CARBOHYDRATE RECOGNITION IN IMMUNE RESPONSES

by

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STATEMENT

Except for the experiments represented in Figures 3.4 and 6.1 which were done by Dr. C.R. Parish, all experiments described in this thesis represent my own work and were done by me.

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ABSTRACT

The material presented in this thesis examines the observation that some simple sugars inhibit immune responses and attempts to analyse the mechanisms underlying the inhibition.

In chapter 1, a survey of the literature relevant to T cell dependent antibody responses is presented, discussing the involvement of macrophages, T cells and B cells in antibody formation and highlighting the role of the H-2 complex and that of soluble "helper" factors.

Chapter 2 contains a description of the materials and methods used in the thesis.

Chapter 3 is the first experimental chapter and describes the initial observation that some sugars selectively inhibit immune responses *in vitro*. N-acetyl-D-galactosamine (Gal NAc), L-fucose (Fuc) and N-acetyl-D-glucosamine (Glu NAc) inhibited secondary IgG responses to hapten carrier conjugates in CBA/H cultures, but had no effect on the primary and secondary IgM responses to hapten-carrier conjugates, mixed lymphocyte reactions or on the generation of cytotoxic T (Tc) cells to alloantigens. Gal NAc inhibited the ability of a T cell replacing factor (TRF) to restore IgG responses to T cell-depleted cultures.

In chapter 4, the observation that some sugars selectively inhibit IgG responses in certain mouse strains was analysed. Inhibition of IgG responses to hapten carrier conjugates was H-2 haplotype dependent; hence Fuc inhibited $H\text{-}2^{k}$ but not $H\text{-}2^{d}$ or $H\text{-}2^{b}$ haplotypes whereas N-acetyl-D-mannosamine (Man NAc) and D-galactose (Gal) inhibited $H\text{-}2^{d}$ and $H\text{-}2^{b}$ but not $H\text{-}2^{k}$ haplotypes. Using recombinant

mouse strains, inhibition by Fuc was mapped to the I-J subregion and the inhibition by Man NAc and Gal to the I-C subregion of the H-2 complex. Although Gal NAc inhibited IgG responses in all mouse strains tested, its structural similarity to Man NAc and Gal suggested that the inhibition by Gal NAc is also under I-C subregion control.

In chapter 5, the nature of the sugar-inhibitable TRF was investigated. The TRF was not antigen specific, was active across H-2 barriers and was active when added late to the cultures of T cell-depleted spleen cells. The sugar inhibition pattern of TRF activity was analogous to that seen in chapter 4, i.e. Fuc inhibited $H\text{-}2^k$ but not $H\text{-}2^b$ or $H\text{-}2^d$ TRF activity whereas Man NAc and Gal inhibited $H\text{-}2^b$ and $H\text{-}2^d$ but not $H\text{-}2^k$ TRF activity; Gal NAc inhibited TRF from all strains. Moreover, the TRF molecule was shown to be an H-2 controlled lectin which recognised a carbohydrate-defined receptor on its target cell.

Inhibition of immune responses by D-mannose was examined in chapter 6. D-mannose inhibited all immune responses tested in all mouse strains. Hence unlike the other sugars discussed, it was nonspecific in its action and appeared to interfere with cellular matabolism.

Finally, chapter 7 contains a summary and concluding remarks of all the experimental chapters.

ABBREVIATIONS

AEF	allogeneic effect factor
B cell	cell with surface immunoglobulin
BSA	bovine serum albumin
CI	cell interaction
CR	complement receptor
DMEM	Dulbecco's modified essential medium
DNP	dinitrophenyl
DTH	delayed-type hypersensitivity
EMEM	Eagle's essential medium
FCS	foetal calf serum
GVHR	graft-versus-host reaction
H-2	histocompatibility-2
HIFCS	heat-inactivated foetal calf serum
HRBC	horse red blood cells
HCY	haemocyanin
Ia	I-region associated
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-2	Interleukin-2
KLH	keyhole limpet haemocyanin
Lad	lymphocyte-activating determinant
LAF	lymphocyte activation factor
MØ	macrophage
MHC	major histocompatibility complex

MLC	mixed lymphocyte culture
MLR	mixed lymphocyte response
MON	monomer of flagellin
NAP	nucleic acid precursor
NIP	4-hydroxy-5-iodo-3-nitrophenacetyl
OVA	ovalbumin
PBS	phosphate-buffered saline
PFC	plaque-forming cell
SRBC	sheep red blood cells
T cell	thymus-derived cell
Thy-1	theta-1 antigen
TNP	trinitrophenyl
Tc	cytotoxic T cells
T_H	helper T cells
Ts	suppressor T cells
TCGF	T cell growth factor
TRF	T cell replacing factor
2-ME	2-mercaptoethanol

SUGARS:

FUC	L-fucose
Glc NAc	N-acetyl-β-D-glucosamine
Glc Met	α-methyl-glucoside
Gal	galactose
Gal NAc	N-acetyl-β-D-galactosamine
Gal Met	α-methyl-galactoside
Man Met	α-methyl-mannoside
Man NAc	N-acetyl-β-D-mannosamine

CHAPTER 1

LITERATURE REVIEW:

"T Cell Dependent Antibody Responses"

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1. INTRODUCTION

For many years the immune system has appealed to biologists as it represents a unique system of differentiation, the administration of endogenous antigen resulting in the rapid differentiation of resting lymphocytes to highly specialized antibody-forming cells or plasma cells. An additional attraction of the immune system is its ability to respond and produce specific antibodies against an almost infinite number of molecular structures, a property that poses intriguing questions for both biochemists and cell biologists.

Interest in immunology has been further stimulated in the last decade by the realisation that the immune system represents a complex network of interacting subpopulations of lymphoid cells. Furthermore, the major histocompatibility complex (MHC), which was for many years thought to be a biological curiosity that frustrated transplantation surgeons, has now been shown to play a fundamental role in cell to cell communication.

The antibody response to T-cell dependent antigens clearly expresses the unique features of the immune system outlined above. This thesis deals with T-cell dependent antibody responses.

2. HISTORICAL OVERVIEW

2.1 Early Evidence for T cell- B cell collaboration

Following the pioneering experiments of Gowans *et al.* (1964) which clearly showed that lymphocytes mediated immune responses, the first evidence for lymphocyte collaboration came from an analysis by Claman and coworkers (1966) of the cellular basis of the humoral response to sheep red blood cells (SRBC). In these experiments, lethally irradiated mice were injected intravenously with varying doses of either spleen, thymus, bone marrow or thymus plus bone marrow cells from normal or imune syngeneic donors. The results clearly showed that at least two classes of immunocompetent cells were needed for optimal antibody responses. Thus neither bone marrow nor thymus cells alone produced anti-SRBC antibodies, whereas a mixture of the two populations resulted in a substantial antibody response. On the basis of these observations, the authors proposed that the bone marrow contained "effector" cells capable of producing antibody but only in the presence of "auxilliary" cells present in the thymus.

Support for collaboration between different cell populations came from Davies *et al.*, (1967), whose work showed that antibody was produced by cells originating from bone marrow and not from thymus. Moreover, they showed that the highest antibody response occurred when both thymus and bone marrow cells were preprimed to the antigen.

Perhaps the clearest evidence for thymus-derived (T) cell - Bursa-equivalent (B) cell collaboration came from Miller and Mitchell (1967) who showed that bone marrow of neonatally thymectomized mice was as effective as that of normal mice in restoring immunological

responsiveness to irradiated mice with an intact thymus. Furthermore, giving neonatally thymectomized mice allogeneic thoracic duct or thymus lymphocytes restored their responsiveness to SRBC. The resultant antibody forming cells could be destroyed by antisera against host cell alloantigens but not by antisera against donor thymus cells. These observations and their subsequent work (Miller and Mitchell, 1968; Mitchell and Miller, 1968; Nossal *et al.*, 1968) established that antibody forming cell precursors are derived from bone marrow and that, as first proposed by Claman, thymus cells recognise and specifically react to antigen, but perform an auxilliary role in the activation of antibody forming cell precursors.

Subsequent studies by a number of workers (Chan *et al.*,1970; Schimpl and Wecker, 1970, 1971; Hartmann, 1970, 1971; Hirst and Dutton, 1970; Munro and Hunter, 1970) have documented, either by the use of anti-theta (Thy-1) antisera for T cell depletion or by the use of specifically primed T cell populations, that the development of both primary and secondary anti-SRBC responses *in vitro* requires the participation of both T and B lymphocytes in a manner analogous to that postulated from *in vivo* experiments.

2.2 Early Evidence for Macrophage Involvement

The development of *in vitro* methodologies by Mishell and Dutton (1967) and Marbrook (1967), provided a highly useful approach for studying cell - cell interactions in antibody responses. Of particular importance was the ability to analyse the participation of other cells, notably nonlymphoid "accessory" cells, in the development of such responses. The first studies of this type were made by Mosier

and colleagues (Mosier and Coppleson, 1968; Mosier *et al.*, 1970) who separated mouse spleen cells into subpopulations based on their relative abilities to adhere to plastic or glass surfaces. The separation of spleen cells into adherent and nonadherent fractions resulted in cell populations that were unable to develop primary *in vitro* antibody responses. However, the antibody responses could be restored by recombining the two cell populations. The adherent cell was shown to be a macrophage (mϕ) and the nonadherent cell population contained both T and B lymphocytes.

2.3 Early Analyses with hapten-carrier Conjugates

Shortly after the initial studies demonstrating the requirement for T cell-B cell-macrophage interactions in the antibody response to SRBC, analogous interactions were demonstrated in the *in vivo* humoral responses to protein antigens and to hapten-carrier conjugates (Taylor, 1969; Chiller *et al.*, 1970; Miller and Sprent, 1971). Furthermore, with the use of hapten-carrier antigens it has been possible to further analyse cell-cell interactions in antibody responses. Since it is this system that is used extensively in this thesis, a more detailed analysis of it will be undertaken here.

The ability to couple hapten onto protein carriers, originally described by Landsteiner (1962), has provided a powerful tool for analysing the interaction between hapten-specific and carrier-specific subpopulations of lymphoid cells. Initially it was found that a hapten only elicited an anti-hapten antibody response when it was coupled to a carrier which was itself immunogenic (Benacerraf *et al.*, 1963; Levine *et al.*, 1963; Green *et al.*, 1966). Optimal secondary

hapten-specific antibody responses only occurred when animals were challenged with the same hapten-carrier conjugate used for priming, a phenomenon that was subsequently termed the "carrier effect". Since the antibodies produced by such immunizations were highly specific for the hapten and, since it was assumed that the serum antibody represented the specificity of immunoglobulin receptors on antibody-forming cell precursors, it was postulated that another cell recognised determinants on the carrier molecule.

The first direct evidence, with an antibody response to a hapten-carrier conjugate, for co-operative participation of two cells with distinct determinant specificities was obtained by Mitchison (1969). In this study it was demonstrated that syngeneic spleen cells primed to 4 - hydroxy - 5 - iodo - 3 - nitrophenacetyl ovalbumin (NIP-OVA) when injected into irradiated recipients, made a seconday anti-NIP antibody response to the homologous conjugate NIP-OVA but failed to respond to a heterologous conjugate, NIP-bovine serum albumin (BSA). However, when spleen cells from donors immunized with NIP-OVA were injected together with spleen cells from donors immunized with bovine serum albumin (BSA), a good secondary anti-NIP response was elicited by the heterologous conjugate NIP-BSA. This basic *in vivo* observation has been confirmed repeatedly by other workers.

On the basis of these findings it was postulated that for the induction of an anti-hapten antibody response, carrier-specific T cells initially interact with the hapten-carrier conjugate and induce hapten-specific B cells to differentiate into antibody forming cells. The similarities between this system and the observed thymus-bone marrow co-operation in anti-SRBC responses was immediately obvious, a point

that was further established by Raff (1970), who showed, with the use of anti-Thy-1 and complement that the carrier-specific co-operating cells or "helper" cells were indeed thymus-derived, whereas the anti-hapten antibody forming cells were not.

The capacity to analyse *in vitro* responses to hapten-carrier conjugates was greatly facilitated by the development of a simple method for coupling the chemical hapten trinitrophenyl (TNP) directly onto red cell membranes (Rittenberg and Pratt, 1969). Based on this method, Kettman and Dutton (1970) obtained a primary anti-TNP antibody response *in vitro* by culturing mouse spleen cells with TNP-SRBC. Using this system, a number of other laboratories soon obtained evidence for co-operation between carrier-specific and hapten-specific cells in the *in vitro* primary antibody response (Katz *et al.*, 1970; Trowbridge *et al.*, 1970; Kettman and Dutton, 1971). Furthermore, the carrier-specific cells were shown to be T cells as helper activity was eliminated by anti-Thy-1 and complement treatment (Dutton *et al.*, 1971).

At about the same time *in vitro* responses to soluble hapten-protein conjugates were reported (Bullock and Rittenberg, 1970a, b). Spleen cells from mice primed previously to TNP-KLH (keyhole limpet haemocyanin) were shown to develop TNP-specific IgM and IgG plaque-forming cells (PFC) when challenged *in vitro* with TNP-KLH. The *in vitro* response to soluble hapten-carrier conjugates also involved co-operative cell interactions since spleen cells only gave secondary responses when the same conjugate was used for *in vivo* priming and stimulation, thus demonstrating that these responses were analogous to the *in vivo* co-operation model (Cheers *et al.*, 1971).

