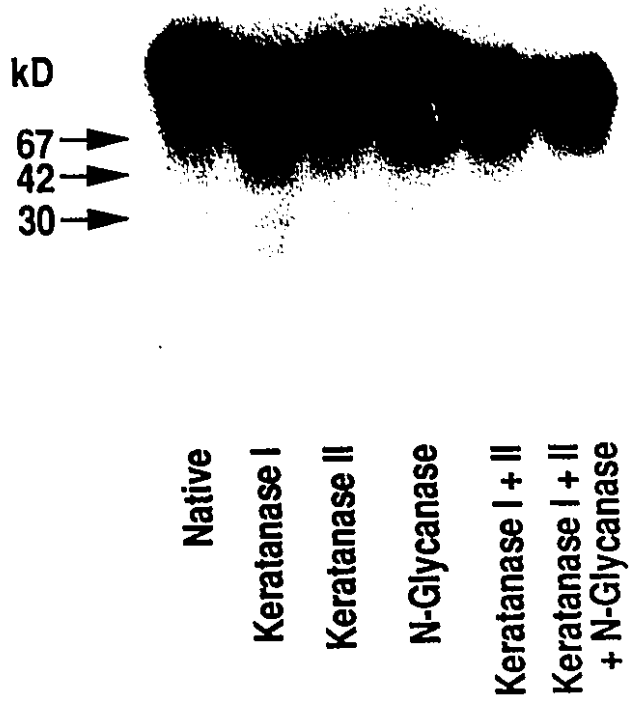

FIGURE 26. SDS-PAGE of Radiolabeled ^{125}I-G1 .II. Influence of Digestion with Other Glycosidases

Native G1 and after treatment with keratanaseI, keratanase II, N-glycanase, a combinaison of the two keratanases, and a combination of both keratanase I and II with N-glycanase, was electrophoresed (10,000 cpm/lane) under reducing conditions on a 12.5 % polyacrylamide gel. The gel was dried and autoradiographed.

This latter enzyme requires sulfation at the 6 position on the glucosamine residue but acts independently of the 6 sulfation of galactose. N-glycanase hydrolyses asparagine-linked oligosaccharides to leave a peptide/protein free of oligosaccharide with an aspartic acid instead of asparagine, and this enzyme is known to cleave all types of N-linked oligosaccharide (Elder and Alexander, 1982). Mobility of native ^{125}I-G1 on SDS-PAGE was then compared to the ^{125}I-G1 after digestion with keratanase I, keratanase II, N-glycanase, a combination of both keratanases and a combination of the two keratanases plus N-glycanase. All of these treatments produced a shift in mobility of G1 (figure 26). The highest shift was observed with keratanase I and the combination of the three enzymes. This shift was only slightly more than what was produced with keratanase II suggesting that the KS chains account for most of the carbohydrate mass. Both N-glycanase and keratanase II treatments resulted in a reduction in Mr of native G1 but less than that seen for keratanase I. N-glycanase results indicated the presence of N-oligosaccharide(s) on G1 as indicated earlier (Lohmander et al., 1980; De Luca et al., 1980; Nilsson et al., 1982). It has been shown recently that KS chain(s) are not the N-linked type (Barry et al., 1992). Had they been N-linked the results for keratanase I and N-glycanase might have been similar.

7.2.2 CNBr PEPTIDES OF BOVINE G1

7.2.2.1 SDS-PAGE AND IMMUNOBLOTTING

Purified bovine G1 in native form, treated with keratanase I or treated with CNBr, was electrophoresed on a 12.5 % polyacrylamide gel and transferred onto a PVDF membrane for aurodye staining and immunoblotting with an anti-G1 + G2 antibody (1C6), and with two anti-KS

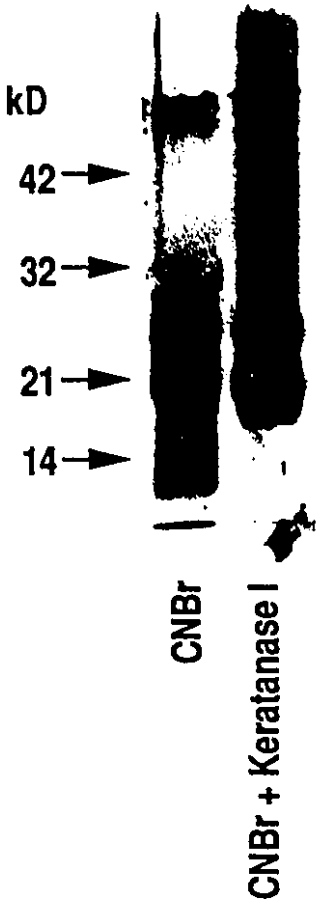

Figure 27. Aurodye Staining of CNBr-treated G1 Before and After Keratanase Digestion

CNBr-treated G1, before and after treatment with keratanase I (20μg/lane), was electrophoresed under reducing condition on a 12.5% polyacrylamide gel. Material was electrotransferred onto a PVDF membrane and stained with aurodye.

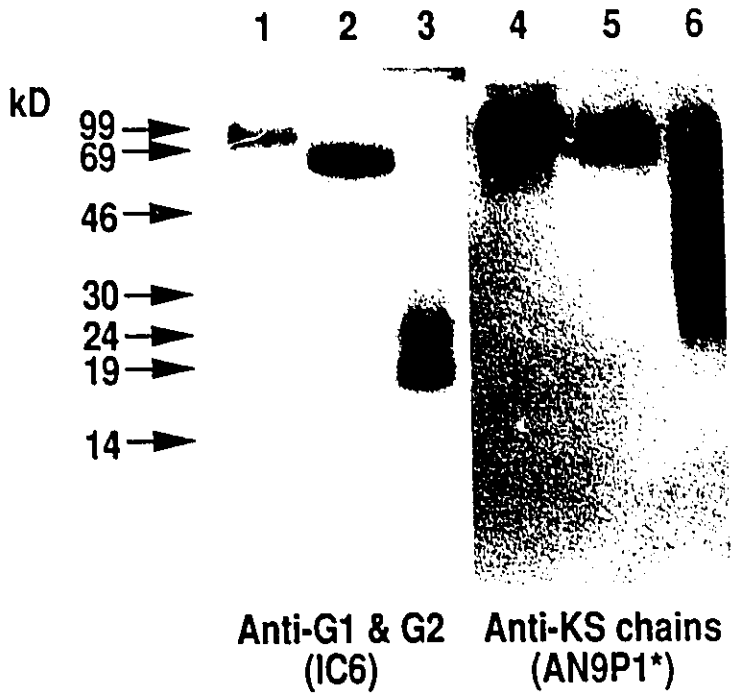

FIGURE 28. Immunoblotting of the Purified Bovine G1

G1 native (lanes 1 and 4) or treated with keratanase I (lanes 2 and 5) or treated with CNBr (lanes 3 and 6) was electrophoresed on a 12.5 % polyacrylamide gel under reducing conditions and transfered onto PVDF membrane which was subjected to Western blotting with the anti-HABR mAb 1C6 (lanes 1 to 3) and with the anti-KS mAb AN9P1 (lanes 4 to 6).

antibodies AN9P1 and 5D4. The aurodye staining of native and keratanase I treated G1 confirmed the reduction in Mr of G1 after keratanase digestion that was observed with radiolabelled G1 (Figure 27). G1 treatment with CNBr (Figure 27) produced multiple fragment peptides of Mr 13, 15, 17, 19.5 and 24 kD with a wide smear beginning at around 30 kD up to 70 kD. When the CNBr treated G1 was also treated with keratanase it was possible to distinguish a faint band within the smear (Figure 27). The presence of KS on G1 was also indicated by alcian blue staining of the native G1 molecule which was markedly diminished after keratanase digestion (data not shown). Western blotting with anti-KS antibody AN9P1 of the CNBr G1 digest revealed much polydispersity of CNBr fragments bearing KS chains (Figure 28). As shown in lane 4 in Figure 28, the anti-KS antibodies stained undigested G1 showing the presence of KS on the purified G1. However after keratanase treatment (Figure 28, lane 5) the anti-KS antibody (AN9P1) is still reactive with the G1. This may be explained by the fact that some KS remains attached to core protein after keratanase and this bears epitopes recognized by the antibody. When analysed by immunoblotting with the 1C6 Ab, the native form of G1 migrated as a single band with an apparent Mr of 71.4 kD, but after treatment with keratanase the G1 migrated with a smaller Mr of 64.5 kD. The treatment with CNBr produced two smaller bands of 19 and 24 kD recognized by the 1C6 antibody (Figure 28, lane 3). IC6 has been shown earlier to bind to only one non-repetitive epitope on G1 (Fosang and Hardingham, 1991). This epitope does not contain methionine residues which can be CNBr cleaved. This suggests that there may be two forms of G1 present in the preparation, perhaps due to differences in post translational modification such as ± N-linked oligosaccharide as is observed with LP (Nguyen et al., 1990). Neither of the lower and higher Mr. bands which stained for the 1C6 epitope stained

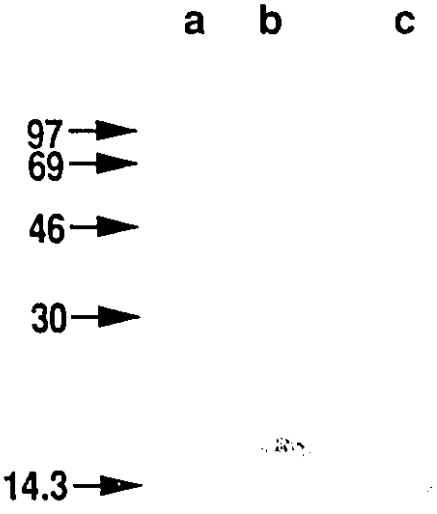

FIGURE 29. Immunoblotting of CNBr-treated G1 After N-Glycanase Digestion
Immunoblotting with Anti-G1 Ab 1C6 of CNBr Treated G1 Before Treatment (lane a) and After Keratanase I (lane b) and Keratanase I Plus N-glycanase (lane c) Digestion

with the anti-KS antibody. The high Mr band was close to the AN9P1 staining, but was 4-5 kD smaller than the lower limit of the smallest KS stained band in other gels. Also keratanase treatment did not alter the mobility of the two 1C6 staining bands (Figure 27), which indicates that these fragments do not contain KS chains. Furthermore N-glycanase treatment of CNBr and keratanase treated G1 (Figure 29 lane c) converted the two 1C6 bands of 19 and 24 kD to one smaller band of about 14 kD. As described below, both bands have the same N-terminal sequence. This indicates that the two bands containing the 1C6 epitope have the same N-terminal sequence but vary in size because of differences in N-linked oligosaccharide content.