As a general rule, the most effective T-B collaboration was observed when both T cells and B cells recognised distinct determinants on the same molecule (Rajewsky *et al.*, 1969; Mitchison, 1971). This important point was clearly demonstrated by Mitchison (1971) in adoptive transfer studies in mice where recipients of hapten-primed and carrier-primed spleen cell populations failed to respond when they were simultaneously challenged with the relevant hapten and the unconjugated carrier protein. Such a result was interpreted to mean that close contact between carrier-specific T cells and hapten-specific B cells is required for successful collaboration and this proximity cannot be achieved when the relevant hapten cand carrier are on separate molecules. This basic observation has been confirmed by other workers both *in vivo* and *in vitro* (Hamaoka *et al.*, 1971; Unanue and Katz, 1973; Askonas and Roelants, 1974) supporting the concept that close proximity of collaborating T and B cells is required, although the regulatory signal may be mediated either by direct cell-cell contact or via elaboration of short-range factor(s).

Although experiments such as these show that in many systems the most efficient T cell-B cell interaction occurs when the respective determinants are present on the same molecule, there are exceptions to this rule. A number of workers have reported substantial secondary anti-hapten antibody responses both *in vivo* (Hamaoka *et al.*, 1973) and *in vitro* (Sulica *et al.*, 1971; Kishimoto and Ishizaka, 1973a) when the hapten and the relevant carrier were present as separate molecules. The most likely explanation for these differences in the ability to elicit antibody responses to linked or nonlinked determinants is antigen concentration. Hence at high concentrations of hapten and unconjugated

carrier, there is a greater probability of close proximity between these two molecules in the membranes of accessory cells, a situation that could facilitate T-B collaboration. In the case of *in vitro* antibody responses an additional factor is that short range and/or unstable T cell factors could accumulate in the culture medium and allow T-B collaboration to occur at a distance.

3. CELLULAR REQUIREMENTS FOR T-DEPENDENT ANTIBODY RESPONSES

At least three cell types are required for the induction of an antibody response to a T cell-dependent antigen. They are a) thymus-derived (T) lymphocytes, b) bone marrow derived (bursa equivalent) antibody-forming cell precursors, (B) lymphocytes, and c) accessory cells. These three cell types are physically and functionally distinct, T cells being classically characterised in the mouse by the Thy-1 cell surface antigen, B cells being characterised by a high density of surface immunoglobulin (Ig) and accessory cells being distinguished from lymphoid cells by their adherent properties. A brief description of these cell types is presented below.

3.1 Accessory Cells

The regulatory role of accessory cell in immune induction has been reviewed in detail by Unanue (1972, 1981) and therefore will only be briefly outlined here. As mentioned above (section 2.2), the active participation of an adherent, non-lymphoid cell is necessary for the generation of antibody responses, and since this cell is frequently only defined by its adherent properties it is usually termed "accessory cell". Classically, cells of the mononuclear phagocyte lineage have been

considered to be the major accessory cell population in most immune responses (Feldmann, 1972a). However, *in vitro* studies indicate that several members of the mononuclear phagocyte system can function as accessory or antigen-presenting cells, namely peripheral blood monocytes (Rosenberg and Lipsky, 1979), peritoneal macrophages (Rosenthal *et al.*, 1976), Kupffer cells of the liver (Lipsky and Rogoff, 1980), splenic adherent cells (Cowing *et al.*, 1978a) and alveolar macrophages (Lipscomb *et al.*, 1981). Furthermore, cells that are not part of the mononuclear phagocyte system, such as dendridic cells and Langerhans cells, may also function as accessory cells. It should be noted, however, that in many cases the assay for accessory cell function has been the ability to support antigen-specific T cell proliferation rather than initiation of antibody responses.

Within these accessory cell populations it has been shown that only those cells expressing I-region associated (Ia) antigens can act as accessory cells (Yamashita and Shevach, 1977; Cowing *et al.*, 1978 b). For example, the primary *in vitro* antibody response to TNP-KLH was shown to be strongly dependent upon phagocytic accessory cells bearing both I-A and I-E/I-C subregion controlled determinants (Hodes *et al.*, 1978; Dickler *et al.*, 1980), a point that is discussed in more detail in section 4.3. In a separate study, Niederhuber and coworkers (1978, 1980) reported that a primary *in vitro* anti-SRBC response was inhibited when accessory cells were pretreated with anti-I-J sera. By contrast, pretreatment of accessory cells with anti I-A and/or anti I-E/I-C sera had no effect: these latter antisera were only effective when added in the presence of complement (Hodes *et al.*, 1978; Dickler *et al.*, 1980). Such data suggest that macrophage Ia determinants

play a key role in immune induction, an interpretation discussed more extensively in section 4.3.

In vitro experiments have also indicated that accessory cells not only act as antigen-presenting cells but can accomplish additional functions. For example, it has been reported that they maintain lymphocyte viability and functional integrity in the absence of antigen activation (Chen and Hirsch, 1972; Ellner *et al.*, 1976). This may involve the production of soluble factors essential for cell growth or viability.

Direct physical contact between lymphocytes and macrophages also appears to be essential for these cells to express many of their biological activities (Lipsky and Rosenthal, 1973, 1975). Thus, macrophage-lymphocyte clusters provide a focus for collaboration between T cells and B cells (Katz and Unanue, 1973), allow the direct presentation of immunologically relevant determinants by antigen-bearing macrophages (Lipsky and Rosenthal, 1975) and may regulate the transmission of information between T and B cells (Ptak *et al.*, 1977). Furthermore, macrophages may also modify lymphocyte reactivity through the action of secreted immunoregulatory molecules, in particular interleukin-1 (IL-1) (see section 5.3). The secretion of soluble factors may be increased or even triggered by the development of physical contact between macrophages and lymphocytes, thus explaining the importance of direct macrophage-lymphocyte contact in lymphocyte activation. In turn, the factors may be at optimal concentrations in close proximity to the cell secreting them.

In summary, the role of macrophages in immune induction is served by two distinct yet interrelated functions: handling and presentation of antigen in association with I region products to lymphocytes, and secretion of lymphostimulatory molecules.

3.2 Lymphocytes

As reviewed in section 2, the past two decades have seen the recognition of two distinct classes of lymphocytes that are required for immune responses. It is generally accepted that one class of lymphoid stem cell from yolk sack, foetal liver or bone marrow migrates to the thymus, acquires immuno-competence in the thymic microenvironment and then migrates to spleen and lymph nodes as mature T cells. The second lymphoid cell class, referred to as B cell, originates in the bone marrow in mammals, although these cells have been shown to originate from a separate lymphoid organ in chickens, namely the Bursa of Fabricius. These cells also migrate to peripheral lymphoid organs such as spleen and lymph nodes and become antibody-forming cell precursors (Claman and Chaperon, 1969; Davies, 1969; Miller and Mitchell, 1969; Katz and Benaceraff, 1972). The general properties of the T and B lymphocytes required for T-dependent antibody responses will be discussed below.

3.2.1 T lymphocytes

The functional subdivision of lymphocyte populations has been greatly assisted by the development of antisera against cell surface antigens (reviewed by McKenzie and Potter, 1979; McKenzie *et al.*, 1981). Thy-1 was the first alloantigen used to distinguish T cells ($Thy\text{-}1^+$) from B cells ($Thy\text{-}1^-$) in the mouse and is now used as a standard cell surface marker for murine T cells (Raff, 1971; McKenzie and Potter, 1979). Following these early studies, additional alloantigens have been described that discriminate between functional subsets of lymphoid cells (McKenzie and Potter, 1979). On the basis of these studies it has become apparent that T lymphocytes represent a highly complex mixture of functional

subsets of cells. Not only can T cells act as "helper cells" for antibody responses, but they can mediate such diverse phenomena as delayed-type hypersensitivity (DTH), lysis of target cells and suppression of immune responses; effects that are usually mediated by different subsets of T cells. Of particular relevance to antibody responses was the realisation that T cell subsets exist that can either "help" or "suppress" antibody formation (Katz and Benacerraf, 1972, Eardley *et al.*, 1978). Although superficially these observations added additional complexity to the system, they were consistent with the notion that T cells regulate antibody production by B cells.

There are a number of excellent reviews and monographs on functional T cell subsets (Cantor and Boyse, 1975; Katz, 1977), particularly suppressor T cells (Parks and Weigle, 1979; Cantor *et al.*, 1978; Asherson *et al.*, 1980) and consequently this discussion will touch on these only briefly. Furthermore, subsequent sections of this review will concentrate on the induction of antibody formation by helper T (T_H) cells rather than the inhibition of humoral immunity by suppressor T (Ts) cells, as the helper function is more relevant to the experimental chapters of the thesis.

In functional terms, T cells may be broadly categorised as either regulatory cells or effector cells, each of these having a number of subcategories. The effector cells are T cells mediating graft versus host (GVH) reactions, cytotoxic killing (Tc) and delayed-type hypersensitivity (T_{DTH}) (review in Katz, 1977). Regulatory cells consist of helper (T_H) and suppressor (Ts) T cells that can modify both humoral and cell-mediated immunity.

Helper T lymphocytes have been differentiated from other T cells by both functional and cell surface characteristics. The most useful cell surface antigens for differentiating functional T cells subsets are the Ly-1 and Ly-2/3 alloantigens (Boyse *et al.*, 1968, 1971), although the use of other antigens such as Ly-6 and Ly-7 is also becoming important (McKenzie *et al.*, 1977a, b).

It was originally believed that T_H and T_{DTH} cells were Ly-$1^+2/3^-$ whereas Ts and Tc cells were Ly-$1^-2/3^+$. However, it appears now that all T cells express the Ly-1 antigen, but Ly-1 density varies greatly between T cell subsets: Ly-$2/3^-$ cells expressing the highest quantity of Ly-1 and Ly$2/3^+$ cells expressing the lowest amounts (Mathieson *et al.*, 1979; Ledbetter *et al.*, 1980; Hogarth *et al.*, 1980). Nonetheless, the Ly alloantigens have been used extensively to discriminate between functional subpopulations of T cells. For example, the Ly phenotype of T_H cell precursors has been examined by some workers and been shown to be either Ly-1^+, $2/3^-$ (Feldmann *et al.*, 1977) or Ly-$1^+2/3^+$ (Shen *et al.*, 1981), depending upon the experimental system used. One explanation for this discrepancy is that the Ly-$1^+2/3^+$ cells are not T_H precursors but aid in the development of T_H cells.

Although the Ly antigens cannot be used to distinguish between Tc and Ts cells, the expression of I-J subregion controlled determinants on Ts cells has become a particularly useful method of differentiating these two activities (Murphy *et al.*, 1976). It should be noted however, that I-J^+ T_H cells have been reported (Tada *et al.*, 1978). An additional method of separating Ts cells has been described by Shearer *et al.*, (1972) based on the affinity of these cells for histamine.

While both T_H and T_{DTH} cells are Ly-$1^+2/3^-$, they can be differentiated functionally. The use of selective immunization regimens by a number of investigators have suggested that the induction of T_H cells may be inversely related to the induction of cells mediating DTH reactions (Parish, 1972). In a typical experiment, immunization with low doses of SRBC induced DTH but failed to induce antibody (Gordon and Yu, 1973). These observations were supported by others (Liew and Parish, 1974; Silver and Benacerraf, 1974) and functionally dissociated T_H from T_{DTH} cells.

Similarly, Dennert and Lennox (1974) developed a system for selective stimulation of Tc or T_H cells. Their findings indicated an inverse relationship between the generation of Tc and T_H cells. Comparable observations by Janeway *et al.*, (1975) clearly demonstrated that T cells mediating helper activity for humoral immune responses are distinct from those participating in cytotoxic reactions as well as those mediating DTH responses.

Thus T_H cells for antibody responses appear to be in many cases physically and functionally distinct from Tc, T_{DTH} and Ts cells. However, even T_H lymphocytes are not a homogeneous class of cells (see section 4.4.1). Heterogeneity within the Ly-$1^+2/3^-$ T_H cells for antibody responses has been demonstrated by a number of laboratories (Marrack and Kappler, 1976; Tada *et al.*,1978; Janeway, 1979; Bottomly *et al.*, 1980; Takatsu *et al.*, 1980). In these studies two helper T cells which may act independently or synergistically were identified: Th_1 which is Ly-$1^+2/3^-$, 1-J^-, 1-A^- and is nylon wool nonadherent and Th_2 which is Ly-$1^+2/3^-$, 1-J^+, 1-A^+ and nylon wool adherent.

3.2.2 B lymphocytes

B cells are the precursors of antibody forming cells, and like T cells, they are diverse in terms of physical properties, display of cell surface markers and responsiveness to various stimuli. This heterogeneity is due in part to cells differing in terms of maturity and antigen experience. In addition, differences in responsiveness may also reflect functional specialisation of B cell subsets (reviewed by Katz, 1977).