7.2.2.2 SEQUENCING

The CNBr -digested bands of G1 transferred to a PVDF membrane were visualized with Coomassie blue and subjected to N-terminal sequencing by techniques described earlier (Matsudaira, 1987). Only the most clearly defined bands below the KS chain smear were submitted to sequencing (Figure 30). The smallest band of 14 kD had the sequence EGEVFYATSP which is the smallest CNBr fragment predicted from the known bovine G1 sequence described by Neame et al. (1987). This CNBr fragment is found C-terminal to the 1C6 CNBr fragment in the B' region. The two close fragments of apparent Mr of 15-16 kD both have the N-terminal sequence: HPVTTAPTTA (Figure 30). This fragment is located in the Ig fold (Doege et al., 1991; Neame et al., 1987) and represents the second CNBr fragment from the N-terminus of the whole G1. There may be three explanations for the existence of these two CNBr fragments. One would be the partial cleavage by CNBr at the first methionine such that there may be differences in the C-terminal end since there are two close methionines (M 104 and M 118, separated by 14 amino

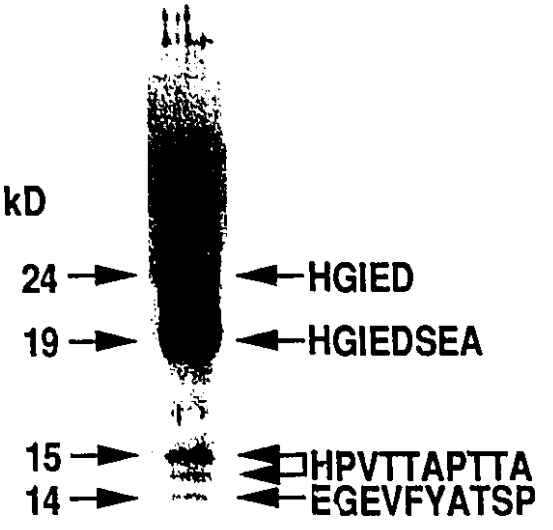

FIGURE 30. N-terminal Sequences of G1 CNBr Peptides

The CNBr-digested G1 was electrophoresed on PVDF membrane after electrophoresis and stained with Coomassie blue. The bands were excised and membrane bound peptide analysed for N-terminal sequence.

acids). The second could be due to two different sequences of the same molecule in this G1 preparation where one would have the methionine at position 104 and the alternate sequence could have a lysine instead, like the human sequence (see figure 33). However the most likely explanation could simply reside in glycosylation differences between the two fragments. The 19 and 24 kD bands showed the same N-terminal sequence as expected. Their different apparent Mr is therefore due to N-linked carbohydrate since treatment with N-glycanase led to a single 1C6 containing band as described above. Because these two bands have the same core peptide length the difference must be explained by differences in size of the N-linked oligosaccharide chains in these peptides perhaps due to differences in branching.

7.2.3. REACTIVITIES OF THE T CELL HYBRIDOMAS TO THE CNBr FRAGMENTS: THE BLOTTED PEPTIDE APPROACH

In order to help identify the epitope recognized by the T cell hybridomas on the G1 domain several techniques were used. Reverse phase HPLC purification of the diffrent fragments (C18 Bondapack, Aplied Biosystem) using TFA and acetonitrile as eluent, was unsuccessful. As were few attempts using ion exchange HPLC chromatography with MONO-Q and MONO-S columns. However the use of membrane bound peptide antigen technique to screen peptide immunoreactivity proved useful (Abou-Zeid et al., 1987; Lamb and Young, 1987). This technique relies upon the capability of SDS-PAGE to separate the CNBr fragments. Instead of eluting the band from the gel, these are transferred to a membrane which is stained, and the bands can be cut out and used directly or as fine particulates in T cell response assays

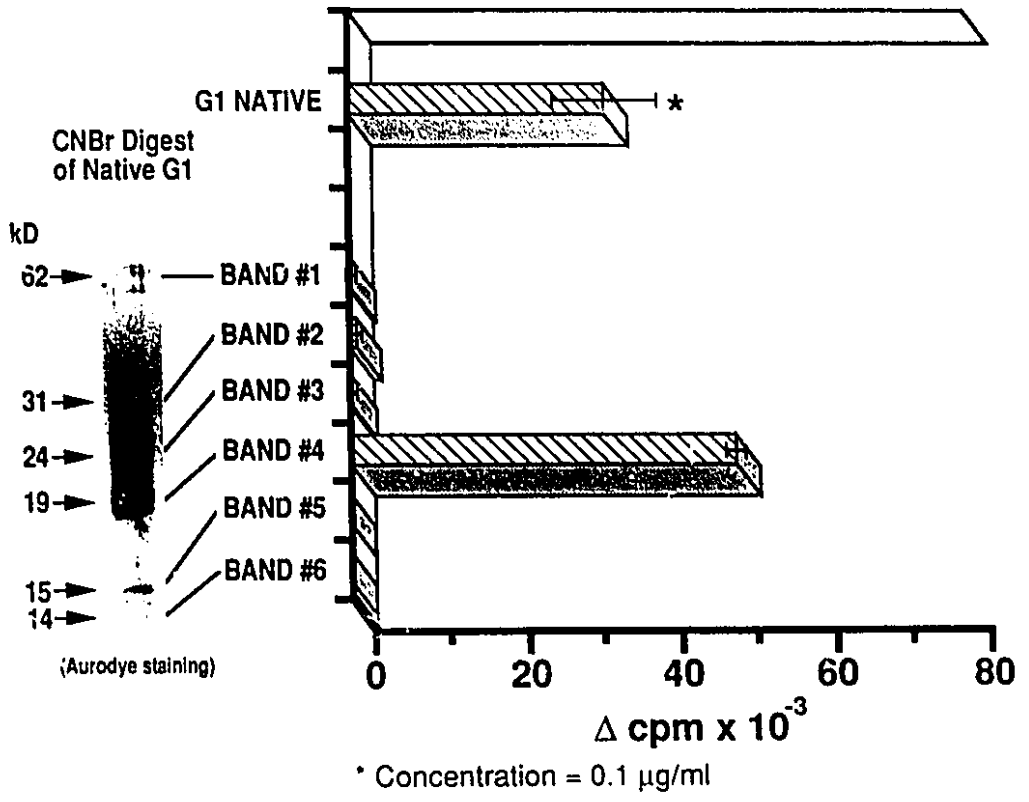

FIGURE 31. Reactivity of TH5 to the CNBr Peptides of the G1 Domain Blotted on PVDF Membrane

(Abou-Zeid et al., 1987). This technique can be used with recombinant antigens where a mixture of proteins is difficult to purify (Lamb et al., 1988). The technique is particularly useful for epitope mapping when it is used with specific chemical and proteolytic cleavages of protein antigens (Demotz et al., 1989). One important limitation of this technique is that the fragments must be of reasonable size so they can be resolved by electrophoresis. Disadvantages included the limited amount of protein that can be loaded on each lane of the gel and the difficulties estimating the exact amount of protein in each band. Figure 31 shows the reactivity of TH5 to the CNBr peptides of G1 using this technique. The data show that TH5 recognized the 19 kD band which has the HGIED sequence and is stained with the 1C6 Ab. Surprisingly TH5 did not recognize the 24 kD band which has the same N-terminal sequence and also stains with 1C6. This was repeated four times with the same results except on one occasion where a small response to the higher Mr. fragment was observed. It is possible that differences in the post-translational modification in these peptides such as N-linked glycosylations as described above may be responsible for the differences in reactivity of TH5 to the two peptides. However treatment of soluble G1 native with N-Glycanase only resulted in lower reactivity of TH5 at suboptimal concentrations of antigens: G1 native at 5 and 50 nM gave the optimal Δ CPM of 10 380 and 9134 respectively compared to 2653 and 11 402 for G1 treated with N-glycanase at the same concentrations. TH14 has shown reactivity to the lowest fragment (14 kD) of CNBr treated G1 in one experiment. This result was not repeated in other experiments. It is possible that the amount of antigen contain in the band is at the limit needed for stimulating the reactivity of the low sensitive TH14 hybridoma.

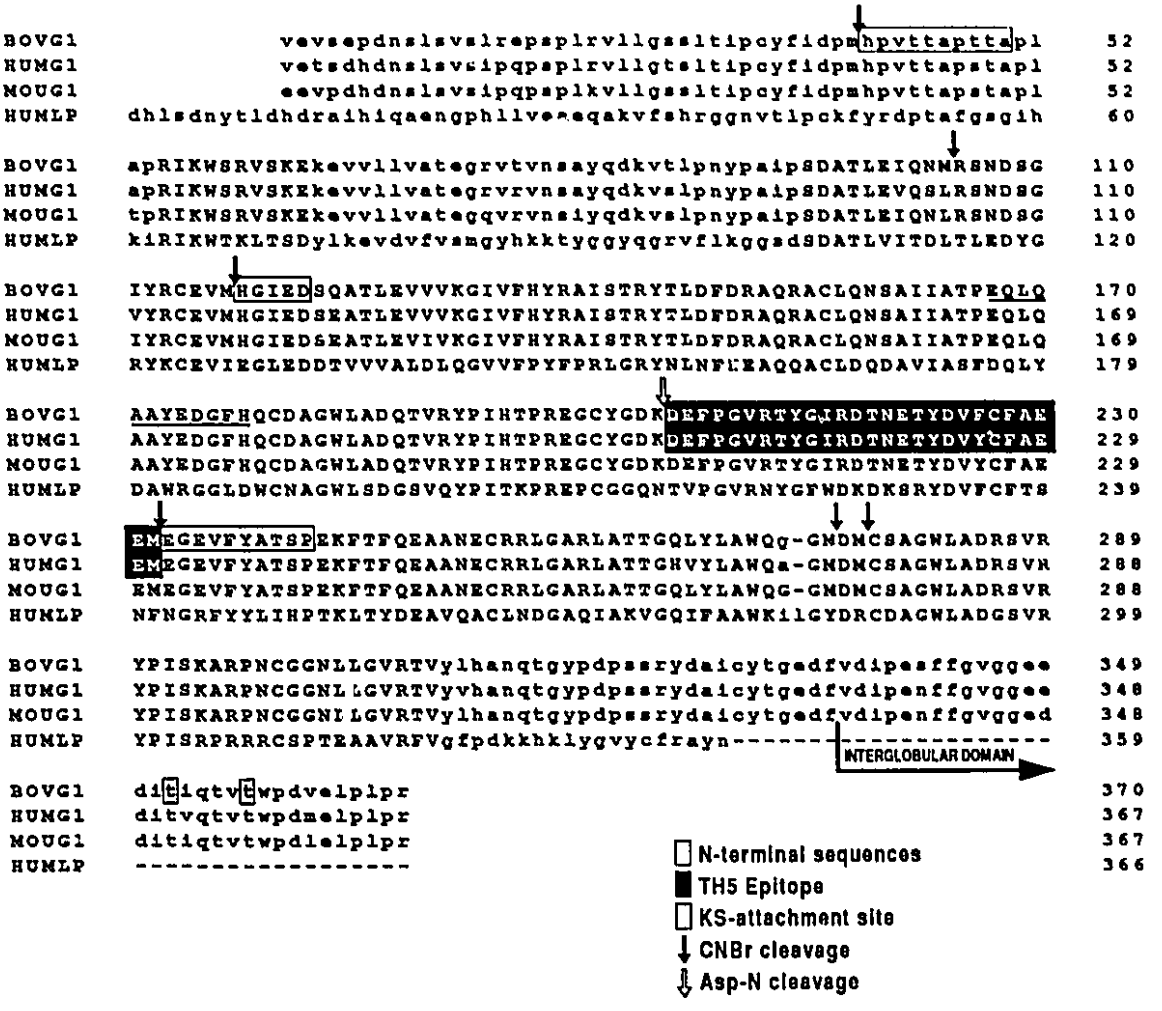

FIGURE 32. Sequence Alignment of Human G1, Bovine G1, Mouse G1 and Human LP

Uppercase letters represent region of homology. Black arrows are CNBr cleavage sites. Open arrow is endo Asp-N identified cleavage site. Shaded area is the region recognized by TH5. Underline is 1C6 epitope. The sequences are from Doege et al.,1990 for Human G1; Neame and Rosenberg for Bovine G1; Glant et al., 1992 for Mouse G1; and from Dudhia and Hardingham, 1990 for Human LP.

7.2.4 SYNTHETIC PEPTIDES

The mapping of T cell epitopes by using the technology of synthetic peptides has proven useful, particularly in combination with other approaches such as recombinant proteins (Lamb et al., 1987). This approach was used mainly for TH5 epitope because of its known location in a specific CNBr fragment. Peptides were synthesized on the basis of two parameters. The first parameter concerned the sequence homology between the known sequence of bovine and human G1 domain and human LP, since TH5 recognized all three species. This was achieved by using the computer program MACAW which can perform sequence alignment and analyse different protein sequences (Schuler et al., 1991) (Figure 32). Secondly it was based on the identification of putative T cell epitopes by the program T sites which predicts T cell epitopes sequences by different models of peptide sequence motif analysis; the Rothbard motif (Rothbard et al., 1988), the amphipathic algorithm analysis (Margalit et al., 1987), and the I-A^d and I-E^d binding sites (Sette et al., 1989) (Figure 33). A series of 7 peptides with lengths varying between 22 and 27 amino acids were synthesized and purified as described in the Materials and Methods, based on the human G1 domain sequence. Depending on their acidic/basic characteristics as well as their hydrophobicity scores, the peptides were dissolved accordingly in the appropriate buffer (acidic or basic). However, most of these peptides were soluble in deionized water at high concentration and were diluted in PBS or culture media before use. On occasion when solubilisation presented a problem, the peptides were dissolved in DMSO first and then diluted with deionized water. The peptide sequence positions in the G1 domain are indicated in Figure 33. None of the seven synthetic peptides revealed any

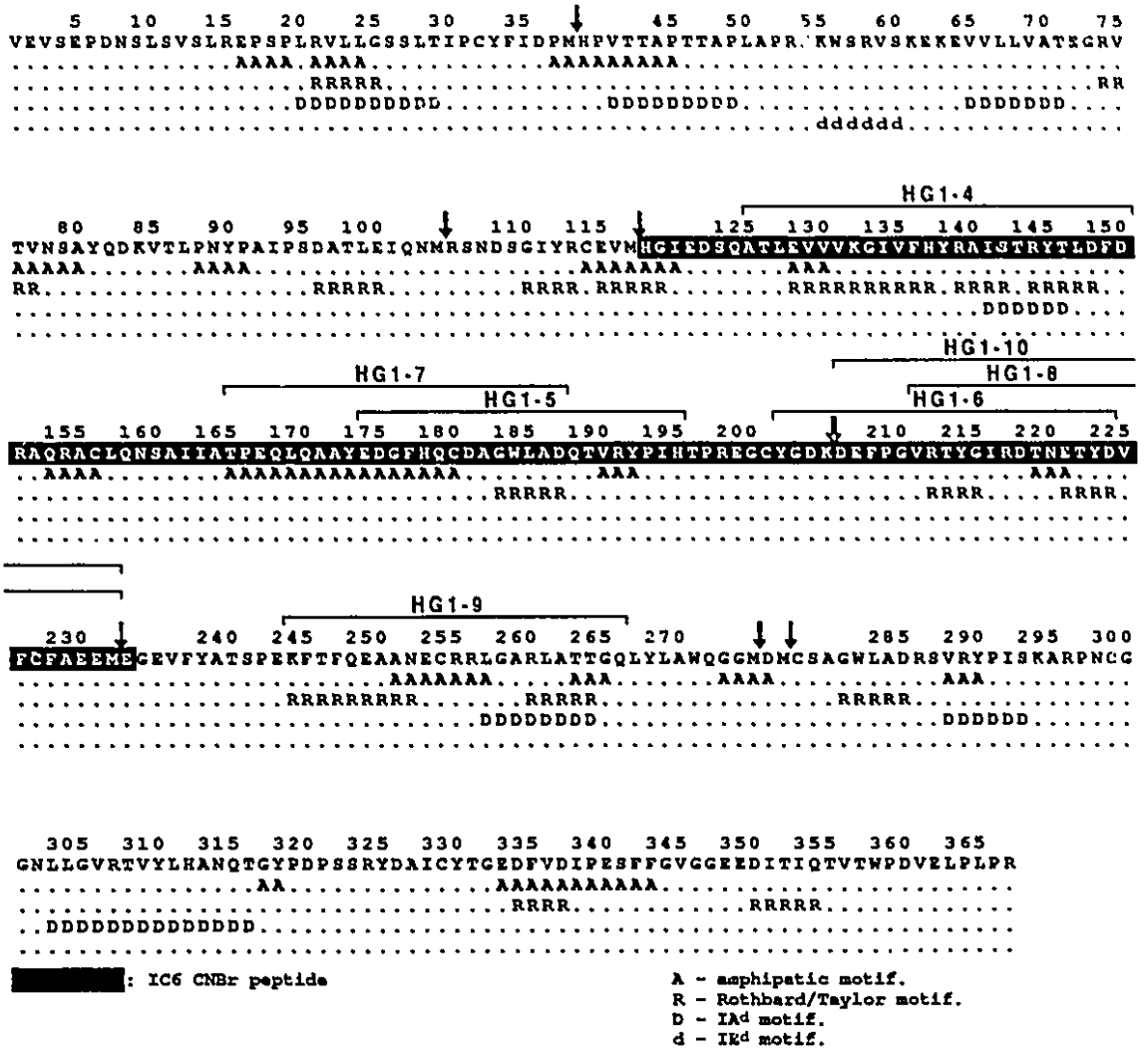

FIGURE 33. Identification of Putative T Cell Epitopes On Bovine G1 Using Program T Sites*

The underlining represents; A: Amphipathic motif; R: Rothbard/Taylor motif; D: IA^d motif; d: IE^d motif. Black arrows are CNBr cleavage sites. Open arrow is endo Asp-N cleavage site identified. ■ : 1C6 CNBr peptide.

*Medimmune, Inc., Gaithersburg, MD.

immunoreactivity with either TH5 nor TH14. Most of them were prepared from sequences in the 1C6 staining CNBr fragment in order to locate the TH5 epitope. These peptides were used in the T cell assays under different conditions. To avoid the possibilities of peptide interference by serum protein such as non-specific binding and masking of the peptides by proteins such as albumin or α2-macroglobulin, serum-free medium was used. None of these attempts led to T cell reactivity. This lack of reactivity of the synthetic peptides could be due to a small difference in peptide sequence where the addition or omission of a single amino acid to the exact T cell epitope can have profound effect on the T cell reactivity as in a recent exemple of HEL peptide 47-61 where the deletion of a single C-terminal amino acid residue increased significantly the T cell reactivity (Kim and Jang, 1992). In addition N-linked oligosaccharide could be part of the T cell epitope as it has been proposed in another instance (Ishioka et al., 1992).

7.2.5 T CELL EPITOPE MAPPING OF G1

Chemical and proteolytic cleavages of known specificity (Carey, 1989; Aitken et al., 1989) were used as an alternative approach in the determination of T cell epitopes. The purified G1 domain was submitted to digestion with O-iodosobenzoic acid (Mahoney et al., 1981), trypsin, chymotrypsin, endo-Glu-C, endo-Arg-C, endo-Asp-N and endo-Lys-C. The specificity of the treatment and the relative reactivity of the two T cell hybridomas TH5 and TH14 are indicated in Figure 34. The reactivities are compared to the native G1 and the CNBr treated G1. The latter treatment was shown to increase the reactivity of both hybridomas. O-iodosobenzoic acid digestion (which cleaves at the C-terminal end of tryptophan), was performed on the TH5 reactive 1C6 staining

Treatment	Cleavage site on core protein	Relative reactivity TH5	TH14
No treatment	—	+++	+++
CNBr	–M↓–X–	++++	++++
O-iodosobenzoic acid	–W (Y)↓–X–	–	N.D.
Trypsin	–R (K)↓–X–	++	–
Chymotrypsin (immobilized)	–F↓–X– (F>W>Y)	–	–
Endo Glu-C (V8) (immobilized)	–D (E)↓–X–	+++	–
Endo Arg-C (immobilized)	–R↓–X–	+++	+
Endo Asp-N	–X–↓D–	+++	++++
Endo Lys-C	–K↓–X–	+++	++++

FIGURE 34. T Cell Epitope Mapping of G1

Single letter amino acid code is presented. Arrows indicate the cleavage site (N or C terminal) of the specific amino acid residue. Amino acid in brackets represent second susceptible residue for cleavage. X represents any amino acid. N.D. = not determined

CNBr band extruded from the nitrocellulose membrane. This digestion was based on the technique described by LeGendre and Matsudaira (1989). The digestion products were assayed with TH5, and this treatment was compared with control untreated 1C6 staining 19 kD CNBr band. O-iodosobenzoic acid treatment abrogated the TH5 reactivity of the membrane bound peptide. Trypsin treatment of G1 partially diminished TH5 reactivity and totally abrogated TH14 reactivity. Chymotrypsin treatment of G1 also abrogated totally the reactivity to G1 of both T cell hybridomas. While endo Glu-C and endo Arg-C did not modify the reactivity of TH5 to G1 they abrogated the reactivity for TH14. Endo Asp-N and endo Lys-C did not change the reactivity to G1 of TH5 but enhanced it for TH14 (figure 34).

The information thus gained has the potential to indicate the location and composition of the T cell epitopes by excluding certain amino acids as part of the epitope because of the cleavage or non-cleavage at a particular site by the proteolytic or chemical treatments. But in reality proteolytic enzymes do not necessarily cleave at every site where a consensus sequence is found. Because of that it is difficult to ascertain amino acid composition of a T cell epitope with certainty with this method alone. In any case chymotrypsin destroyed the T cell epitope for TH5 and TH14 showing that phenylalanine or tyrosine residues were important in these epitopes. The destruction was not due to toxicity of chymotrypsin since a chymotrypsin bound column was used. Also the eluated peptide was boiled and the addition of the digest to G1 did not decrease the reactivity of the hybridomas. Chemical cleavages seem to be more reliable in predicting important amino acid residues since extensive treatment should result in cleavage at all the specific sites. The data obtained with digestion by O-iodosobenzoic acid also indicated that tryptophan or possibly a tyrosine is part of the epitope for TH5.

The protease digests were also used for testing other cleavage products by the peptide-membrane bound method. Endo Asp-N gave limited bands to be tested this way. A band of 34 kD from an endo Asp-N digest was found to be immunogenic to TH5 and was sequenced. The N-terminal sequencing of this band revealed the sequence DEFPGVRTY which is 28 amino acids N-terminal to the C-terminal end of the 19 kD 1C6 staining CNBr digested peptide (Figure 32). This narrowed down the putative T cell epitope to 27 amino acids, starting at the endo-Asp-N N-terminal cleavage site to the predicted C-terminal end of the 1C6 staining CNBr peptide (Figures 32 and 33). The double digestion of G1 with CNBr and endo-Asp-N preserved the antigenicity of the G1 digest to TH5. A synthetic peptide of the same sequence and size was then synthesized and tested for reactivity. However this peptide, HG1-10 (Figure 33), was not found to stimulate TH5 cells. The difference in TH5 reactivity to the natural and the synthetic peptide can only be explained by postranslational diferences as discussed below.

7.3 DISCUSSION

The purified bovine G1 domain was shown not to contain CS chains because digestion of G1 with chondroitinase ABC did not produce a shift in the mobility pattern of G1 on gel electrophoresis. The presence of KS chain(s) on G1 was shown in several ways. Firstly G1 was also stained by alcian blue dye which is specific for highly negatively charged molecule such as GAG chains. Secondly digestion with either specific keratanase I or II enzyme increased the mobility of G1. Thirdly, specific Ab to KS chains stained the G1 molecule in immunoblotting.