Surface immunoglobulin (Ig) is the most widely used marker for identifying B cells. B cell subsets expressing one or more of the known Ig classes, namely IgM, IgD, IgG, IgA and IgE have been identified. IgM is the first Ig class expressed on immature cells, subsequently followed by IgD and then other classes. This differentiation is probably antigen driven, although there is some evidence that differentiation can occur in absence of exogenous antigens (reviewed by Kincaide, 1981). Usually the Ig specificity for antigen expressed on B cell surface represents the specificity it will synthesise following antigenic stimulation. However, B cells do have an ability to switch Ig class they synthesise, whilst maintaining the same specificity for antigen. The manner in which Ig genes are rearranged is now well documented, although the actual mechanisms of gene splicing and the factors which regulate this process remain unknown (Cory and Adams, 1980; Weigert *et al.*, 1980).

In addition to Ig, B cells express a variety of cell surface markers (reviewed in Katz, 1977). Some of these are found only on B cells, namely (a) alloantigens such as Lyb - 4, 5, 6, 7, 8 (b) heteroantigens such as mouse-specific B cell antigen (MBLA) and mouse specific plasma cell antigen (MSPCA) (reviewed by Katz, 1977). Examples of other surface

structures found on B cells but not exclusive to them are Fc receptors for different Ig classes (Basten *et al.*, 1972), receptors for the third component of complement (CR) and antigens of the major histocompatibility complex.

Functional subsets of B lymphocytes have been identified on the basis of some of the cell surface markers listed above. Thus Parish and Chilcott (1975) demonstrated that antigens vary in their ability to activate CR^+ and CR^- B cells to synthesise specific antibody. Polymeric antigens such as DNP-POL and horse red blood cells were found to activate both CR^+ and CR^- B cells to secrete antibody whereas soluble antigens such as DNP-MON triggered only CR^+ B cells. It was concluded that polymeric nature rather than T cell dependence is the factor that determines whether an antigen can provoke CR^- B cells to secrete antibody since the response to HRBC is T cell dependent while the response to DNP-POL is not. Complement receptors may facilitate T-B interaction by stabilizing the antigen on immunoglobulin receptors on CR^+ B cells. Polymeric antigens, on the other hand, bind avidly to Ig receptors without such a mechanism.

The CBA/N mouse strain has been particularly useful in delineating B cell subpopulations as it lacks a functional subset of B cells carrying the Lyb 5 alloantigen. Asano *et al.*, (1981) and Singer *et al.*, (1981) have shown that both Lyb 5^+ and Lyb 5^- subsets respond to T cell dependent antigens *in vivo* and *in vitro*; however, while the activation of the Lyb 5^+ subset did not require the T cell recognition of MHC determinants on its cell surface, the Lyb 5^- subset can only be activated by T cells which do interact with MHC antigens on its membrane.

A subset of B cells can be activated to antibody secretion without T cell help (Playfair and Purves, 1971) to haptenated "T cell

independent antigens" such as Ficoll, T4, *Brucella abortus*, and LPS. Whether responses to these antigens are truly T cell independent or whether they are very efficient inducers of T_H cells is still a contentious issue (Letvin *et al.*, 1981) discussed in detail by Katz (1977).

4. CENTRAL ROLE OF THE MHC IN HUMORAL IMMUNITY

4.1 General Considerations

The general requirements for cell-cell interaction in the development and regulation of immune responses have been extensively investigated during the past 10-12 years. During this time a large amount of evidence has been obtained which indicates that gene products of the major histocompatibility complex (MHC) play an important role in lymphocyte function (reviewed by Katz and Benacerraf, 1975, 1976; Rosenthal and Shevach, 1976; Solliday-Rich and Rich, 1976). The original studies which implicated the MHC, suggested that the interaction of macrophages with T cells (Rosenthal and Shevach, 1973) and T cells with B cells (Kindred and Shreffler, 1972; Katz *et al.*, 1973) occurred only if these cells shared MHC determinants. These observations led to the proposition that cell-cell collaboration in immune responses is controlled by MHC-linked cell interaction (CI) genes coding for cell surface molecules which mediate these interactions (Katz and Benacerraf, 1975).

Initially, CI genes were thought to serve a purely physiological role, enabling T cells and B cells to interact. The role of the MHC in immune responses became more clear cut when Zinkernagel and Doherty (1974) demonstrated that virus-specific cytotoxic T cells only lysed virus-infected target cells which were MHC compatible. This phenomenon became known as "MHC (H-2 in the mouse) restriction". Subsequent work established an immunologically important role for MHC antigens, showing that T cell

recognised a foreign antigen in association with a self-MHC antigen (Zinkernagel and Doherty, 1979).

4.2 The Murine MHC

The phenomenon of "MHC restriction" has been studied most extensively in the mouse due to the well characterised MHC of this species. Before discussing MHC involvement in the humoral immunity, the general properties of the murine MHC will be considered.

4.2.1 Genetic Arrangement

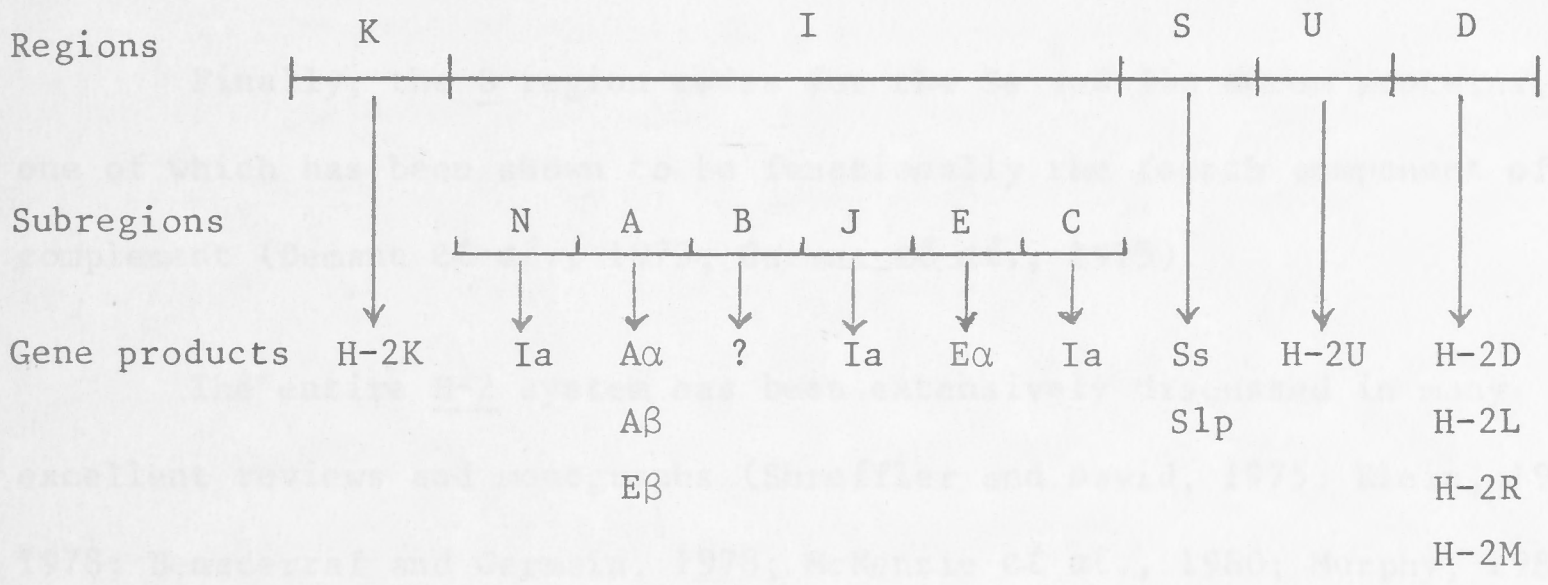
The MHC, known in the mouse as the histocompatibility 2 complex (H-2), (originally defined by Gorer, 1936), is known to affect a spectrum of immune functions such as cell-mediated immunity, collaborative interactions between T cells and B cells and proliferative responses to various antigens (reviewed by Klein, 1975, McKenzie *et al.*, 1980). The H-2 complex also controls a variety of nonimmunological functions such as serum testosterone levels, mating preference and cortisone-induced cleft palate (Klein, 1978).

The analysis of this gene complex has been greatly advanced by the availability of H-2 congenic and intra-H-2 recombinant strains of mice (Klein, 1975) which have enabled the division of the H-2 complex into distinct regions and subregions, each defined by a marker locus. The H-2 complex can be divided into four major regions, namely H-2K, H-2I, H-2S, and H-2D (see Figure 1).

The H-2K and H-2D regions code for serologically defined H-2K and H-2D antigens which serve as strong transplantation antigens and are involved in the activation of T cells in GVH reactions, and in the induction of cell-mediated cytotoxicity (Klein, 1975). More recently,

FIGURE 1

Genetic map of the murine MHC



a sixth sub-region I-N has been assigned on the basis of serological and MLR data (Hayes and Bach, 1980). Also, a new region termed H-2U has been recently defined at the D end of the H-2 complex that appears to control Ia-like antigens and may have arisen from gene duplication of a primordial I region (O'Neill and Parish, 1981a).

Finally, the S region codes for the Ss and Slp serum proteins, one of which has been shown to be functionally the fourth component of complement (Demant *et al.*, 1973; Curman *et al.*, 1975).

The entire H-2 system has been extensively discussed in many excellent reviews and monographs (Shreffler and David, 1975; Klein, 1975, 1978; Benacerraf and Germain, 1978; McKenzie *et al.*, 1980; Murphy, 1980a) consequently the section below will only briefly describe the H-2K and H-2D antigens and will focus largely on the I region encoded antigens.

4.2.2 H-2K and H-2D Antigens

The H-2K and H-2D region coded antigens are expressed on virtually all tissues. Some 70 antigenic specificities are controlled by these regions indicating the high degree of complexity and polymorphism of this antigenic system (Klein *et al.*, 1978; Morgan *et al.*, 1980). Biochemical studies have shown that both molecules are glycoproteins with a molecular weight of 45,000 daltons and, in contrast to the Ia antigens, associate with β_2 microglobulin (11,500 daltons). While studies on the chemical composition and biochemical properties suggest that the K and D gene products are proteins, evidence for carbohydrate-defined specificities for H-2K antigens also exists, suggesting the existence of two types of antigenic determinants (O'Neill *et al.*, 1981; O'Neill and Parish, 1981b).

As mentioned earlier, there is now good evidence that the H-2K and H-2D antigens can act as strong transplantation antigens and

lymphocyte activating determinants and, also, represent the "self" structures involved in the "H-2 restricted" recognition of target cells by Tc cells.

4.2.3 Ia Antigenic System

The I-region associated (Ia) antigens of the mouse are a highly polymorphic system of antigens which are controlled by a cluster of genes located within the H-2 complex (Shreffler and David, 1975; David, 1976). Whether these Ia antigens represent the products of Ir genes is at present controversial, although recent studies with an I-region mutant mouse strain suggests that this is indeed the case (McKenzie *et al.*, 1981). The Ia antigens undoubtedly play a role in MLR (Schwartz *et al.*, 1976a; Okuda and David, 1978) and T-mϕ interactions (Schwartz *et al.*, 1976b; 1978). They are also associated with several immunoregulatory molecules such as helper factors (Armerding *et al.*, 1974; Munro and Taussig, 1975), and suppressor factors (Tada *et al.*, 1976; Theze *et al.*, 1977) of antibody formation.

It is generally accepted that the Ia antigens are glycoprotein molecules of molecular weight 58,000 daltons, which dissociate into two polypeptide chains of mol. wt. 33,000 (α chain) and 25,000 daltons (β chain) (Cullen *et al.*, 1976). The antigenic determinants of these molecules appear to reside in their protein portion. However, considerable evidence has now been obtained which suggests that Ia antigenicity can also reside in carbohydrate determinants (reviewed by Parish and McKenzie, 1980). The carriers of these antigens appear to be gangliosides (Parish *et al.*, 1976a, b, c), and they have been detected on cell surfaces as well as on soluble T cell factors. Together, the evidence suggests that two classes of Ia antigens exist, one whose anti-

genic determinants are defined by protein, the other by carbohydrate.

Unlike the H-2 antigens, Ia antigens have a restricted cellular distribution, possibly reflecting their different functional role. They are expressed in highest concentration on B cells and macrophages, but can also be detected on T cells especially when these cells become activated (reviewed by McKenzie and Potter, 1979).

Genetic control of the expression of Ia antigens can involve interaction between more than one I subregion. Recent studies by Jones *et al.* (1978) suggest that the Ia molecules controlled by the I-E subregion arise by complementation between gene mapping in both the I-A and I-E subregions. Subsequently, Cook *et al.* (1979) demonstrated considerable heterogeneity in the β chains of I-E molecules detected in recombinant strains differing at the I-A subregion. Since no heterogeneity was found in the α chains of the recombinants, these results suggest that the I-E controlled molecules are hybrids, the α chain being the product of I-E and the β chain being coded for by the I-A subregion. The β chain also appears to be essential for insertion of the molecule into the plasma membrane (Jones *et al.*, 1978).

As already mentioned, the I-region can be subdivided into six genetic regions, I-N, I-A, I-B, I-J, I-E, I-C which will be considered here separately. Each subregion is associated with one or more genes which control different immunological phenomena, although whether some of these phenomena are effects of one or several genes mapping to a particular subregion is not yet known.

I-N subregion

The I-N subregion maps between H-2K and I-A and has been only recently identified by serologically defined Ia specificity and MLR stimulating determinants (Hayes and Bach, 1980).