CNBr digestion of G1 proved to be valuable in both the biochemical study of the G1 molecule as well as in determining the T cell epitope because it did not destroy the T cell epitopes and gave reasonably large fragments of known sequence . These fragments could be analysed biochemicaly with different Ab of known specificity in immunoblotting studies. These fragments were visualized with aurodye staining and could therefore be used in T cell reactivity assays. With all the data obtained, it became clear that two different forms of G1 may coexist in the bovine G1 preparation. Two bands of 19 and 24 kD reactive with the 1C6 Ab were observed on gel electrophoresis and both have the same N-terminal sequence. This heterogeneity of the peptide does not appear to be due to differences in KS chains content since no KS chain(s) was found in these two bands, as evidenced by the lack of effect of keratanase digestion on their electrophoresis migration as shown in Figure 27. Instead they were found to contain N-linked oligosaccharide since N-glycanase converted the two bands into one (Figure 29). This result is in agreement with the predicted substitution sites of N-linked oligosaccharide with a consensus sequence of Asn-X-(Ser/Thr)-Y, where X and Y can be any amino acid except proline (Wilson et al., 1991), for this type of oligosaccharide at position 220 (Doege et al., 1987). It is intersting in this regard that two G1 bands of high Mr (64 and 72 kD) are observed on high resolution gels (Dr. Rosenberg personal communication). Similar findings of molecular size differences due to oligosaccharides have been made with LP, where three LP species were described based on their different mobilities on SDS-PAGE. The two first LP: LP1 and LP2 have been found to vary in size because of different substitutions by N-linked oligosaccharides: LP1 has an extra chain at the N-terminal end of the molecule (Le Glédic et al., 1983; Mort et al., 1985). Though it is known that the bovine LP possesses a high mannose type of

oligosaccharide (Caterson et al., 1985) and that human LP may contain the complex type (Mort et al., 1985), it is not known if differences in length of the chains exist in these cases.

The nature of the T cell epitope is however not clear yet. Most T cell epitopes described so far are small linear peptides which usualy adopt an helical configuration with an amphipathic characteristic, allowing them to bind to MHC molecules and form stabilized complexes, within the groove of MHC class I or II molecules, with the TCR of the T cells. It is believed that they are uniquely protein in nature (Schwartz, 1985; Berzofsky et al., 1897). This concept is strenghtened by data showing that polysaccharides do not bind to class II MHC molecules (Harding et al., 1991). However the influence of carbohydrates on recognition of T cell epitopes has been reported recently (Ishioka et al., 1992). In addition, unusual examples of T cell reactivity to various carbohydrate structure have been reported in the recent years: streptococcal carbohydrate (Jackson et al.,1984), ganglioside (Bellamy et al., 1986), tumor-associated glycoprotein (Henningsson et al., 1987), bacterial peptidoglycans (Katsuki et al., 1987), and lipophosphoglycan (Moll et al., 1989) are examples. The TH5 cell recognize only the lower Mr form of the 19 kD 1C6 staining CNBr peptide but not the upper 24 kD 1C6 staining peptide which differ from the lower one probably in their oligosaccharide chains which can be removed by N-glycanase. Since TH5 did not recognize the synthetic peptide that would represent the peptide generated by the combined digestion of CNBr and endo Asp-N it is possible that carbohydrates, possibly N-linked oligosaccharide, may form an important component of the epitope recognized by TH5. However the length and/or qualitative differences of the chain may be critical in determining the reactivity of TH5. This may be why a long or qualitatively different N-linked oligosaccharides on the 24 kD 1C6

reactive peptide could not be recognized by TH5. This carbohydrate chain may be an integral part of the T cell epitope or it may sit outside and act distantly on the stability, conformation or in the interaction with the TCR as has been observed for amino acid residues outside the determinant region of hen egg lysosyme (HEL) which can have a profound effect on T cell activation (Shastri et al., 1986; Gammon et al., 1987). However G1 treated with N-glycanase or with both N-glycanase and keratanase only resulted in a slight diminution of TH5 reactivity, it did not abbrogate TH5 reactivity. These remain unanswered questions. In order to prove the importance of the N-linked oligosaccharide for TH5 recognition, it will be necessary to isolate the two forms of the 1C6 CNBr peptides, to show the differences in glycosylation pattern and to show that cleavage of the carbohydrate chain of the isolated glycopeptide can influence the T cell reactivity. If the identity of N-linked oligosaccharide chain is known it will be interesting to look at restitution of immunoreactivity of the synthetic peptide by chemically coupling carbohydrate to it.

CHAPTER VIII CHARACTERIZATION OF THE EPITOPES: INFLUENCE OF GLYCOSAMINOGLYCAN ON T CELL REACTIVITY

8.1 INTRODUCTION

Earlier studies have shc vn that removal of the chondroitin sulfate (CS) chains attached to the core protein of PG could markedly increase the efficiency of induction of the disease as compared with the native molecule (Glant et al., 1987, Mikecz et al., 1987). The frequency of the disease and anti-PG immune responses were much higher when the antigen used for immunization was CS-depleted PG as compared to immunization with native PG. The disease-enhancing effect of CS depletion was observed with human fetal PG (HFPG), but very little with human adult PG (HAPG) which is antigenically different to HFPG. HFPG was found to be much more arthritogenic than HAPG. Structural differences are known to exit between PG of different sources which includes the loss of intact PG with aging due to increasing amounts of degradation of PG (Roughley and White, 1980; Webber et al., 1987; Paulsson et al., 1987); alternate splicing of the G3 domain of the core protein of PG in some species (Doege et al., 1991; Baldwin et al., 1989) and age-related changes in protein epitope (Glant et al., 1986) are also observed. Some of the major differences between the HFPG and HAFG molecules are in the nature and degree of glycosylation (Roughley and white, 1980). HAPG has higher keratan sulfate and chondroitin 6-sulfate contents than HFPG (Roughley and White, 1980; Glant et al., 1986). The above data suggested that glycosaminoglycan chains, which include keratan sulfate and chondroitin sulfate, could potentially influence the responsiveness of PG-reactive T cells.

To investigate the potential role of the presence of GAG chains attached to the core protein on the reactivities of the T cells, PG and G1 were treated with several glycosidases, prior to examining their recognition

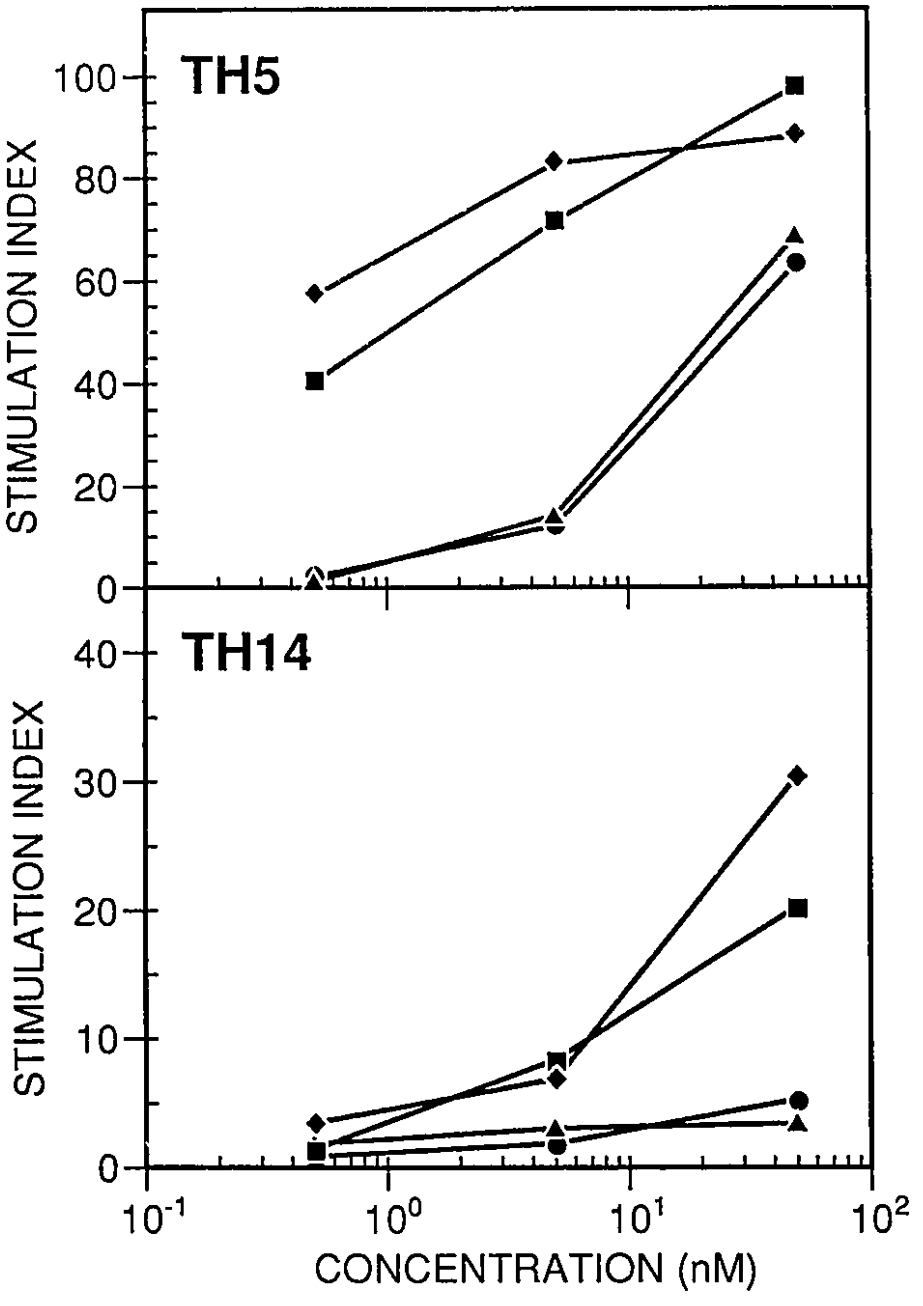

FIGURE 35. Reactivities of the T Cell Hybridomas TH5 and TH14 to HFPG After Glycosidase Treatments

Native HFPG (●); ABC chondroitinase-digested HFPG (▲); keratanase I-digested HFPG (■); combination of chondroitinase and keratanase I digested HFPG(◆), were added at various concentrations in assay. Supernatants were harvested 24 h later and assayed for IL-2 in a CTLL proliferation assay.

by the T cell hybridomas TH5 and TH14. Depletion of KS chains on PG and G1 by keratanase digestion was found to increase the reactivities of the T cells to these molecules.

8.2 RESULTS

8.2.1 EFFECT OF CS AND KS DEPLETION OF PG ON T CELL REACTIVITY

The reactivities of the two T cell hybridomas (TH5 and TH14), were studied before and after treatment of HFPG with two different glycosidases that cleave glycosaminoglycans (GAG). Treatment of HFPG with chondroitinase ABC, which cleave off the chondroitin sulfate chains and leaves only a pentasacharride moiety on the core protein (Oike et al., 1980), had no effect on the reactivity of the two T cell hybridomas (figure 35). However the treatment of HFPG with keratanase I, which cleaves off the partially sulfated moiety of KS chains attached to the core protein of PG (Oike et al., 1980), increased the reactivity of both hybridomas when compared to the untreated HFPG or the CS-depleted HFPG (Figure 35). The CTLL responses to IL-2 released by TH5 were always significantly higher (40X, 7X, and 1.5X) with KS-depleted PG as compared to native PG at three different doses (0.5, 5, and 50nM respectively). With TH14 cells the CTLL responses were low and not much different between native & KS-depleted PG at lower doses (0.5 and 5nM). However, at the higher dose of 50nM, KS-depleted PG stimulated the TH14 cells significantly more than native PG, with CTLL responses being 4 times more with the former as compared to the latter. Additional digestion with chondroitinase ABC either before (Figure 35) or after (data not shown) keratanase digestion did not further

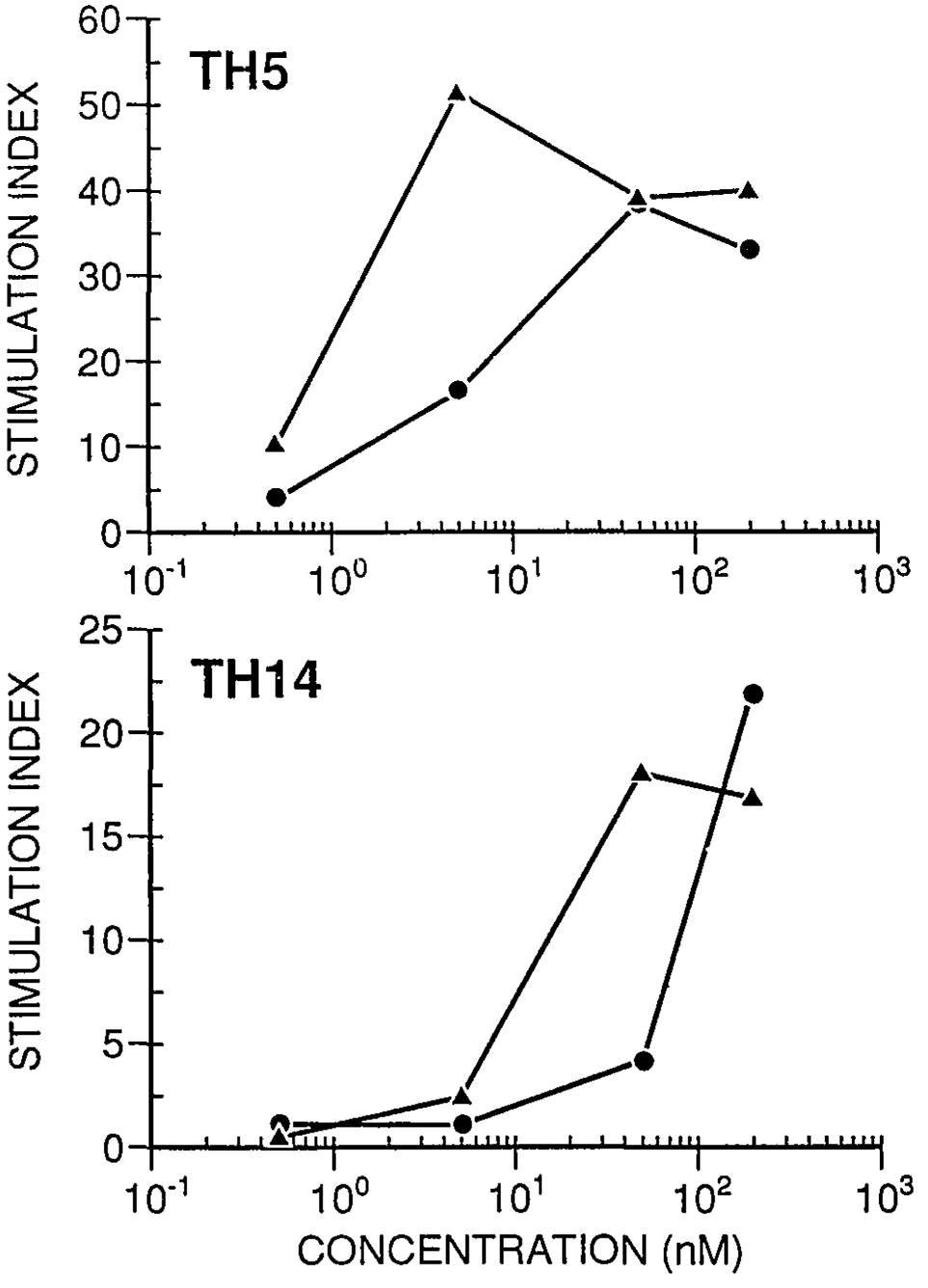

FIGURE 36. Reactivities of the T Ceil Hybridomas TH5 and TH14 to G1 After Keratanase I Treatment

Native G1 (●); keratanase I-treated G1 (▲), were used in assay as in figure 1.

increase the responses of either TH5 or TH14. Similar results were obtained in at least two other separate experiments. As mentioned earlier TH14 responses were always found to be lower than TH5 responses to all antigens suggesting that TH14 had lower sensitivity to activation by its epitope than TH5.

8.2.2 EFFECT OF KERATANASE TREATED G1 ON T CELL REACTIVITY

The responsiveness of TH5 to keratanase digested G1 was significantly higher than to native G1 at 0.5 & 5 nM antigen (Figure 36) These differences were not observed at 50 nM. The responsiveness of TH14 to G1 was somewhat less sensitive to the keratanase effect as was earlier observed with the whole PG molecule. Again, a significant difference between responses to native and KS-dep G1 was observed at 50nM antigen but not at 0.5 or 5nM (Figure 36). Similar data were obtained on two other separate occasions.

Addition of free corneal KS chains did not influence the reactivities of T cell hybridomas in the absence of antigen or with G1 or KS-depleted antigen present.

8.2.3 REACTIVITY TO CNBr DIGESTED G1

CNBr digested fragments of native G1 stimulated both T cell hybridomas significantly more than native G1 (Figure 37). Furthermore the CNBr-peptide could be presented to both the T cell hybridomas by fixed APC (Table 7), indicating that the epitope bearing peptide did not need internalisation and processing This suggested that neither KS nor

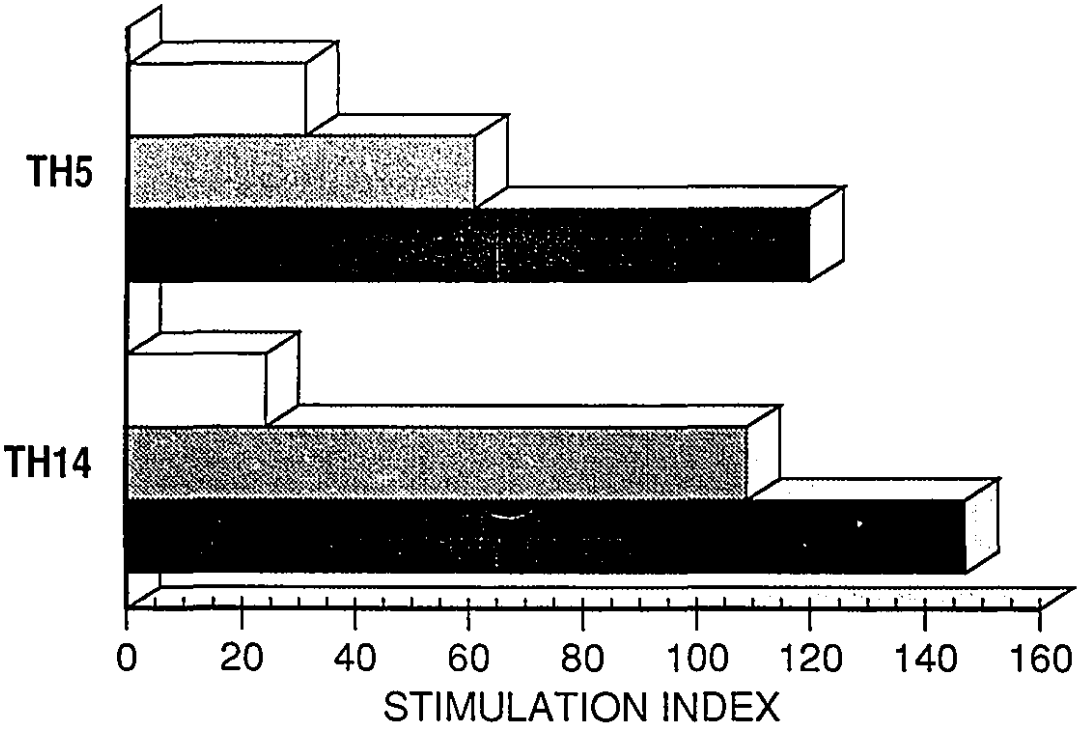

FIGURE 37. Reactivity of the T Cell Hybridomas to G1 After Treatment with CNBr
5 nM of antigen was used for TH5 and 50 nM of antigen for TH14. G1 native : white bar; G1 treated with keratanase : shaded bar; G1 treated with CNBr : black bar

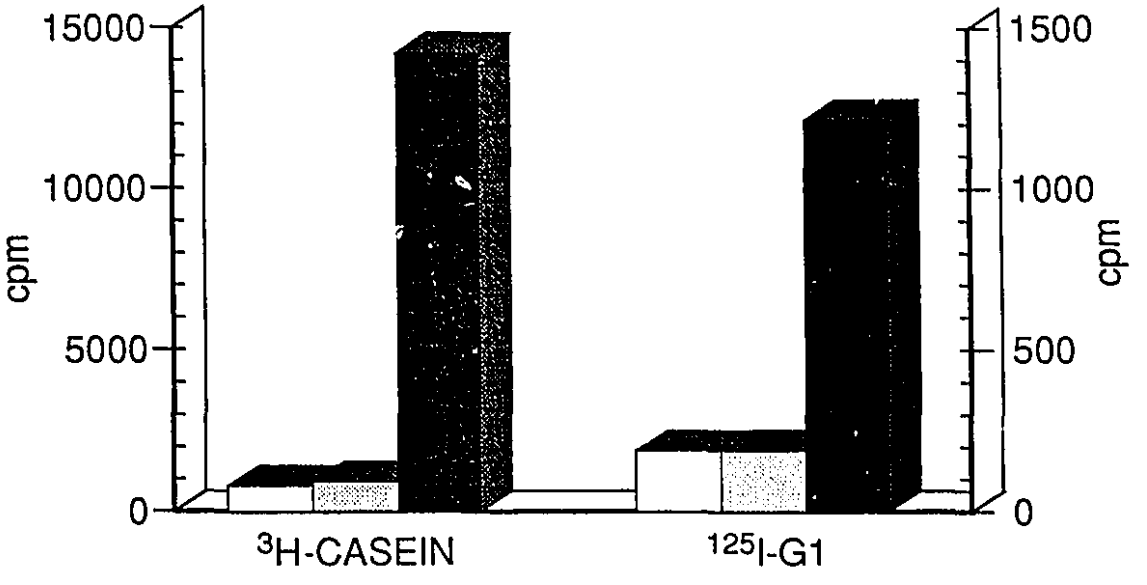

FIGURE 38. ^{3}H-casein and ^{125}I-G1 Degradation Assays

^{3}H radiolabeled casein and ^{125}I-G1 were incubated in keratanase digestion buffer alone (white bar), with keratanase I (grey bar) or with trypsin (black bar) for 24 h at 37°C. Supernatants, containing non-precipitable degraded peptides, were counted after TCA precipitation. The same results were obtained on two separate occasions.

oligosaccharide was interfering with antigen presentation by being located within the region of the T cell epitope itself, since in that case the CNBr cleaved fragment containing the epitope would not have been efficiently presented by APC. The observations suggest that carbohydrate attachment site(s) on G1 are probably located further away from the T cell epitope than the nearest methionine residue(s) which is the site of the CNBr digestion. In fact two sites of O-linked KS site are known at present at the end of the B' region (Figure 32) which is C-terminal to the suspected locations of the T cell hybridomas epitopes (L. C. Rosenberg and P. Neame, personal communication).

8.2.4 THE INCREASED REACTIVITY OF THE T CELL HYBRIDOMAS TO GLYCOSIDASE TREATED PG OR G1 IS NOT DUE TO PROTEASES CONTAMINATING THESE ENZYMES.

The keratanase I preparation in the presence of proteinase inhibitors did not contain any detectable proteolytic activity as revealed by a sensitive casein degradation assay (Figure 38). In addition no degradation of G1 was seen when the keratanase I treatment was performed in the presence of inhibitors, as detected with both ^{125}I radiolabelled G1 and by colloidal gold (aurodye) staining of G1 analyzed by SDS-PAGE (Figure 27 and 28). Furthermore, the KS-depleted G1 preparation could not be presented by fixed APC to the T cells, ruling out the possibility of the release of small immunostimulatory peptides containing the T cell epitope(s) being produced by proteolytic digestion of G1 by proteolytic contaminants in the keratanase preparation, which could be presented directly by the APC without antigen processing (Table 7).