I-A subregion

The I-A subregion contains the Ia-1 locus coding for Ia specificities found mainly on B cells (Hammerling, 1976; Abbas *et al.*, 1976) and less often on T cells (Murphy *et al.*, 1976b; Stout *et al.*, 1977; Fujimoto 1978; Marrack *et al.*, 1980), splenic macrophages (Cowing *et al.*, 1978b), epidermal cells and Langerhans cells (Frelinger *et al.*, 1978). However, current evidence suggests that some of the Ia specificities on T cells differ from those on B cells (Stout *et al.*, 1977; Hammerling *et al.*, 1979; Marrack *et al.*, 1980), suggesting that at least two Ia loci exist in the I-A subregion.

I-A subregion antigens have also been found on several immunoregulatory factors such as helper (Thf) and augmenting (Taf) T cell factors (Taussig *et al.*, 1975; Tokuhisa *et al.*, 1978; Howie *et al.*, 1979), allogeneic effect factor (Armerding *et al.*, 1974) and macrophage helper cell inducing factor (Erb *et al.*, 1976). The I-A subregion also codes for antigens on B cells acting as acceptor sites for some of these Ia-bearing factors (Taussig *et al.*, 1976), thus implying that Ia antigens can mediate cell-cell communication.

A great deal of evidence suggests that products of the I-A subregion (probably the I-A antigens) play an important role in the induction and regulation of immune responses. Some of this evidence is listed below briefly.

. Cellular interactions between T cells and B cells (Sprent and Alpert, 1981; Asano *et al.*, 1981) and T cells and macrophages (Erb and Feldmann, 1975) are genetically restricted, requiring I-A subregion compatibility for successful interaction.

. Genes mapping in the I-A subregion code for lymphocyte-activating determinants (Lad-1) which induce strong MLR (Klein, 1975; Lonai and McDevitt, 1977). Furthermore, anti-I-A sera that react with the stimulator cells can specifically block the induction of the MLR.

. A locus leading to graft rejection maps to the I-A subregion (Klein, 1975). As with the MLR above, a positive graft rejection response correlates with the presence of an Ia antigenic difference (McKenzie and Henning, 1977). Anti-Ia sera enhance graft survival.

. Anti I-A subregion sera and anti I-A subregion monoclonal antibodies can specifically block antigen-induced T cell proliferation (Schwartz *et al.*, 1978; Nepom *et al.*, 1981) and T-dependent antibody responses (McDevitt *et al.*, 1976; Sprent, 1980a).

Taken together, these points suggest that the I-A subregion controlled antigens play a major role in the induction and regulation of immune responses, a point of view strongly supported by the I-A subregion mutant, B6.C-H-2^{bm12}, which simultaneously express modified Ia antigens, transplantation antigens, Lad determinants and Ir genes (McKenzie *et al.*, 1979; Michaelides *et al.*, 1981).

I-B subregion

This region was initially defined by the presence of immune response (Ir) genes controlling responsiveness to an IgG protein (Klein, 1975). One other Ir gene effect controlled solely by this subregion is recorded: it controls responsiveness to the male-specific H-Y antigen for DTH and graft rejection (Hurme *et al.*, 1978; Liew and Simpson, 1980). So far, no Ia antigens are known to be associated with I-B.

I-J subregion

This region, containing the Ia-4 locus, was originally defined by alloantisera which, in combination with complement, removed suppressor T cells for antibody responses (Murphy *et al.*, 1976b). the I-J subregion antigens are difficult to detect serologically, possibly because only a small number of lymphoid cells bear this marker (Parish and McKenzie, 1977).

Initially, the I-J antigens were associated with suppressor function in a range of immune responses. Thus, I-J determinants were demonstrated on carrier-specific, idiotype-specific and tumour-specific suppressor T cells, since treatment of the cell populations with anti-I-J sera and complement, removed these activities (Murphy, 1980). Additionally, the I-J antigens have been detected on T cell derived, antigen specific suppressor factors for humoral (Tada *et al.*, 1976) and cellular (Greene *et al.*, 1977) immunity as well as being associated with the acceptor sites for suppressor factors on T cells (Tada *et al.*, 1977).

Although I-J determinants are widely used as a marker for Ts cells, they have been detected on other cell types. A macrophage subpopulation required in the generation of primary *in vitro* antibody responses is $I\text{-}J^{+}$, and anti I-J sera also blocks its activity (Niederhuber and Allen, 1980). Furthermore, a subpopulation of T_H cells has also been shown to carry I-J determinants (Tada *et al.*, 1978) although whether this antigen is directly involved in mediating helper function remains to be determined. to date, all B cells are apparently $I\text{-}J^{-}$. In addition it appears that the I-J determinants found on accessory cells and helper T cells are distinct from those found on suppressor T cells (Murphy *et al.*, 1981), suggesting that at least two loci exist in the I-J subregion, an assumption supported by the observation that the I-J subregion codes for determinants expressed on suppressor factors as well as for

the acceptor site on target cells for these factors.

Thus, as with I-A subregion antigens, the I-J determinants appear to be regulatory structures used for cell communication both in a positive and negative mode.

I-E subregion

The I-E subregion is defined by the Ia.7, Ia.22 and Ia.23 specificities, all of which are present on one Ia molecule and are therefore controlled by the one locus (Shreffler *et al.*, 1977; Cullen *et al.*, 1980). I-E determinants are found on most B cells (Abbas *et al.*, 1976; Shreffler *et al.*, 1977), on some splenic macrophages (Cowing *et al.*,1978b) but not on resting T cells. Recent studies have demonstrated the presence of a second locus in the I-E subregion, coding for determinants found on Con A-activated T cells but not on B cells (Hayes and Bach, 1978).

As mentioned above, the β chain of the I-E molecule is coded for by the I-A subregion whereas the α chain is an I-E subregion product. The I-E subregion coded part appears to regulate the cell surface expression of the I-A subregion product (David and Cullen, 1978). More recently, it has been shown that a gene mapping in I-A-I-J regulates the quantitative expression of the I-E subregion controlled α chain (Murphy *et al.*, 1980). These findings demonstrate that I region gene interactions may be essential for the expression of some Ia antigens and provide a rational explanation for complementing Ir genes in the I-A and I-E/C subregions (Benacerraf and Germain, 1978).

I-C subregion

This region is defined by the Ia.6 specificity which is only found in two haplotypes (David and Shreffler, 1974). Some difficulty

has been encountered in demonstrating the existence of the I-C subregion and for a long time the I-C and I-E subregions were considered to be a single subregion I-E/C (Murphy, 1980). However, recent work of Sandrin *et al.*, (1981) has confirmed the original observations of David & Shreffler (1974) and provided good evidence for the existence of an Ia.6 specificity and hence a distinct I-C subregion.

The I-C subregion has been detected on a subpopulation of T cells and mitogen-stimulated T cell blasts (David *et al.*, 1976; McKenzie *et al.*, 1980). Ia specificities controlled by a gene mapping in the I-C subregion have also been detected with the use of xenogeneic antisera. These determinants represent carbohydrate-defined specificities and have been detected mainly on B cells (McKenzie *et al.*, 1980), suggesting the existence of a second locus in the I-C subregion. In addition, the I-C subregion codes for Lad determinants expressed on stimulator T cells in the MLR (Okuda and David, 1978), and antigens on suppressor factors generated in a MLR (Rich *et al.*, 1979).

In summary, the different I subregions appear to control a variety of related effects such as determinants on helper and suppressor cells or factors, Lad determinants and Ir gene action. The ability of the appropriate anti-Ia reagents to block these various functions suggests a direct role for Ia antigens in these effects.

As mentioned above, it is now generally accepted that MHC antigens (H-2K, H-2D, and Ia) play a key role in the presentation of foreign antigens to the immune system, T cell recognising an association of antigen with MHC gene products. In the case of virus-specific Tc cells it has been suggested that H-2K and H-2D restricted recognition

ensures that Tc cells identify and kill virus-infected cells while ignoring an excess of free virus (Howard, 1980). On the other hand, since Ia antigens have a limited cellular distribution, I-region restricted recognition may control cell-cell collaboration in immune responses. Nonetheless, of all immune responses, the role of MHC gene products in antibody responses remains the most controversial. The sum of evidence suggests however, that MHC restriction in responses to T-dependent antigens applies at two different levels: first, during activation of T helper cells (a manifestation of T-macrophage interaction) and second, during T cell-B cell collaboration per se.

4.3 MHC involvement in T cell-macrophage interaction

There is general agreement that the macrophage presentation of antigen to T lymphocytes in a range of systems is H-2 restricted (Rosenthal and Shevach, 1973; Erb and Feldmann, 1975; Paul *et al.*, 1977; Sprent, 1980). Although little is known of the exact role of macrophages in the antigen presentation, it seems that small fragments of antigen become associated with macrophage MHC determinants (usually Ia antigens). T cells then recognise this association, are stimulated, and henceforth can only be restimulated by antigen presented in association with the same MHC determinants.

A number of workers have demonstrated that the response in primary *in vitro* antibody responses to hapten-carrier required that T cells and macrophages share the I-A subregion of the H-2 complex (Singer *et al.*, 1977, 1978). Using a somewhat different approach, Kappler and Marrack (1976, 1978) reached the same conclusion: in their system it was found that F_1 (P_1 x P_2) T_H cells primed to antigen on parent 1 macrophage could subsequently only be activated by antigen on P_1 but not P_2 macrophage.

Analysis of a range of recombinant macrophages revealed that the I-A subregion of P_1 was required for antigen presentation. The demonstration that Ia^+ macrophages are required for *in vitro* antibody responses (Yamashita and Shevach, 1977; Cowing *et al.*, 1978a) is consistent with Ia involvement in these responses (see section 3.1). However, using a primary anti-SRBC response, Niederhuber (1978, 1980) reported that although macrophages carrying I-A, I-E/C and I-J determinants were required, treatment of macrophages with anti-I-J but not anti-I-A or I-E/C antibodies, completely blocked their ability to present antigen to T cells. This result appears to be inconsistent with those mentioned above, since it implies that the I-J, not the I-A, determinant is the restricting element in T-macrophage interactions. One possible differene is that Singer *et al.*, Kappler and Marrack tested the restriction in a secondary (primed) situation whereas Niederhuber examined a primary response.

It should be noted that in some studies, I region restricted interactions between lymphocytes and macrophages were not found (Katz and Unanue, 1973; Unanue, 1978). In these experiments allogeneic macrophages were used to present TNP-KLH to carrier-primed spleen cells. However, the primed spleen cells were unfractionated and therefore contained syngeneic macrophages. Unanue (1978) raised the possibility of antigen transfer from allogeneic to syngeneic macrophages, a possibility with is supported by the work of Pierce and Malek (1980).

In vivo evidence for MHC-restricted interaction between helper T cells and macrophages has been elegantly demonstrated by Sprent (1978a, b). In his system, purified F_1 (P_1 x P_2) T cells were primed to antigen in an irradiated parent 1 strain. The resultant T_H cells were shown subsequently

to only be activated when exposed to antigen in the presence of P_1 but not P_2 accessory cells. Furthermore, the priming process could be blocked by the injection of monoclonal anti Ia P_1 antibodies (1980b).

4.4 MHC Involvement in T cell-B cell interaction

While it is generally believed that the T cell-macrophage interaction in antibody responses is I- region restricted, the unresolved question until very recently was whether T cell clones which respond to antigen in association with a particular macrophage Ia antigen will only collaborate with B cells bearing the same Ia antigens.

Thus, cellular interactions between T_H cells and B cells in the generation of primary and secondary IgM and IgG responses both *in vivo* and *in vitro* have variously been reported as being restricted by the MHC (Katz *et al.*, 1973; Sprent, 1978b, c; Swierkosz *et al.*, 1978) or not being MHC restricted (McDougal and Cort, 1978; Erb *et al.*, 1979; Singer *et al.*, 1979, 1981; Shih *et al.*, 1980).

The explanation for these differences has not been apparent. Variables such as the antigen-primed status of the responding population of cells, the predominant Ig isotype of the response and the use of *in vivo* or *in vitro* response systems have failed to correlate with the presence or absence of a requirement for H-2 restricted recognition of B cells.

Although a large number of workers have tried to resolve the question of MHC restriction between T and B cells since Katz *et al.*, (1973), first reported it, much of the early work is difficult to evaluate, either because the reported lack of H-2 restriction may have been due to allogeneic effects between H-2 incompatible cell

populations or, the apparent presence of restriction could have been due to the absence of the appropriate H-2 bearing macrophages, thus preventing helper T cells from being reactivated.

Possibly the two best experiments resolving this issue were done by Sprent *in vivo* (1978c) and by Asano *et al.* (1981) and Singer *et al.* (1981) both *in vivo* and *in vitro*. Sprent used F_1 (P_1 x P_2) into parent (P_1) chimeric T_H cells primed to SRBC in the context of P_1 MHC antigens. He injected these T_H cells into irradiated F_1 (P_1 x P_2) hosts (which therefore contained radioresistant macrophages able to present antigen in the context of either the P_1 or P_2 H-2 type) together with either P_1 or P_2 derived B cells and antigen and demonstrated that such T cells could help only P_1 B cells but not P_2 B cells. His system avoided the allogeneic effect and supplied the appropriate antigen-presenting cells for reactivation of primed T cells thus unequivocally demonstrating that T-B interaction during a secondary antibody response to SRBC is H-2 restricted.

Asano *et al.* (1981) used F_1 (P_1 x P_2) into P_1 chimeric T_H cells which were cultured *in vitro* with appropriate F_1 or P_1 strain macrophages) in the presence of either P_1 or P_2 B cells and TNP-KLH. It was demonstrated that for responses to low concentrations of antigen the T_H cells only collaborated with B cells of the P_1 H-2 haplotype. In contrast, at higher antigen concentrations, the T_H cells were unrestricted, collaborating with both P_1 and P_2 B cells.

In contrast to the findings of Sprent, Singer *et al.* (1981), using a cell transfer system to examine a primary *in vivo* antibody response to SRBC demonstrated that in normal animals the response was restricted by the I-A subregion at the T cell-B cell level whereas in CBA/N mice no

H-2 restriction occurred, although both of these responses were restricted by the I-A subregion at the T cell-macrophage level.

Underlying the controversy of H-2 restriction at the T cell-B cell level is the possibility that both T_H cells and B cell populations are functionally heterogeneous. Indeed, it has been suggested that two distinct subpopulations of T_H cells exist, one of which is H-2 restricted at the T-B level while the other is not (Janeway, 1975; Marrack and Kappler, 1976; Tada *et al.*, 1978; Takatsu *et al.*, 1980, etc.). Similarly, some reports suggest that at least two distinct subpopulations of B cells with identical antibody repertoires but different activation requirements exist (Asano *et al.*, 1981; Singer *et al.*, 1981). One of these B cell subpopulations interacts in an MHC-restricted manner with T_H cells whereas the other B cell subpopulation does not.

4.4.1 Heterogeneity in T helper cells

A number of laboratories have now shown that more than one kind of T_H cell is required for B cell responses to protein-coupled antigens (Feldmann, 1972b; Schimpl and Wecker, 1972; Waldmann *et al.*, 1976; McDougal and Gordon, 1977; Woodland and Cantor, 1978). This is in direct contrast to reports that B cell responses to red cell bound antigens probably only require one helper T cell activity (Schimpl and Wecker, 1972; Harwell *et al.*, 1976), although there may also be a role for macrophage-derived factors in these responses (Hoffmann *et al.*, 1979). It is quite possible that different forms of antigen have different T cell requirement, since some antigens are thought to be able to induce antibody responses in the absence of T_H cells.

The involvement of two T_H cells in the anti-TNP, antibody response to TNP-protein conjugates has been demonstrated in a number of ways.

Janeway (1975) obtained unexpected slopes on log-log plots of antibody obtained versus T cells of transferred in *in vivo* cell transfer experiments which indicated that responses to hapten-carrier conjugates indeed two T cell populations and one B cell population. More recently, Tada *et al.* (1978) showed that two types of T_H cells were involved in these responses, one of which was $I\text{-}J^+$ and nylon wool adherent whereas the other lacked these characteristics. Marrack *et al.* (1980) also reported the existence of two T_H subsets, one of which expressed I-A subregion antigens while the other did not. the T cell subset carrying I-A determinants was resistant to lysis by conventionally raised anti-Ia sera plus complement, but was depleted with sera raised against the Ia antigens on Con A blasts (Marrack *et al.*, 1980).

The two sets of T_H cells were designated Th_1 and Th_2 by Tada *et al.* (1978). Marrack and coworkers defined the two classes of T_H cells in the following experiment: F_1 (P_1 x P_2) into P_1 chimeric T cells, when antigen primed, could only help P_1 but not P_2 B cells even when the appropriate macrophage type was present, thus demonstrating that an essential T_H cell can only interact with H-2 compatible B cells. In the second part of the experiment it was shown that the T_H cells could collaborate with both P_1 and P_2 B cells in the presence of a con A-induced supernatant, but only if P_1 strain macrophages were also present. The ability of the Con A supernatant to over-ride the H-2 restricted interaction of T_H cells with B cells but not with macrophages suggested that two helper activities were required for an anti-TNP antibody response, one involving the MHC-restricted interaction of T cells with macrophages and the other resulting from a MHC-restricted T-B interaction.

An attempt will be made here to summarise the different characteristic of the two types of T_H cells, i.e. Th_1 and Th_2 (see Table 1).

TABLE 1

CHARACTERIZATION OF T_H SUBSETS

Trait tested	Th_1	Th_2
Ly antigenic phenotype	Ly-1^+, $2/3^-$	Ly1^+ $2/3^-$
Ia antigens	I-A^-	I-A^+
nylon wool adherence	No	Yes
Antigen specificity	Yes	Yes
Requires an antigen bridge	Yes	No
Requires Ig for priming	No	Yes
Required for *in vivo* antibody responses	Yes	No?
Required in anti-SRBC antibody response	No	Yes
MHC restricted at T cell-macrophage level	Yes	Yes
MHC restricted at T-B level	No?	Yes?

The Th_1 subset of T_H cells is $Ly1^+2/3^-$, $1\text{-}A^-$, $I\text{-}J^-$ and nylon wool nonadherent and acts early in the B cell response to TNP-protein conjugate (Marrack and Kappler, 1976; Tada *et al.*,1978; Kappler *et al.*, 1979; Marrack *et al.*, 1979). It recognises macrophage bound carrier molecules in an H-2 restricted manner and requires a hapten-carrier bridge in order to activate B cells (Tada *et al.*, 1978; Janeway *et al.*, 1980), suggesting that this T cell subset must be physically close to a hapten-specific B cell to activate it either directly or by release of close-range T cell factor(s). Whereas the Th_1-macrophage interaction is H-2 restricted, the Th_1-B cell interaction is not (Marrack *et al.*, 1980). It is this subset of T_H cell which is active in the *in vivo* adoptive cell transfer experiments to hapten-carrier conjugates, but is absent in anti-SRBC responses (Janeway *et al.*, 1980; Marrack *et al.*, 1979).

The Th_2 subset is $Ly\text{-}1^+2/3^-$, $I\text{-}A^+$, $I\text{-}J^+$ and nylon wool adherent (Tada *et al.*, 1978; Marrack *et al.*, 1980). It is required late in the response to hapten-carrier and is the only T_H cell needed in anti-SRBC responses (Marrack *et al.*, 1980). Th_2 is antigen-specific at the level of the T cell-macrophage interaction, but does not appear to need an antigen bridge to activate B cells, i.e. the relevant hapten and carrier need not be on the same molecule (Marrack and Kappler, 1975; Tada *et al.*, 1978). Like Th_1, Th_2 recognises the carrier in association with Ia on the macrophage membrane in an H-2 restricted manner (Marrack *et al.*, 1980). However, unlike Th_1, Th_2 cell interacts with hapten-specific B cell in an H-2 restricted manner, this interaction resulting in the secretion of a nonspecific helper factor from Th_2 akin to those factors found in Con A supernatants (Marrack *et al.*, 1980).

Do Th_1 and Th_2 act independently or in unison in responses to hapten-carrier? The answer appears to vary: Janeway *et al.* (1980) and Bottomly *et al.* (1980) claim that Th_1 subset alone is sufficient for these responses both *in vivo* and *in vitro*. In contrast, Marrack *et al.* (1979) and Kappler *et al.* (1979) suggested that both types of T_H cells can act independently, but best responses occur when these two cells synergise.

The existence of two T_H cell subsets can resolve a number of apparently contradictory observations in the literature: namely, the genetic restriction observed between T cells and B cells and the ability to obtain responses to linked and unlinked hapten-carrier conjugates. Hence when responses were obtained only to a hapten linked to the relevant carrier, or in the absence of histocompatibility between T and B cells, the authors must have dealt with the Th_1 subset. On the other hand, responses requiring strict histocompatibility between T cells and B cells or triggering of hapten-specific B cells by unlinked carrier involved the action of Th_2 subset.

In addition to characteristics ascribed to the Th_2 subset, Bottomly *et al.* (1980) showed that Th_2 cells cannot be induced in mice depleted since birth of B cells (and Ig), suggesting that the induction of Th_2 is Ig-dependent. Although this was interpreted to mean that Th_2 has receptors for Ig, it would mean that Th_2 cells must have at least 3 separate recognition capabilities: one for antigen, one for self-MHC and another for Ig. Alternatively it may be postulated that an inducer of Th_2 must recognise Ig: this "third" cell may be involved in regulating Ig class secreted by B cells as postulated by Rosenberg (1980).

A possible model for the role of the two T_H cell subsets in an antibody response to a TNP-protein conjugate is as follows: Th_1 cell interacts with carrier-Ia antigen complexes on macrophages in an I-region restricted manner and become activated. In turn, the Th_1 cells activate the linked, TNP-specific B cells that have bound antigen, either through cell contact (not H-2 restricted) or by short-range T cell factors. Once these B cells are activated, they become capable of interacting with Th_2 cells in an H-2 restricted fashion. The Th_2 cells, like Th_1, are initially activated by the recognition of carrier-Ia antigen complexes. Possibly another Ig-recognising cell regulates Th_2 activation. The activated Th_2 cells then interact with B cells through recognition of H-2 molecules and may release nonspecific T cell factors which support the differentiation of B cells into plasma cells.

How then, are anti-SRBC antibody responses activated? Evidence suggests that the Th_1 subset is not needed, Th_2 cells alone providing sufficient help. If this is true, then the B cells in an anti-SRBC response obviously must either not need the first signal from the Th_1-macrophage-B cell complex or the nature of this signal must be different. For example, there may be a role for macrophage derived factors in this response (Hoffman *et al.*, 1979).

4.4.2 B lymphocyte heterogeneity

In addition to the existence of heterogeneous T_H cell subpopulations which differ in their requirements for recognition of MHC determinants on B cells, recent findings have demonstrated that B lymphocytes differ in their inductive requirements, some requiring recognition of surface MHC antigens by T cells for activation whereas others being induced by T cells without MHC involvement. The following section deals with the existence of such B cell subpopulations and discusses their role in T cell-dependent antibody responses.

The existence of distinct B cell populations has been established on the basis of their expression of a number of cell surface markers including Ia, Ig, Fc receptors, complement receptors and most recently by the differentiation antigens Lyb-3, Lyb-5 and Lyb-7 (Huber *et al.*, 1977; Ahmed *et al.*, 1977; Subbaro *et al.*, 1979). (see section 3.2.2). B cells can be separated into two equally sized subpopulations based on their expression of the Lyb-5 antigen, which is a cell surface antigen encoded by a single locus with two allelic forms. B cells expressing the Lyb-5 antigen are a late-appearing subpopulation in mice and are virtually absent in mutant CBA/N mice. The Lyb-5^+ and Lyb-5^- B cell subpopulations may represent two distinct B cell lineages or two functionally distinct differentiation stages of the same B cell.

Using CBA/N or Lyb-5-depleted B cells as a source of the Lyb-5^- B cell population and normal B cells containing both populations, Singer and coworkers (Singer *et al.*, 1981; Asano *et al.*, 1981) demonstrated that Lyb-5^- and Lyb-5^+ B cells differ, both *in vivo* and *in vitro*, in their MHC requirements for activation by T_H cells. In an adoptive cell transfer system similar to that used by Sprent (1978c) chimeric T cells (F_1 (P_1 x $P_2 \rightarrow P_2$) restricted to see P_1 MHC were assayed for their ability to activate unprimed SRBC-specific P_2 B cells in an irradiated P_2 host containing F_1 (P_1 x P_2) macrophages. When the B cells consisted of both Lyb-5^+ and Lyb-5^- subpopulations, no H-2 restricted interaction between T cells and B cells were observed, i.e. T cells restricted to see P_1 MHC determinants delivered help to P_2 B cells. However, when the B cell population contained only Lyb-5^- cells, the restricted T_H cells could no longer help P_2 B cells even in the presence of P_1 macrophages. This implies that for T_H cells to activate Lyb-5^- B cells, recognition of B cell MHC antigens is essential, whereas Lyb-5^+ B cells do not require such an MHC-restricted interaction for activation.

A similar experiment *in vitro* produced comparable findings (Asano *et al.*, 1981). At low concentrations of hapten-carrier, H-2 restricted interaction between chimeric T cells and normal B cells was observed. In contrast, at higher antigen concentrations, T-B collaboration was not H-2 restricted. It was then demonstrated that low concentrations of antigen induced Lyb-5^- B cells, but at high concentrations, Lyb-5^+ B cells were activated. These findings were consistent with the *in vivo* experiments indicating that the activation of Lyb-5^- B cells requires direct T_H cell recognition of B cell H-2 antigens, whereas such a recognition does not occur when T_H cells interact with Lyb-5^+ cells.

In conclusion, the controversy over the MHC requirement for T cell-B cell collaboration can probably be explained by the B cell subpopulations that differ in their MHC requirements. Restriction at delivery level occurs in responses which activate Lyb-5^- B cells; lack of restriction reflects the induction of Lyb-5^+ B cells. In this context, it may be suggested that the *in vivo* experiment of Sprent (1978c) in which the induction of an anti-SRBC antibody response was, unlike Singer's *et al.* (1981), MHC restricted, induced Lyb-5^- B cells, whereas Singer induced Lyb-5^+ B cells. A key difference between the two experimental systems was that Singer examined a primary response, whereas Sprent analysed a secondary response. This difference could have resulted in different B cell subsets being activated.