TABLE 7. Presentation of G1 to T Cell Hybridomas by Fixed and Unfixed APC

	TH5 CPM (mean±SEM)		TH14 CPM (mean±SEM)	
Antigens	Non-fixed APC	Fixed APC	Non-fixed APC	Fixed APC
Medium	429 ±133	854 ±345	716 ±269	674 ±430
G1 Native	7020 ±532	755 ±648	2972 ±663	510 ±237
G1 KS-depleted	21987 ±1400	208 ±6	12914 ±1069	399 ±313
G1 CNBr-digested	30034 ±1060	20508 ±1277	n.d.	10718 ±1700

TH5 and TH14 reactivity to G1 in native form and after treatment with keratanase I and CNBr (with concentrations of 5 nM for TH5 and 50 nM for TH14) were compared between non-fixed and formaldehyde (1%) fixed APC.

n.d. = not determined.

8.3 DISCUSSION

The two PG-reactive T cell hybridomas TH5 and TH14 used in this study recognize possibly two different epitope(s) on the G1 domain of HFPG and HAPG and have different TCR. This study provides evidence that the glycosaminoglycan keratan sulfate attached to the core protein can diminish both T cell hybridoma responses to the whole PG as well as a to the G1 domain of PG.

The G1 domain of porcine PG is reported to possess one or more keratan sulfate chains (Bonnet et al., 1985; Fosang and Hardingham, 1991; Barry et al., 1992). Recently the fetal bovine G1 domain has also been reported to possess KS chains (L. C. Rosenberg, and P. Neame unpublished data). This was confirmed in the present study by immunoblotting with the monoclonal antibody AN9P1 which is specific for KS chains (Poole et al., 1989a). Moreover there was also a decrease in apparent MW of 7 kD after keratanase I digestion of G1. This may indicate the presence of 1-2 KS chains on G1 as was observed for porcine G1 (Barry et al., 1992). More recent data have provided evidence for the presence of two O-linked KS chains at the C-terminal end of the bovine G1 molecule (L. C. Rosenberg and P. Neame, unpublished data).

The G1 specific monoclonal antibody 1C6 recognizes two CNBr digested fragments of G1 which possess the same N-terminal sequence (Figure 28 and 30). The 1C6 Ab is known to bind only to one epitope on G1 (Fosang and Hardingham, 1991). The difference between the two 1C6 CNBr-peptides from the G1 preparation was shown to be due to differences in N-linked oligosaccharide chains substitution similar to that already observed in LP (Nguyen et al., 1990) (see chapter VII). However this does not exclude the

possibility of heterogeneity in the G1 also due to different KS substitution. This has been shown earlier in other laboratories (Fosang and Hardingham, 1991).

The G1 domain in porcine PG is believed to contain about 25% (w/w) carbohydrate, including N-linked oligosaccharides and O-linked keratan sulphate (Bonnet et al., 1985). Carbohydrates can interfere with the binding of monoclonal antibodies to core protein. The 1C6 antibody was recently found to be poorly reactive to its epitope on the G2 domain in the native porcine PG molecule, but on removal of KS chains with keratanase I it was shown to be strongly reactive with the antibody (Fosang and Hardingham, 1991). It was suggested that an O-linked KS chain substituted close to the epitope sequence in the G2 domain prevented antibody access to the epitope and thus masked its detection (Fosang and Hardingham, 1991). A rabbit anti-serum to mouse EGF which recognizes epitopes contained in the EGF-like region of the G3 domain, can also bind to its epitope (by both immunoblotting and ELISA) only after digestion of the PG with chondroitinase ABC (Stanescu et al., 1991). In the above instances, GAG chains have an important influence on antibody binding, and this masking effect might be due to steric hindrance of Ab binding to the epitope on the core protein. Though these two reports constitute examples of the effect of carbohydrates on Ab recognition of epitopes on glycoproteins, the present study show that the presence of carbohydrate (KS) on core protein can also influence the reactivities of T cell hybridomas to such molecules.

The fact that T cell reactivity is not suppressed by the KS chain at high concentrations could be explained by the heterogeneity of the G1 preparation such that some molecules are substituted with KS whereas others are not. The reactivity of the T cells to the native G1 may be due to the reactivities to

the portion of G1 molecules not substituted with KS. Removal of KS from the G1 preparation probably allows the T cells to react with all G1 molecules. Such heterogeneity in G1 molecule has been shown by others (Fosang and Hardingham, 1991; L. C. Rosenberg and P. Neame, personal communication) for the KS chains.

There is always the possibility that small T cell epitope containing peptides are generated by proteolytic degradation of G1 by protease contaminants in the keratanase preparation and that they could then be more efficiently presented by the APC. This possibility was ruled out. Firstly, fixed APC could not present KS-digested G1 preparations to the PG reactive T cell hybridomas. Peptides containing T cell epitopes have been shown earlier to be presented by fixed APC to relevant TCR on T cells (Berzofsky et al., 1988). These fixed APC could, however present the CNBr digested G1 fragments to TH5 and TH14 showing that the CNBr peptides were small enough to be presented by the fixed APC to TH5 and TH14. These responses were MHC class II restricted. At first sight the CNBr peptide presented by the APC appears to be too large (apparent Mr of 19 kD and up to 118 residues) to be presented by MHC class II molecules on fixed APC. It is believed that one important physiological function of antigen processing is to unfold proteins so they can be presented by MHC molecules (Berzofsky et al., 1988). Denatured HEL can be presented without further processing to T cell hybridomas (Allen and Unanue, 1984) and the large fibrinogen molecule can be presented without any processing (Lee et al., 1988). In contrast a small molecule such as apamin (18 a.a.) with two disulfide bounds needs to be processed (Régnier-Vigouroux et al., 1988). Thus the CNBr peptide recognized by TH5 must fulfil the requirements for T cell recognition. There is also the unlikely possibility that cell-surface enzymes (Semple et al., 1989;

Naquet and Pierres, 1991) are still functional after fixation of APC's and could process further the CNBr fragments and allow presentation by MHC class II molecules.

Hence, the mechanism of enhancement of T cell responses by keratanase I digestion was not simply by generation of immunoreactive T cell epitopes bearing peptides from G1 but involved removal of KS. A second point in favor of this argument was that no fragmentation of unlabelled G1 or radioiodinated G1 after keratanase digestion could be demonstrated on gradient SDS-PAGE by sensitive gold staining or autoradiography Thirdly, no digestion of radiolabelled casein was observed after digestion with keratanase I. Casein degradation assays have been used earlier to detect protease activities (Cawston and Barrett, 1979). It must be noted here that all keratanase I digestions were done in the presence of a cocktail of protease inhibitors.

The KS on G1 may inhibit antigen processing involving enzymatic digestion of exogenous proteins in the endosomes and lysosomes which have been shown to be crucial for antigen presentation by APC to the TCR (Unanue, 1984). Carbohydrate residues on glycoproteins have been shown to protect glycoproteins from proteolytic degradation by preventing accessibility of the proteases to the protein core (Olden et al., 1979; Kozarsky et al., 1988). The KS chains could similarly inhibit, at some stage, antigen processing of G1 by endosomal/lysosomal enzymes in APC. The cleavage of G1 by CNBr to produce a small peptide may have circumvented this crucial intracellular digestion step. The ability of fixed APC to present CNBr digested G1 also suggested that the interference in antigen presentation by KS was not due to steric hindrance of binding to MHC class II molecules and presentation to TCR. CNBr digested native G1 could be presented equally better than KS-

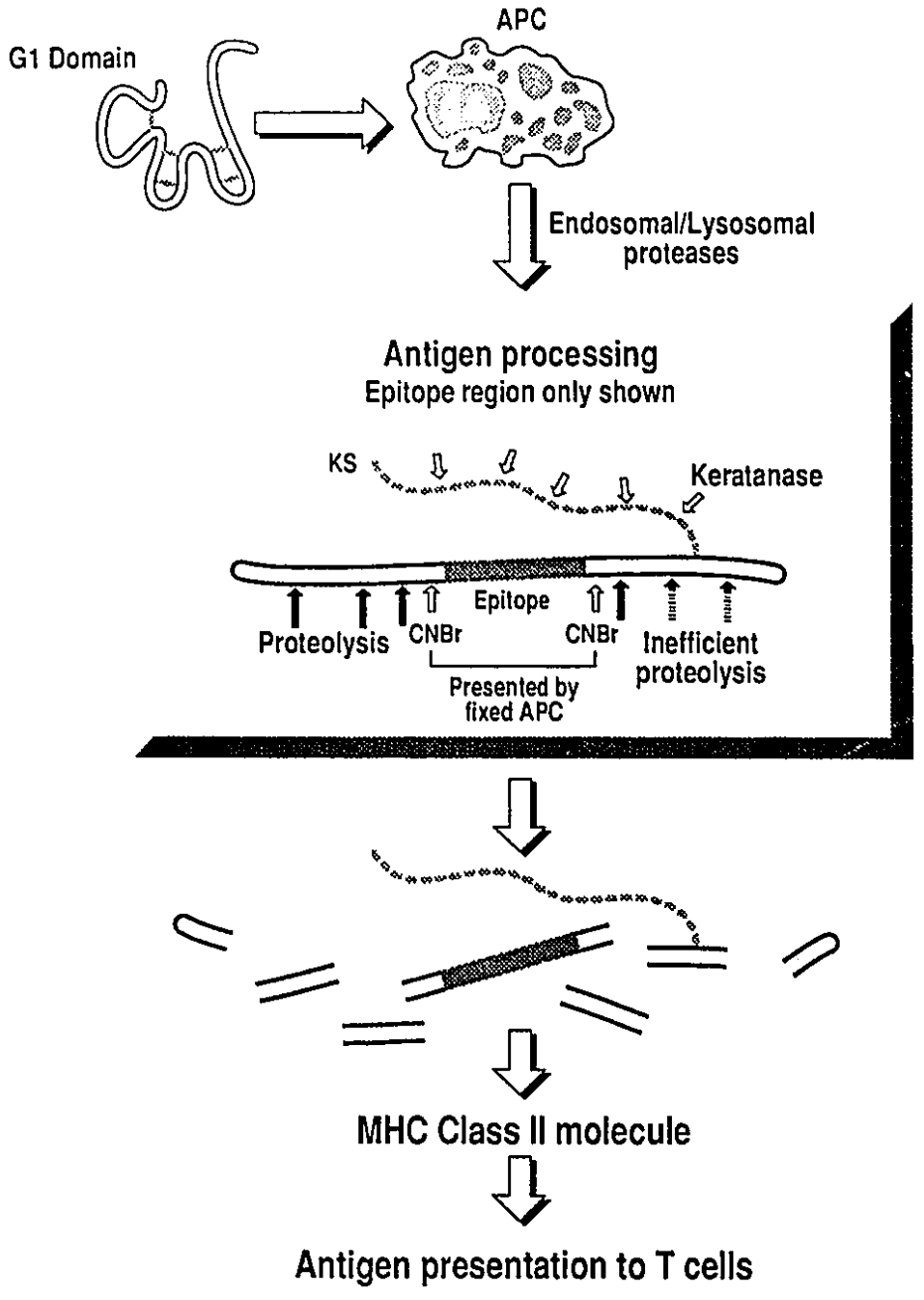

FIGURE 39. Hypothetical Mechanism of Influence of KS on Antigen Presentation

depleted G1 to the T cells and the CNBr digestion of KS depleted G1 was not better presented than the CNBr digested G1 alone suggesting again that KS is probably not present on this CNBr peptide and that the KS effect on T cell reactivity is not due to the presence of KS on the immunoreactive peptide. The mode of inhibition of T cell recognition of PG/G1 by KS is in contrast to the steric interference by GAG chains of Ab binding to antigen shown earlier (Fosang and Hardingham, 1991; Stanescu et al., 1991). The inhibition mechanism is also believed to be different from a unique recent report on the interference of T cell activation by glycosylation of peptides (Ishioka et al., 1992). In this report the addition of an oligosaccharide to a known peptide T cell epitope (OVA 323-339), destroyed both MHC binding and T cell recognition by steric hindrance. However if the attachment of the carbohydrate was outside the MHC-binding region no effect would be observed which is in contrast to our observation with the presence of KS on the G1 molecule. The hypothetical mechanism of action of the KS effect is depicted in Figure 39. The long KS chain may interfere with the accessibility of the core protein substrate to processing enzyme. This may prevent cleavage or reduce the rate of catalysis. Influence of KS chains on endocytosis of G1 by APC has not, however, been be ruled out. Studies are in progress to narrow down the exact mechanisms of interference of antigen presentation of G1 by KS chains.