5 ROLE OF SOLUBLE HELPER FACTORS IN T CELL DEPENDENT ANTIBODY RESPONSES

Ever since it was established that immune responses involve complex interactions between T cells, B cells and macrophages a large number of workers have been attracted to the idea that such interactions are mediated by soluble factors that act as communication signals between the cells producing them and other cells carrying relevant acceptor sites.

Evidence for the existence of such mediators came initially from the demonstration by Dutton *et al.* (1971) that supernatants obtained from short term cultures of histoincompatible mouse cells contained a nonspecific biologically active mediator capable of markedly affecting *in vitro* antibody responses to T-dependent antigens. Since this observation, a number of different factors that appear to have biological relevance to T-B cell collaboration have been described by a plethora of investigators (Feldmann and Basten, 1972; Schimpl and Wecker, 1972; Rubin and Coons, 1972; Watson 1973; Armerding and Katz, 1974a; Munro *et al.*, 1974; Marrack and Kappler, 1976).

A multitude of soluble helper factors can be generated by stimulation with specific antigens or mitogens. They are produced by T cells or macrophages and may be antigen-specific or nonspecific, bear or lack MHC determinants and are either MHC restricted or unrestricted in their action. The bewildering array of factors in the literature is in part a reflection of the methods which have been used to identify and classify lymphokines over the past decade: each has been defined by its biological activity in a particular *in vitro* assay and many have been given acronyms. The factors are difficult to purify due to their lability

and very low starting concentrations in biological preparations. As a result, the term "factor" is not a clear definition, merely designating a heterogeneous group of molecules whose molecular identity is not yet known. Many of the factors discussed here have not been isolated or characterized beyond localization of activity in column effluents.

Soluble mediators exerting a positive effect on antibody responses will be considered here in three separate sections:

(i) antigen-specific T cell derived helper factors

(ii) antigen-nonspecific T cell factors, and finally

(iii) macrophage-derived factors.

It should be emphasized at the outset that a variety of different molecules are capable of exerting virtually indistinguishable biological effects. Hence the expression of a given biological activity cannot be taken as an identification of any one factor; the ultimate proof of identity requires biochemical and immunological characterization. It should also be noted that soluble mediators, as a rule, serve the role of a "second signal" in cell activation and exert activity only when antigen-specific receptors on lymphocytes have bound antigen ("first signal").

A summary of factors discussed in this chapter is presented in Table 2.

TABLE 2

AUTHOR	DERIVATION	ASSAY SYSTEM	PROPERTIES PHYSIOCHEMICAL	TARGET	CARRIES H-2 ANTIGENS	H-2 RESTRICTED
(1) IgT						
Feldman and Basten, 1972	*In vivo* education with protein antigens followed by culture with antigen.	*In vitro* IgE response to hapten carrier DNP-KLH or DNP-FGG	180,000 daltons monomeric IgM k^+, μ^+	Macrophage		
Tamaguchi and Tada, 1974	*In vivo* education with carrier, *in vitro* culture with antigen.	*In vivo* IgE response to ascaris	100-200,000 daltons monomeric IgM?	B cells		
(2) Ia						
Taussig and Munro, 1974	*In vivo* education to synthetic polypeptides, *In vitro* 8 hr culture with antigen.	*In vivo* IgM & TGAL response	1.50,000 daltons 2.Two subunits 45,000 and 70,000 daltons	B cell	$I\text{-}A^+$	NO
Isac and Mozes, 1974	*In vivo* education to synthetic polypeptides *In vitro* culture with antigen.	*In vivo* & PGAL, HGAL	∿50,000 daltons	B cell (absorbed)	$I\text{-}A^+$	NO

(Table 2 continued)

AUTHOR	DERIVATION	ASSAY SYSTEM	PROPERTIES PHYSIOCHEMICAL	TARGET	CARRIES H-2 ANTIGEN	H-2 RESTRICTED
Howie and Feldmann, 1977	*In vitro* education with proteins or synthetic polypeptides, *in vitro* 24 hr culture with antigen.	*In vitro* IgM to KLH or TGAL	50-60,000 daltons reacts with anti-Ig	B cell or macrophage	$I\text{-}A^+$	NO
Howie ., 1979	*In vivo* education with TGAL, *in vitro* culture	*In vitro* anti-GAT response	50,000	B cell	$I\text{-}J^+$	NO
Tokuhisa	*In vivo* education with antigen, *in vitro* culture with antigen.	*In vitro* anti-KLH response		T cell	$I\text{-}A^+$	YES I-A
(3) Nonspecific						
AEF (Armerding and Katz, 1974)	*In vivo* education with irradiated allogeneic cells, *in vitro* culture with target.	*In vitro* anti-hapten response	40,000 12,000 - β_2	B cell	$I\text{-}A^+$	NO
Delovitch	"	"	35,000 68,000	B cell	$I\text{-}A^+$	YES I-A

(Table 2 continued)

AUTHOR	DERIVATION	ASSAY SYSTEM	PROPERTIES PHYSIOCHEMICAL	TARGET	CARRIER H-2 ANTIGEN	H-2 RESTRICTED
TRF (Schimpl and Wecker, 197	Antigen, alloantigen or mitogen-induced T cells	*In vitro* anti-SRBC or anti-hapten response	30-35,000	B cell	NO	NO
(4) Monokines						
K-1 Hoffmann, etc.	*In vitro* activation with LPS, activated T cells	*In vitro* IgM and IgG responses	∿16,000 daltons	T cells B cells?	NO	NO
GRF Erb and Feldmann		*In vitro* response to KLH, TGAL	55,000 + 3,000 complex including antigen	T cell	NO	NO

5.1 Antigen-specific helper factors

One of the key questions in T-B collaboration is how an antigen-specific T_H cell can successfully activate rare clones of antigen-specific B cells. One means of explaining this dilemma is that the T_H cells secrete antigen-specific helper factors. Theoretically, such factors have the advantage that they only interact with B cells that have bound the relevant antigen (particularly if MHC restricted recognition is involved) and therefore greatly increase the probability of an interaction between rare antigen-specific T_H and B cells. Work during the last decade has, in fact, resulted in the identification and characterisation of such antigen-specific helper factors produced by T cells. These factors can bind antigen (or idiotype in some cases) and are capable of activating other cells. Besides helper factors, T cells have been shown to secrete antigen-specific suppressor molecules for both humoral and cellular immunity, but these will not be discussed here (reviewed by Tada and Okumura, 1979; Rocklin *et al.*, 1980).

The antigen-specific helper factors, released by primed T cells upon challenge with specific antigen, induce B cells and plasma cells to produce immunoglobulin that is directed exclusively against the antigen used to elicit the helper factor. In experimental systems where hapten-carrier conjugates have been used to induce a secondary antihapten antibody response, these factors have been shown to possess specificity for the carrier component.

Antigen-specific helper factors for antibody responses may be subdivided into two separate groups: those that carry Ig determinants and those which bear Ia antigens.

5.1.1 Helper factors bearing Ig-like determinants

The first demonstration of an antigen specific helper factor with biological activity was made by Feldmann and coworkers (reviewed by Feldmann and Nossal, 1972; Feldmann, 1974a, b) using Marbrook culture vessels. These authors separated T cells from B cells with a nucleopore membrane and showed that carrier primed T cells, when incubated with a hapten-carrier conjugate, released a mediator that passed through the nucleopore membrane and activated hapten-primed B cells. The effect was carrier-specific, since antibody responses to unrelated antigens were not seen. Furthermore, the soluble mediator could be removed by anti-μ-chain and anti-κ-chain antibody absorbents (Feldmann, 1972a, b) and its molecular weight was about 180,000 daltons, which is close to that of monomeric IgM (Feldmann *et al.*, 1973). Together, these results were taken to mean that the factor was T cell released Ig ("IgT") and that "IgT" had similarities to monomeric IgM. This hypothesis was strongly supported by the coinciding demonstration of monomeric IgM on T cells (Marchalonis *et al.*, 1972). Subsequent studies revealed that the factor was cytophilic for macrophages: the factor-antigen complex bound to macrophages and could subsequently trigger B cells without T cells (Feldmann, 1972b).

It has been hypothesised that this T cell-derived mediator may have a number of functions in cell cooperation. Thus (i) when bound to macrophages and antigen it triggers B cells, (ii) when the factor occupies the receptor for IgT on macrophages, responses to unrelated antigens are abrogated explaining "antigenic competition" and finally (iii) in the absence of macrophages, factor-antigen complexes may interact directly with B lymphocytes, resulting in specific T cell suppression (Feldmann, 1973, 1974c, Feldmann and Schrader, 1974).

Besides these *in vitro* experiments, some reports suggested that IgT-like molecules enhance *in vivo* antibody responses (Taniguchi and Tada, 1974). In these studies thymocyte extracts from hyperimmunized animals reconstituted the antibody response of T cell deficient animals against the relevant antigen. The active molecule in the thymocyte extract carried Fab and μ chain determinants and had a molecular weight of between 100-200,000 daltons, thus resembling IgT. However, it is not known whether this mediator is T cell derived, and, in fact, there is some evidence that carrier-specific B cells produce the "IgT" molecule (Kirov and Parish, 1976a, b).

The validity of the IgT molecule *in vivo* and *in vitro* is yet to be established. A number of other workers have had great difficulty in detecting μ and κ chain determinants on T cell factors. Moreover, the presence of Ig determinants on T cells is also unresolved (Marchalonis and Warr, 1979; Vitetta and Uhr, 1975).

5.1.2 Helper factors bearing Ia antigens

The first antigen-specific T_H cell factor not bearing Ig determinants was described by Taussig (1974). Spleen cells from irradiated recipients reconstituted with thymocytes and challenged with synthetic copolymer antigens elaborated soluble mediators when cultured for 6 hours with the relevant antigen. This supernatant could replace antigen specific T cells when injected with unprimed bone marrow cells and antigen into irradiated mice, an IgM response being generated. The factor was a T cell product (Taussig *et al.*, 1976) with a molecular weight of 35,000-60,000 daltons. The activity could not be removed by an anti-mouse Ig immuno-absorbent, but was completely removed by absorbents coupled with the specific antigen (Munro *et al.*, 1974). The most

important feature of this factor is that it contains antigenic determinants coded for by genes in the H-2 complex. Specifically, the activity of this factor was removed by anti-I-A subregion immuno-absorbents, indicating that I-A subregion controlled determinants are carried by this antigen-specific helper factor (Taussig *et al.*, 1975).

Similar antigen-specific T cell factors were generated against three other multichain synthetic polypeptides to which responses in mice are controlled by Ir-1 genes (Taussig *et al.*, 1974; Isac and Mozes, 1977). These factors have a very similar physiochemical and immunochemical properties to the factor first described by Taussig (1974), and are all products of the I-A subregion.

The putative B cell receptor for these factors appears to be coded for by genes in the I region, since treatment of B cells with anti-Ia antisera prevented the cells from absorbing the mediator (Munro and Taussig, 1975). However, Munro *et al.* (1974) using crossreactive anti-Ia antisera, demonstrated that the I-region determinants on the helper factors were different from B cell Ia antigens. It should be emphasized, however, the despite the fact that these antigen-specific T_H cell factors carry I-A subregion controlled determinants, they are capable of cooperating with allogeneic B cells, i.e. they are not H-2 restricted in their action (Taussig and Munro, 1976).

These helper factors probably act directly on B cells since they can be absorbed by bone marrow cells even in the absence of antigen. However, B cells of nonresponder strains were unable to absorb the factor suggesting a lack of the I-A subregion acceptor in these mice (Munro and Taussig, 1975). B cells treated with this factor and antigen *in vitro* mounted a good antibody response *in vivo* when they are

injected into lethally irradiated recipients with additional antigen. If antigen was ommitted *in vitro*, no response was obtained, suggesting that B cell triggering is initiated by Ig receptors on the B cell binding antigen followed by factor-acceptor interaction, the antigen-specific factor binding antigen on the B cell surface as well as interacting with its I-A subregion controlled acceptor. This latter interaction provides the second signal for B cell differentiation.

The generation of Ia-bearing antigen specific helper factors has also been demonstrated *in vitro* (Howie and Feldmann, 1977). *In vitro* induced T_H cells specific for the synthetic polypeptide (T,G)-A-L elaborated a factor which could help B cells to secrete antibody against the relevant antigen. Interestingly, while this factor shared characteristics with Munro and Taussig's factor, it had some properties of Ig T as well. Like Munro and Taussig's factor, this molecule was between 50-60,000 daltons in molecular weight, could be absorbed by columns coupled with homologous antigen (Howie *et al.*, 1977) and by immuno-absorbents coupled with anti-I-A subregion antibodies against the haplotype of the donor of the T cell factor (Howie *et al.*, 1979). Like IgT, the activity of this factor required the presence of macrophages and, more importantly, was removed by a chicken anti-mouse Ig immuno-absorbent, suggesting that the T cell factors may bear structures resembling Ig.

In an additional study, Howie and coworkers (1979) analysed an *in vitro* generated antigen-specific factor against another synthetic polypeptide, GAT. The basic properties of this factor are the same as of the one described above. However, whereas the TGAL-specific factor carried I-A subregion controlled determinants, the GAT-specific factor

was only absorbed by anti-I-J subregion immuno-absorbents. Furthermore both the TGAL-specific and GAT-specific helper factors were shown to be removed by immuno-absorbents against the carbohydrate-defined Ia antigens (see section 4.2.3), indicating that these factors carry carbohydrate Ia specificities.

Whereas the studies mentioned above were performed using synthetic polypeptide antigens whose antibody responses are under Ir gene control, a number of workers have demonstrated antigen-specific T cell factors against protein antigens that augment the antibody responses to haptens coupled to these antigens (Gordon and Yu, 1973; Tokuhisa *et al.*, 1978; Howie and Feldmann, 1977). Thus, using the same procedure as making helper factors against synthetic polypeptides, Howie and Feldmann (1977) produced a KLH-specific helper factor that specifically augmented the B cell response to DNP-KLH-*in vitro*. This factor has properties identical to those of the (T,G)-A-L specific helper factor.

Tokuhisa *et al.* (1978) also reported a carrier-specific T cell factor capable of enhancing a T-cell dependent antihapten antibody response. Their factor is secreted by Ly-$1^{+}2/3^{-}$ T cell and carries I-A subregion controlled antigens which are different from the I-a determinants on B cells. However, this factor is clearly different from that described by Taussig *et al.* and Feldmann *et al.* Firstly, the factor was isolated from T cell extracts. Secondly, the target for the factor is a T cell rather than a B cell. Thirdly, the enhancing molecule is not capable of replacing T cells since addition of the factor to T cell depleted spleen cells did not induce an anti-hapten antibody response. Fourthly, a very important characteristic of this factor is the very strict H-2 requirement for its action: a factor derived from one strain of mice

could only augment the response of another strain if there was I-A subregion identity.

In conclusion, the majority of these T cell factors carry Ia antigens, have a molecular weight of about 50,000 daltons, interact with B cells and, despite bearing Ia determinants and probably interacting with an I-region controlled acceptor, are not I-region restricted in their action. IgT is the exception to the rule, but some similarities with the above factors exist, such as the target of IgT being a B cell and some Ia bearing factors may express Ig-like determinants. On the other hand, Tokuhisa's factor may be more appropriately classed with antigen-specific factors which augment T cells rather than being termed an antigen-specific helper factor for B cells.

5.2 Antigen nonspecific helper factors

Since Dutton's first demonstration that non-antigen specific T cell replacing supernatants can be obtained from cultures of histo-incompatible cells (Dutton *et al.*, 1971), numerous studies have shown that such supernatants can augment the *in vitro* antibody responses unfractionated spleen or can reconstitute the *in vitro* response of T cell depleted spleen to T-dependent antigens. These helper supernatants could be generated by (i) stimulation of primed T cells with specific antigen (Rubin and Coons, 1971; Geha *et al.*, 1973; Kishimoto and Ishizaka,1973b; Rosenthal *et al.*, 1973; Waldmann and Munro, 1975; Harwell *et al.*, 1976; Marrack and Kappler, 1976; Takatsu *et al.*, 1980), (ii) stimulation of lymphoid cells with alloantigen (Andersson *et al.*, 1972; Katz, 1972; Schimpl and Wecker, 1972; Gorzinski *et al.*, 1973; Watson, 1973; Armerding and Katz, 1974; Hunter and Kettman, 1974; Farrar, 1975) or (iii) simulation of lymphoid cells with T cell mitogens (Watson *et al.*, 1973; Rich and Pierce, 1974).

Culture supernatants from activated T cells have multiple biological functions. Besides augmenting antibody responses, they can facilitate the response of thymocytes to mitogens, expand Tc cell responses and, under certain conditions, suppress humoral responses (Chen and Di Sabato, 1976; Pierce and Tadakuma, 1977). Hence two possibilities exist: either all of these activities are mediated by a family of closely related molecules, or supernatants of activated T cells contain a large number of different factors mediating different activities.

Non-antigen-specific T cell helper factors may be divided into three groups: The allogeneic effect factors (AEF), early acting T cell replacing factors (TRF) and late acting TRF's.

5.2.1 AEF

The AEF's have been implicated in a range of immunological phenomena such as augmenting (Armerding and Katz, 1974) and suppressing (Gisler and Fridman, 1975) antibody responses, suppressing MLR (Rich and Rich, 1975), and in inducing self reactive cells (Altman and Katz, 1980). The factors are obtained from culture supernatants of short term *in vitro* MLR between *in vivo* alloantigen-activated T cells and irradiated stimulator cells. So far AEF is the only non-antigen-specific factor described that expresses Ia determinants (Reviewed by Katz, 1977; Altman and Katz, 1980). Furthermore, like many antigen-specific factors, AEF substitutes for T cells in primary anti-SRBC antibody responses and in secondary IgG anti-hapten responses by acting directly on B cells (Armerding and Katz (1974).

AEF is a protein molecule which is devoid of Ig determinants but carries Ia antigens and β_2 microglobulin (Armerding *et al.*, 1977). While some workers showed these Ia determinants to be of responder cell origin (Armerding *et al.*, 1974b) others demonstrated that they were derived from stimulator cells (McDevitt *et al.*, 1976), suggesting that stimulator cells release biologically active molecules as well. Controversy also exists over whether AEF action is H-2 restricted. While AEF showed no H-2 restricted action in primary *in vitro* antibody responses (Katz *et al.*, 1976), strict restriction to the I-A or I-J subregions of the stimulator haplotype was reported by Delovitch's group for secondary IgG responses *in vitro* (Delovitch and McDevitt, 1977; Delovitch *et al.*, 1978; Delovitch and Sohn, 1979).

The molecular weight of AEF has been reported to be 30,000-40,000 daltons, and it is associated with β_2 microglobulin (10-12,000 daltons) which is an integral part of the active moiety (Armerding *et al.*, 1977). However, Delovitch and coworkers (1981) were able to resolve their AEF activity into two active components. One component, of about 30,000-35,000 molecular weight, possesses identical biochemical and biological properties to murine T cell growth factor (TCGF or Il-2) and is not genetically restricted in its activity. The other component has a molecular weight of about 68,000 daltons, differ both biochemically and biologically from TCGF and is I-A subregion restricted in its helper activity for B cells of the stimulator haplotype. Delovitch suggested that this second component is a T cell receptor molecule that recognises the I-A subregion antigens of the stimulator cells. Furthermore, he found that the purified 68,000 dalton AEF lacked Ia determinants and he proposed that culture supernatant AEF represents a complex of T cell receptor- Ia antigen and this complex is disrupted during AEF fractionation.

Hence obvious differences exist between the AEF described by Armerding and Katz and that of Delovitch. Delovitch *et al.* (1981) suggested that the AEF of Armerding is a TCGF molecule. However, TCGF molecules do not carry Ia determinants and, therefore, a more likely possibility is that the two groups are looking at different molecules.

5.2.2 Early Acting TRF

Factors which replace T cells in antibody responses are called T cell replacing factors (TRF). They may be generated by antigen, alloantigen or mitogen-stimulated Ly-$1^{+}2/3^{-}$ T cells and although not unequivocally demonstrated, all seem to act directly on B cells. TRFs do not carry Ia determinants and do not appear to be H-2 restricted in their action on B cells.

Two kinds of TRFs have been described that differ from each other in their time of action: one TRF must be present at the initiation of an *in vitro* antibody response to exert its action whereas the other TRF appears to act late in the differentiation of B cells into plasma cells.

Initially, early acting TRF will be considered. Supernatants obtained from cultures of KLH-primed T cells or splenocytes, after reexposure of these cells *in vitro* to KLH, were shown to be capable of facilitating B cell responses to SRBC (Waldmann and Munro, 1973, 1974, 1975; Marrack and Kappler, 1976; and Harwell *et al.*, 1976). The factor had a molecular weight of between 25,000 and 60,000 daltons (Waldmann and Munro, 1975; Harwell *et al.*, 1976) and was most effective biologically when added at the beginning of the culture, in contrast to the late TRF (see section 5.2.3) which had optimal activity when added later.

Subsequent studies indicated that while this factor could reconstitute antibody responses to SRBC or TNP-erythrocytes, it could not resture responses to soluble TNP-protein conjugates (Waldmann, 1975; Harwell *et al.*, 1976). From these data it was proposed that the nonspecific factor analyzed could not be the sole mediator of T-B cell collaboration since it was unable to enhance the antibody response to hapten-carrier conjugates. It was therefore proposed that this factor cannot activate hapten-specific B cells unless a carrier-specific helper factor derived from carrier-specific T_H cells is also present. This theory in fact agrees with the observation (see section 4.4.1) that only one T cell subpopulation (Th_2) is required for anti-SRBC antibody responses whereas responses to soluble hapten-carrier conjugates require two T cell subpopulations, i.e. Th_1 and Th_2. Furthermore, it is conceivable that the Th_1 cells could secrete antigen-specific helper factors. However, the relationship between early and late acting TRF is still not clear.

5.2.3 Late acting TRF

The majority of workers, when discussing TRF, refer to the TRF described by Schimpl and Wecker and associates (reviewed by Schimpl and Wecker, 1975). This TRF is a T cell product, produced within 24 hrs of T cell stimulation. The optimal enhancing effect on both the IgM and IgG responses to SRBC is observed when TRF is added after 2 days of culture rather than at culture initiation. In fact, even when given as late as 84 hrs after culture initiation, TRF still exerted substantial biological activity. This observation suggested that TRF facilitates the final stages of B cell differentiation following initial B cell activation by antigen and possibly macrophage derived

factors. Furthermore, TRF acts in an H-2 unrestricted manner, does not carry H-2 determinants and has a molecular weight of between 25,000 and 35,000 daltons (Schimpl *et al.*, 1977; Hubner *et al.*, 1978).

Purification of material present in culture supernatants of activated cells by ion exchange chromatography and isoelectrofocusing suggested that the various biological activities such as TRF activity in antibody responses, induction of MLR in nude mouse cells, costimulator activity and T cell growth factor (TCGF) activity were all mediated by a family of closely related molecules with a molecular weight of about 30,000 daltons and isoelectric points of 4.3 or 4.9 (Farrar *et al.*, 1978; Watson *et al.*, 1979a, b, c). The term interleukin-2 (IL-2) was suggested to describe this T cell derived molecule(s) with the biological activities above (Aarden *et al.*, 1979).

Watson *et al.* (1979c) and Gillis *et al.* (1980) suggested that all the biological assays described above measure the clonal expansion of activated T cells. Even in antibody responses of nude or T cell depleted culture, IL-2 could act by driving the differentiation of pre-T cells into mature T_H cells. This hypothesis is supported by the finding that (i) T cells but not B cells express receptors for IL-2 (Bonnard *et al.*, 1979; Smith *et al.*, 1979), (ii) nude mice can be activated by IL-2 to manifest T cell alloreactivity (Gillis *et al.*, 1979; Wagner *et al.*, 1980) and (iii) IL-2 containing supernatants can induce the differentiation of pre-T cells into Thy-1^+ T cells (Jones-Villeneuve *et al.*, 1980).

However, others have shown that biologically active molecules with IL-2 properties act directly on B cells. A recent study found that B cells of DBA/2Ha mice do not respond to antigen induced TRF because of

the lack of an appropriate acceptor site (Tominaga *et al.*, 1980), thus implying that TRF can act directly on antigen primed B cells. Additionally, Schimpl *et al.* (1980) were able to distinguish IL-2 and TRF on the basis of biochemical criterion, complete separation of these two molecules being achieved on the basis of affinity for Lentil lectin coupled Sepharose and isoelectric focusing behaviour in 4M urea with a pH gradient from 4-6. Moreover, TRF-containing preparations augmented antibody synthesis by the plasmacytoma MOPC 315 and since B cells do not possess IL-2 receptors, classical IL-2 could not be responsible for this effect. Together, these results suggest that IL-2 and TRF are different molecules and that IL-2 acts on T cells and TRF on B cells.

It must be kept in mind, however, that many TRF containing preparations probably contain IL-2 activity. As a result, these supernatants will exert helper activity on T cell depleted cultures in two ways: TRF will directly stimulate B vells and IL-2 will recruit and cause proliferation of immature or remaining T cells, thus indirectly helping B cells. Swain and coworkers (1981) have, in fact, demonstrated synergy between IL-2 and TRF.

In summary, a heterogeneous group of molecules perform the function of delivering antigen-nonspecific helper signals to B cells. The AEF molecule of Armerding *et al.* has some similarities to antigen-specific helper factors as does the larger component of Delovitch's factor. It remains to be seen, however, whether the TCGF-like component isolated from AEF preparations (Delovitch *et al.*, 1981) is IL-2 which acts on T cells or is TRF. The TRF molecules are more straight-forward in their action in that they act on already activated B cells and possibly provide the last signal required for antibody secretion.

Is there any relationship between the different T cell derived helper factors? The nonantigen specific helper factors, except for AEF, appear not to carry Ia antigenic specificities and are not MHC-restricted in their action. In contrast, the antigen-specific factors carry Ia determinants, but also are generally not H-2 restricted in their action. In terms of biological activity, all express T-cell replacing activity.

It is possible that the Ia positive genetically restricted and/or antigen specific factors have a common lymphokine "backbone", such as IL-2, which is complexed with separate molecular component expressing Ia determinants and/or carrying antigen-binding capacity. Support for such an association comes from the work of Taniguchi and Takei (1980) who analysed an antigen-specific, I-J subregion restricted suppressor factor for B cells. The authors found that extracts prepared from a T cell hybridoma contained two separate molecules, one $I\text{-}J^{+}$ and one KLH-specific. Neither molecule alone expressed biological activity, but mixtures of the two were strongly suppressive for B cell responses.