Addition of exogenous carbohydrates like Ficoll and sucrose and other bulky carbohydrate has been shown to decrease antigen presentation by APC by interference with lysosomal digestion (Leyva-Cobian and Unanue, 1988) However, addition of free KS chains at concentrations found to inhibit antigen presentation in the study by Leyva-Cobian and Unanue did not influence the reactivity of the T cell hybridomas to G1-native nor to G1-KS

depleted. However, commercially available corneal KS may not simulate the effect of endogenous KS chains on the core protein of G1. Hence inhibition of lysosomal pathway antigen presentation (Leyva-Cobian and Unanue, 1988) by KS on G1 may still be a possibility.

This study highlights the influence of KS chains present on PG on the antigen presentation of PG to T cells in vitro. This could be of importance in the generation of pathogenic autoimmune responses to PG *in vivo*. As mentioned earlier, CS-depleted PG is more arthritogenic than native PG in mice. Since $CD4^+$ T cells have been shown to be important in the pathogenesis of this disease, it is possible that CS chains may interfere with antigen presentation of arthritogenic epitope(s) on PG to pathogenic $CD4^+$ T cells *in vivo* by mechanisms similar to those described above. This has to be formally proven by T cells reactive with the CS-region of PG, as opposed to the T cells reactive with the G1 region used in the present study. Likewise KS depletion of adult PG may enhance arthritogenicity of this molecule.

CHAPTER IX PATHOPHYSIOLOGY OF THE T CELLS

9.1 INTRODUCTION

T cells are known to be essential for the development of PG-induced arthritis (Mikecz et al., 1990). Spleen cells from mice with PG-induced arthritis (primary arthritis) can transfer the disease into naive irradiated mice (Mikecz et al., 1990). The time of appearence of arthritis in these mice from passive transfer of cells (secondary arthritis) is shorter and even shorter in the passive transfer of a tertiary arthritis. (Mikecz et al., 1990). This adoptive transfer of disease was successful when PG was injected along with the cells or when the spleen cells were stimulated with PG in vitro. The addition of IL-2 or immune sera from arthritic animals accelerated the onset of arthritis (Mikecz et al., 1990). *In vitro* depletion of the T cell populations prevented the development of the disease. *In vitro* depletion of both $CD4^+$ and $CD8^+$ T cell populations prevented the transfer of the disease. Depletion of B cells *in vitro* also prevented the transfer of the disease showing that B cells and/or possibly PG specific antibody were also important in the development of the disease (Mikecz et al., 1990) although antibody alone could not induce the disease (Dayer et al., 1990). T cell depletion studies *in vivo* have shown that depletion of the $CD4^+$ subset during immunization with PG prevented the appearance of arthritis while depletion of the $CD8^+$ subset did not (Banerjee et al., 1992a) showing the importance of the $CD4^+$ T helper subset in this disease.

The TH1 subset of $CD4^+$ T cells can induce DTH *in vivo* (Mosmann et al., 1986) and are thought to be important in the pathogenesis of RA (Klasen et al., 1990). The T cells used in the present study probably belong to the TH1 subset of $CD4^+$ T cells since they secrete IL-2 and not IL-4 characteristic of TH1 type cells (Mosman et al., 1986). The pathogenic potential of the T cells was studied by injecting these T cells into joints of naive mice. For this, naive

mice, which were either irradiated or non-irradiated, served as hosts for the injection of the cells by different routes such as i.v., i.p. and intra-articular (i.a.) routes. The i.a. route should be able to provide the most interesting results since it enables one to look directly at the pathological effects of these T cells in the joint. An intraarticular injection of cells bypasses all the biological barriers that cells encounter when injected by the other routes.

9.2 RESULTS

9.2.1 TRANSFER OF THE DISEASE

The T cell hybridomas, TH5 and TH14 cells were injected i.v. and i.p.into female BALB/c naive mice which were irradiated with 600 rads 24 h prior to injection. Also non-irradiated BALB/c female mice were injected. There were four groups of 5 mice each in this experiment. The individual groups received 5×10^6, 10×10^6, 50×10^6, 100×10^6 cells per injection The mice were examined for clinical appearance of inflammation in the four paws every 2-3 days for a month. The mice injected i.p. with the cells were reinjected with the same number of cells 10 days after the first injection. However no clinical signs of arthritis and/or inflammation was observed in any mouse.

These experiments were repeated with the two T cell lines JY.A and JY.D and compared with a control ovalbumin-reactive T cell line. Because of the limited number of cells available fewer cells were used in these experiments. Only one group of mice was used for each T cell line. Cells were injected at 1×10^4, 1×10^5 and 1×10^6 cells i.p.. Arthritis was not detected in any group.

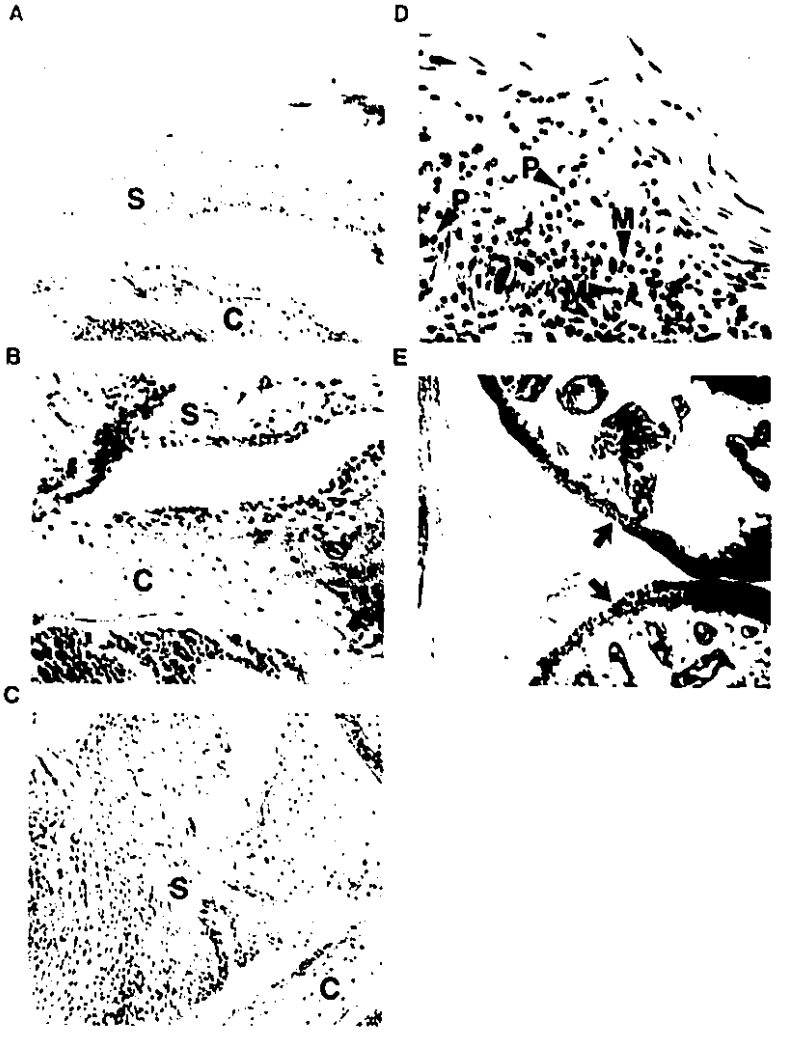

FIGURE 40. Histology of Knee Joints from Mice Injected i.a. with the T Cell Lines JY.A and JY.D

H/E staining : A-D. A): PBS injected (normal joint); B): JY.D; C): JY.A; D): JY.A at higher magnification (36x); E): JY.D Toluidine blue stained.

Cartilage (C), Synovium (S), Polymorphonuclear leukocyte (P), Mononuclear cells (M). Large arrows in panel E show GAG depletion.

However because of the low number of cells obtained, the route of injection used, and other reasons such as the downregulation of "homing" receptors such as the L-selectin coupled to the possible effects of long term culture of T cells, there was the possibility that the cells may not have reached the joint in sufficient quantities to induce inflammation. The cells were thus injected directly into the joint. The cells were first activated *in vitro* with either ConA or PG, and washed extensively prior to i.a. injection. The cells were stimulated with 2.5 μg/ml of ConA in the presence of feeder cells for 48 h, washed extensively and live cells free of ConA were separated from the dead cells and debris on Lympholyte M, and recultured for another 48 h without any feeder cells or ConA. The cells were then washed in PBS and 2 x 10^4 cells in 20-25 μl were injected i.a.. In one experiment 3 mice were injected with the Con A-activated T cell line JY.A in the right knee while the left knee was injected with the Con A-activated T cell line JY.D. The mice were sacrificed 10 days later and the knee joints processed for histology. Only JY.A injected knees showed visible redness and swelling, this was particularly prominant in one mouse. The histology of this mouse is shown in Figure 40. The JY.A injected knee showed hyperplasia of the synovial membrane, early formation of pannus over bone and infiltration of both the capsule and the synovial membrane with polymorphonuclear cells (Figure 40). Mononuclear cells were also observed in this synovial membrane which could be the injected cells or endogenous cells. The joint injected with JY.D showed no clinical signs of inflammation, and only minimal hyperplasia of the synovial membrane without polymorphonuclear cell infiltration on histopathology (figure 40). Knee joints injected with PBS alone served as a normal joint controls and did not show any clinical or histological changes. Staining with

toluidine blue revealed depletion of PG from cartilage in joints injected with JY.D.

The same type of experiment was repeated with the T cell lines JY.A, JY.D and the irrelevant ovalbumin reactive T cell line with *in vitro* stimulation of cells with 25 μg/ml of HFPG instead of ConA. There was no clinical evidence of inflammation in any of these mice. Due to technical difficulties, the histopathology sections could not be studied in these joints.

9.3 DISCUSSION

Transfer of T cell hybridomas was attempted despite the fact that the hybrids were made from mixed haplotype cells (spleen cells from BALB/c are H-2^d and BW5147 thymoma cells are H-2^k, derived from AKR mice). Therefore absence of evidence of disease on adoptive transfer would not necessarily mean that the T cell hybridomas do not have the arthritogenic potential since these cells were probably killed by allogeneic cytotoxic T cells in the BALB/c mice. Transfer of these cells would have to be repeated in [BALB/c x AKR]F1 hybrid mice. In addition T cell hybridomas may lack homing receptor.

The failure of transfer of disease with the T cell lines by the i.v. or i.p. route may still not necessarily signify that these cells do not have the potential to transfer disease because the number of cells transferred was very low. The number of cells used in adoptive transfer experiments in other autoimmune studies, like adjuvant arthritis, varied from 2×10^7 cells of specific T cell clones injected in irradiated rats (Holoshitz et al., 1984) to 10×10^9 cells in EAE (Urban et al., 1989). With the i.v. route, a large proportion of cells may not reach the joint because they migrate to the lung, liver and

spleen (Bischoff et al., 1986). In the CIA mouse model of arthritis, transfer of arthritis with T cell lines produced only a transient disease (Holmdahl et al., 1985) or a low incidence of disease with up to 10^8 cells (Kakimoto et al.,1988).

Since only a limited number of cells could be obtained, the i.a. injection protocol seemed to be the most efficient route. The cell line JY.A showed its potential to induce synovitis when injected by this route. However this was demonstrated by stimulating the cells with a non-specific mitogen (ConA) and still needs to be demonstrated with in vitro HFPG-stimulated cells. Due to technical reasons however histopathologic sections could not be studied. The T cell line JY.D induced limited synovial hyperplasia. It is not clear if this is specific for the T cell line JY.D since the control ovalbumin reactive T cell line was not used in the intraarticular injection studies.

The vaccination potential of the two T cell lines remains to be examined. The potential for protecting against the induction of an autoimmune disease by vaccinating animals with attenuated T cell clones which normally can transfer disease, was first described by Irun Cohen's group (Holoshitz et al., 1983; van Eden et al., 1987). This has been shown to be effective in a number of other autoimmune disease animal model such as EAE (Lider, 1988) and CIA (Kakimoto et al., 1988).

CONCLUSIONS AND FUTURE WORK

In this study, PG-reactive T cells derived from PG-induced arthritic mice were investigated for their pattern of reactivity, phenotypes, specificity and the influence of GAG on their reactivity

The reactivity profiles of spleen-derived T cells indicated differences in recognition of human fetal and adult PG molecules by the T cells. Qualitative differences between the two molecules may explain the differences in reactivities between HFPG and HAPG.

The early T cell lines also showed differences in age-related T cell responses to PG. The T cells showed reduced reactivity to HFPG aggregate (A1) compared to PG monomer. This is possibly because large aggregate of HFPG, LP and HA is not efficiently endocytosed by APC and/or that some epitopes are protected from antigen processing preventing immune recognition. Proteolytic or chemical cleavage of aggregate increased the reactivity of the T cells to this aggregate perhaps by liberating immunoreactive fragments from the large aggregate which could be presented more efficiently by the APC. The use of A1 preparation need to be further studied with the G1 specific T cell hybridomas in order to study the influence of such molecular interactions on T cell immunoreactivity

Two patterns of antigenic reactivities were recognized at the T cell line/hybridoma levels. The first pattern was characterized by reactivities to HFPG, HAPG, BFPG, the bovine G1 domain and LP (T cell line JY.D and T cell hybridomas TH5 and TH14). It is presumed that the epitope(s) on human PG and bovine G1 recognized by these PG-reactive T cells reside in

the regions of the G1 domain homologous with LP. The lower reactivities to bovine LP by the three bovine G1-reactive T cells is suggestive of the fact that the T cell epitope(s) is processed differently to PG and G1 and is less efficiently presented to the T cell receptor. Delineation of the other T cell epitopes need to be continued. Purification of the soluble TH5 peptide generated by the combination of endo Asp-N and CNBr is essential for the analysis of carbohydrate content and its influence on TH5 reactivity.

The G1 domain on PG bears immunodominant epitope(s). Because of previous observations on the presence of free G1 this may be of some pathological significance in arthritis by eliciting T cell responses in the joints (Chapter 6).

The second pattern of PG-reactive T cell responses (T cell line JY.A) was characterized by reactivity against HFPG, but not to HAPG nor to LP and the other above antigens.

This studies clearly showed that different T cell epitopes on human PG are recognized by the two T cell lines JY.A and JY.D, and that fetal and adult human PG express different T cell epitopes. Again identifying the different T cell epitopes will allow further understanding of these age-related differences in PG T cell reactivity.

Neither of the T cell hybridomas which recognize the G1 domain recognized the 65 kD HSP indicating that a crossreactive epitope(s) is not recognized by these T cells.

The two $CD4^+$ PG-reactive T cell lines and two $CD4^+$ PG reactive T cell hybridomas apparently belong to the TH1 subset since they secrete IL-2 but not IL-4 and are MHC class II restricted. The two hybridomas and the T cell line JY.D being predominantly $I\text{-}A^d$ restricted while JY.A is predominantly $I\text{-}E^d$

restricted The hybridomas were shown to bear Vβ4 (TH5) and Vβ8 (TH14) TCR.

It is possible that glycosaminoglycan chains attached to the core protein of PG could interfere with the antigen processing of PG by antigen-presenting cells. This may explain why the development of arthritis is observed much less often after hyperimmunization with native human PG than with the human PG treated with chondroitinase.

HAPG, which possesses more KS chains on its core protein than HFPG is a better stimulating antigen for the G1-domain reactive T cells lines and hybridomas but is yet less arthritogenic. This may be explained by the fact that the increase in KS substitution of HAPG is in the KS rich region while in HFPG there may be KS substitution in or closer to the G1 domain as is known for the pig and bovine G1 (Chapter 8).

The lack of reactivity of the G1 reactive T cells to MPG was not due to degradation of MPG during purification and is not believed to be due to sequence differences between human and mouse PG in the G1 region . The epitopes on MPG may not be recognized because these are cryptic peptides and the lack of reactivity to the native molecule *in vitro,* is possibly due to differences in processing by APC *in vitro* and *in vivo* . The only apropriate APC which can present autologous MPG may reside in the joint itself (Chapter 6). Therefore non-reactivity to self-antigens *in vitro* , in this case MPG, does not necessarily signify that the T cells may not be pathogenic *in vivo.* This lack of recognition needs to be further studied by using and comparing different source of APC with APC derived from joints.

The purified bovine G1 domain was shown not to contain CS chains but KS chain(s) were found to be present on G1 as evidenced by alcian blue

staining, and digestion with either specific keratanase I or II enzymes which increased the mobility of G1 on electrophoresis, and by immunoblotting staining with specific Ab to KS chains. This was confirmed in the recent finding of the presence of two O-linked KS chains at the C-terminal end of the bovine G1 molecules (Chapter 7).

CNBr digestion of G1 generated fragments that preserved T cell epitopes which could be analysed biochemicaly with different Ab of known specificity in immunoblotting studies. Two peptides of 19 and 24 kD reactive with the 1C6 Ab were observed on gel electrophoresis and both had the same N-terminal sequence. This heterogeneity of the peptide was not due to differences in KS chain content but was due to differences in N-linked oligosaccharide since N-glycanase converted the two bands into one smaller band. This result is in agreement with the predicted substitution sites of N-linked oligosaccharide with a consensus sequence at position 220.

The nature of the T cell epitope is however not clear yet. The TH5 cell recognizes only the lower Mr form of the 19 kD 1C6 staining CNBr peptide but not tne upper 24 kD 1C6 staining peptide which differ from the lower only in their oligosaccharide chain which can be removed by N-glycanase. Since TH5 did not recognize the synthetic peptide that would represent the peptide generated by the combined digestion of CNBr and endo Asp-N it is possible that carbohydrate, possibly N-linked oligosaccharide, may form an important component of the epitope recognized by TH5. However the length and/or qualitative differences of the chain may be critical in determining the reactivity of TH5. This may be why a long or qualitatively different N-linked oligosaccharides on the 24 kD 1C6 stain peptide could not be recognized by TH5. However G1 treated with N-glycanase, or with

both N-glycanase and keratanase, only resulted in a slight diminution of TH5 reactivity. This should be further studied by using purified endo Asp-N/CNBr TH5 peptide, and to analyse the carbohydrate content and type.

The two PG-reactive T cell hybridomas TH5 and TH14 used in this study appear to recognize two different epitope(s) on the G1 domain of HFPG and HAPG and have different TCR. This study also provides evidence that the glycosaminoglycan keratan sulfate attached to the core protein can diminish both T cell hybridoma responses to the whole PG as well as to the G1 domain of PG. The possibility that small T cell epitopes containing peptides are generated by proteolytic degradation of G1 by protease contaminants in the keratanase preparation and that there could be more efficiently presented by the APC was ruled out. Digestions were done in the presence of a cocktail of protease inhibitors. No fragmentation of unlabelled G1 or radioiodinated G1 after keratanase digestion could be demonstrated on gradient SDS-PAGE by sensitive gold staining or autoradiography and no digestion of radiolabelled casein was observed after digestion with keratanase I. Fixed APC could not present keratanase digested G1 but could present the CNBr digested G1 fragments to TH5 and TH14.

The KS chains could inhibit, at some stage, antigen processing of G1 by endosomal/lysosomal enzymes in APC. The cleavage of G1 by CNBr to produce a small peptide may have circumvented the ordinarily crucial intracellular digestion step. The ability of fixed APC to present CNBr digested G1 also suggested that the interference in antigen presentation by KS was not necessarily due to steric hindrance of binding to MHC class II molecules and presentation to TCR. CNBr digested native G1 could be

presented as well as KS depleted G1 to the T cells suggesting again that KS is probably not present on this CNBr peptide and that the KS effect on T cell reactivity is not due to the presence of KS on the immunoreactive peptide. The mode of inhibition of T cell recognition of PG/G1 by KS is in contrast to the steric interference by GAG chains of Ab binding to antigen or from TCR binding to MHC class II and peptides. The hypothetical mechanism of action of the KS effect is proposed in the following: the long KS chain interferes with the accessibility of the core protein as substrate to processing enzyme. This may prevent cleavage or reduce the rate of catalysis. Influence of KS chains on endocytosis of G1 by APC has not, however, been be ruled out.

This study highlights the influence of KS chains present on PG on the antigen presentation of PG to T cells *in vitro*. This could be of importance in the generation of pathogenic autoimmune responses to PG *in vivo*. As mentioned earlier, CS-depleted PG is more arthritogenic than native PG in mice. Further studies are needed to narrow down the exact mechanisms of interference of antigen presentation of G1 by KS chains. These includes semi quantitative assays of binding and uptake of radiolabelled G1 in native and KS-depleted forms.

Since $CD4^+$ T cells have been shown to be important in the pathogenesis of this disease, it is possible that CS chains may interfere with antigen presentation of arthritogenic epitope(s) on PG to pathogenic $CD4^+$ T cells *in vivo* by mechanisms similar to those described above. This has to be formally proven by T cells reactive with the CS-region of PG, as opposed to the T cells reactive with the G1 region used in the present study.

Likewise KS depletion of adult PG may enhance arthritogenicity of this molecule.

The cell line JY.A showed its potential to induces synovitis when injected intraarticularly. This was demonstrated by first stimulating the cells with a non-specific mitogen (ConA). Pathogenesis still needs to be demonstrated with *in vitro* HFPG-stimulated cells. The T cell line JY.D induced limited synovial hyperplasia. It is not clear if this is specific for the T cell line JY.D since the control ovalbumin reactive T cell line was not used in the intraarticular injection studies.

The vaccination potential of the two T cell lines remains to be examined.

STATEMENTS OF ORIGINAL CONTRIBUTIONS

These studies:

Are the first to have isolated T cell lines and generated T cell hybridomas of the TH1 subset against aggrecan from PG-induced arthritic mice.

Show age-related epitopes differences in T cell recognition , with specific T cell lines.

Demonstrate that the G1 domain contains one or more immunodominant T cell epitopes.

Shows that some of the T cells recognize cross-reacting epitopes on both G1 domain and link protein.

Are first to show that glycosaminoglycan attached to core protein can influence the level of reactivity of T cells to its epitope.

Show that glycosaminoglycans influence T cell reactivity by acting outside the T cell epitopes, probably at the level of antigen processing.

Are the first to show that a PG-specific T cell line can cause early pathological features of RA when injected directly into the joints of mice.

LIST OF PUBLICATIONS

The majority of the work done in this thesis is based on the following publications two of which are in preparation.

Leroux, J.Y., A.R. Poole, C. Webber, V. Vipparti, H.U. Choi, L.C. Rosenberg, and S. Banerjee. 1992. Characterization of proteoglycan-reactive T cell lines and hybridomas from mice with proteoglycan-induced arthritis. Journal of Immunology 148: 2090.

Leroux, J.Y., A.R. Poole, H.U. Choi, L.C. Rosenberg,, and S. Banerjee. Keratan-sulfate chains can influence antigen presentation of proteoglycan to T cells. Submitted to Journal of Experimental Medicine. January 1993.

Leroux, J.Y., A.R. Poole, H.U. Choi, L.C. Rosenberg,, and S. Banerjee. Characterization of T cell epitopes on aggrecan with proteoglycan-reactive T cells. In preparation.

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