Hence antigen-specific and/or Ia bearing factors may in fact be the classical IL-2 molecule (or TRF, i.e. 30-35,000 daltons) coupled with a separate molecule carrying antigenic specificity and, if the Ia antigens are carbohydrate-defined (Howie *et al.*, 1979), coupled with a carbohydrate side-chain bearing Ia antigenicity.

5.3 Macrophage-derived factors

Mononuclear phagocytes release a variety of biologically active products, some of which influence the functional behaviour of lymphocytes. Secretory products include a number of enzymes, complement components, interferon, growth factors and prostaglandins. The factors not affecting lymphocytes have been reviewed elsewhere (Unanue *et al.*, 1976a; Page *et al.*, 1978; Gordon, 1980) and will not be considered here.

Some of the molecules secreted by macrophages are potent stimulators of T and B cell proliferation and, moreover, are released following macrophage-lymphocyte interactions regulated by the MHC (Unanue *et al.*, 1976b; Farr *et al.*, 1977; 1979). The first clear demonstration of a mitogenic activity secreted by macrophages came from Gery and coworkers (Gery *et al.*, 1972; Gery and Waksman, 1972). They described release of a "factor" from a 24-hour culture of human adherent cells which promoted the response of murine thymocytes to the T cell mitogen phytohaemagglutinin-A (PHA). The factor was named lymphocyte-activating factor (LAF). Following this demonstration, a large number of activities, defined in various assays have been attributed to factors derived from macrophages. These include such molecules as mitogen-protein (Unanue and Kiely, 1977), T cell replacing factor III (Watson, 1979), T cell replacing factor M (Hoffmann and Watson, 1979), helper peak-1 (Koopman *et al.*, 1978), B cell activating factor (Wood, 1979), and B cell differentiation factor (Hoffmann *et al.*, 1979).

It appears now that all these activities are really manifestations of one (or one class of) molecule released by macrophages, possessing a wide range of biological effects. This molecule is defined as interleukin 1 (IL-1) (Aarden *et al.*, 1979).

IL-1 (or LAF) is defined as an activity in macrophage culture supernatants that, when added to thymocytes together with or in the absence of PHA or Con A, increases, after 3-4 days of culture, their uptake of tritiated thymidine. The best evidence for IL-1 being a macrophage product is the fact that a macrophage line P388D1 releases this molecule (Mizel *et al.*, 1978a, b). The most potent inducers of IL-1 secretion are immune T cells which must interact with macrophages in an antigen-specific, I-A restricted manner (Farr *et al.*, 1977, 1979; Ziegler and

Unanue, 1980), and bacterial lipopolysaccharide (LPS) which is often used as an inducer of Il-1 release (Meltzer and Oppenheim, 1977).

IL-1 is a protein with a molecular weight of 13,000-16,000 daltons (Gery and Handschumacher, 1974; Calderon *et al.*, 1975; Mizel, 1979) and with pI values ranging from 4.5 to 6.5 (Economu and Shin, 1978). High molecular forms of IL-1, which may be protein aggregates, have also been observed (Togawa *et al.*, 1979). Furthermore, IL-1 is not strain specific in its action and does not carry H-2 antigens (Calderon *et al.*, 1975).

As already mentioned, IL-1-like molecules have been shown to enhance the generation of antibody-forming cells. There are two ways in which this can happen: IL-1 may stimulate T cells or precursor T cells which in turn help B cells and/or the monokine may stimulate B cells directly. The next two sections will consider the evidence for these two possibilities.

5.3.1 Effects of IL-1 on T cells

IL-1 has two major effects on T cells: one is associated with the proliferation and maturation of immature T cells, the other deals with the induction of secretion of IL-2 by T cells which in turn stimulates the continuous cycling of immunocompetent T cells.

There is no question that IL-1 affects thymocytes. Directly assaying proliferative activity (without PHA) established that peripheral T cells obtained from spleen or lymph nodes responded poorly to IL-1, whereas thymocytes were stimulated markedly by the monokine (Gery *et al.*, 1972; Calderon *et al.*, 1975). The thymocytes also responded to IL-1 by producing an augmented proliferative response to PHA whereas lymph

node T cells responded to IL-1 plus PHA but not as well as thymocytes (Gery *et al.*, 1972). The direct mitogenic action of IL-1 requires a cell to be in an "activated" state, this state usually being induced in peripheral T cells by an antigen or a mitogen (Beller and Unanue, 1979). Hence IL-1 is probably an important endogenous regulator of thymocyte differentiation in the thymus, exercising a powerful role in the intrathymic maturation of T cells.

While IL-1 enhances lectin-initiated thymocyte proliferation it does not support continuous T cell growth (Oppenheim *et al.*, 1980). Several investigators have proposed that IL-1 enhances T cell growth not directly but through an enhancement of a T cell released and T cell specific growth factor (IL-2). A number of groups have provided support for this hypothesis (Smith *et al.*, 1979; Larsson *et al.*, 1980; Shaw *et al.*, 1980) reporting that in the absence of macrophages or IL-1 T cell enriched populations release little or no IL-2. Additionally, IL-1 can convert the nonproducer T cell line (1A5 cells) to a state where subsequent mitogen stimulation triggers production of IL-2 (Gillis and Mizel, 1981). Only a brief exposure (2-4 hrs) of these cells to IL-1 was required for conversion to IL-2 production. Moreover, 1A5 cells could absorb IL-1 from cultures, suggesting the existence of IL-1 receptors on potential IL-2 producer cells. The implications of these results are that IL-1 serves as an early signal for T cell proliferation by promoting the production of a second, T cell derived, soluble factor that actually mediates T cell proliferation and function.

5.3.2 Effects of IL-1 on B cells

A number of investigators have obtained evidence which suggests that macrophage-derived factors can directly stimulate antigen-driven B cell differentiation. The molecule responsible appears to be similar if not identical to IL-1.

Initially Schrader (1973) reported that spleen cells from athymic (nude) mice developed an antibody response if cultured with macrophage-conditioned medium, an observation supported by Calderon *et al.* (1975). Supernatants from stimulated macrophages contained this activity even when the macrophages were first treated with anti-Thy-1 antibody plus complement, or were obtained from athymic mice. The results were taken to mean that a macrophage product in combination with antigen, directly stimulates the differentiation of B cells into antibody forming cells. However, the ability of a macrophage-derived product to do this seems puzzling. Athymic mice are known to have activated macrophages (Zinkernagel and Blanden, 1975) and yet do not respond well to T-dependent antigens. Why should the addition of a macrophage-derived factor (whose induction is T cell independent) alter this state?

Supernatants from activated macrophages also have been shown to restore antibody responses to T-dependent antigens in T-cell-depleted spleen cell cultures (Calderon *et al.*, 1975; Unanue *et al.*, 1976b; Wood *et al.*, 1976). While this was also interpreted as the ability of IL-1 to directly affect B cells, another explanation is also feasible. IL-1 may be stimulating residual mature or immature T cells to secrete IL-2 which in turn induces T cells to divide. The T cells and/or IL-2 may then stimulate B cells; hence the IL-1 could be acting indirectly on B cell differentiation.

Additional studies in the Kumuro-Boyse system (Hoffamnn *et al.*, 1976c, Hoffmann, 1980) clearly indicated that macrophage derived products can induce B cell differentiation from immature bone marrow cells into a state where they can enter the immune response. However, the macrophage factor responsible has not been characterized and hence may not be IL-1.

Finally, a striking synergy between T cells and macrophage product(s) resulting in heightened antibody responses has been shown by a number of workers (Calderon *et al.*, 1975; Hoffmann *et al.*, 1979; Wood *et al.*, 1976). Furthermore, Farrar and associates (1977) have demonstrated that MLC supernatants contain a number of T-cell-replacing activities for an *in vitro* anti-SRBC response. TRF activity was associated with molecules of an apparent molecular weight of 15,000 and 40,000 daltons, which acted synergistically when added together. The 15,000 dalton molecule may well represent IL-1 (Koopman *et al.*, 1977). In support of this cooperativity, recent studies have indicated that macrophage products can enhance the activity of TRF preparations (Hoffmann and Watson, 1979; Hoffmann, 1980). However, these studies say nothing about whether the macrophage product directly acts on B cells.

Controversy exists over the relationship between IL-1 and the molecule which stimulates B cells in culture. A number of studies indicated that the B cell stimulatory molecule, like IL-1, has a molecular weight of 15,000 daltons (Calderon *et al.*, 1975; Wood *et al.*, 1976; Wood, 1979). In one study, molecules having both activities chromatographed in the same position (Calderon *et al.*, 1975) but this was not the case in studies using human material (Wood *et al.*, 1976)

which indicated that thymocyte stimulatory activity and B cell stimulatory activity were mediated by separate molecules. Hence this question remains to be resolved. However, it is possible that the IL-1 molecule is responsible for multiple functions since it is known, for example, that IL-1 also acts as an endogenous pyrogen (Dinarello *et al.*, 1977; Murphy *et al.*, 1980b).

In summary, evidence suggests that during the initial interaction of T cells with antigen on macrophage surfaces, macrophages may be stimulated to secrete IL-1 molecules which activate both T cells and B cells to differentiate. Whether IL-1 alone is enough to induce B cells to secrete antibody is uncertain, but the involvement of other T cell factors in antibody responses and the demonstration of H-2 restricted interactions between T cells and B cells suggests that this is not so.

5.3.3 Genetically Restricted Factor (GRF)

In addition to IL-1 or IL-1-like molecules, at least one other monokine has been shown to influence the generation of antibody responses. Erb and Feldman (1975) generated T_H cells to KLH in a double-chamber culture where the macrophages were separated from T cells by a nucleopore filter. This suggested that macrophages operated by releasing a soluble mediator which is capable of activating T cells without cell contact. The soluble macrophage product was named "genetically restricted factor" (GRF) because it would only induce T cells with the same I-A haplotype as the macrophages which made it. Briefly, GRF is about 55,000 daltons, can be retained on an anti-I-A subregion immunoabsorbent (Erb *et al.*, 1976) and contained antigen, KLH (Puri and Lonai, 1980). Hence, GRF appears to be a complex between Ia and antigen and apparently binds directly to T_H cells but not B cells.

This seems to be the only instance where T cells can be activated in absence of accessory cell. The prevailing evidence indicates that T cells must see Ia plus antigen on the macrophage cell surface (Unanue, 1981). On the other hand, it is possible that the presence of GRF reduces the number of macrophages needed for T cell activation. Thus in the system described above, the macrophage depletion of T cells was not absolute and the addition of GRF in the presence of a small number of macrophages and the antigen resulted in T cell activation.

6 CONCLUDING REMARKS

Since the original definition of T-B collaboration in the late 1960's, many laboratories have attempted to further characterize this interaction. As with most biological systems, this interaction has proven to be much more complex than was originally thought. At least two different types of T_H cells have been identified and T cells with suppressor, rather than helper properties have also been described. Furthermore, the regulation of induction of T_H and Ts cells involves a number of complex interactions. All these findings lend support to the concept that induction of effector cells (e.g. plasma cells, Tc or T_{DTH} cells) is tightly regulated by a complex network of cell-cell interactions.

The demonstration that the MHC plays a key role in cell-cell interactions and antigen-recognition has provided a potential genetic and molecular basis for these processes. However, at the molecular level, the mechanism of T-B collaboration is still not well understood. Obviously, soluble factors (non-specific and possibly antigen-specific) play an important role but the chemical nature of these factors and the way they interact with and activate target cells is still unknown. Furthermore, how MHC antigens are actually recognised by T cells and mediate MHC-restricted interactions between cells remains a mystery. Obviously the molecular basis of cell-cell communication in the immune response represents a fascinating area of future research.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

All animals were bred and maintained in the Animal Breeding Establishment of the John Curtin School of Medical Research.

Inbred and outbred mice were watered and fed *ad libitum* with Mecon rat and mouse cubes (Fidelity Feeds, Murrumburrah). Donors of spleen cells were used between the ages of 6-14 weeks. The H-2 haplotypes of the inbred mouse strains used in this study are listed in Table 2.1 (obtained from Klein *et al.*, 1978).

2.2 ANTIGENS

Monomeric flagellin (MON), molecular weight 40,000 was prepared from the flagella of *Salmonella typhimurium* SL 870 (Ada *et al.*, 1964) Haemocyanin (HCY), a pentamer of molecular weight 450,000 daltons was recrystallized from the haemolymph of the South Australian crayfish *Jasus lalandii* (Moore *et al.*, 1968) as described below. These proteins were dinitrophenylated according to the method of Eisen (1964) (see section 2.2.2), the conjugation ratios used being $DNP_{1:1}$ MON and DNP_{26-42}HCY. SRBC were stored in Alsevers solution and washed four times in normal saline before use.

2.2.1 Purification of haemocyanin (HCY)

Haemolymph was obtained from two crayfish and, clotted overnight serum was exhaustively dialysed against DDW at 4°C. The serum-containing dialysis tubing was placed into buffer pH4.6, ionic strength 0.015 (containing 0.005M sodium acetate, 0.005M acetic acid and 0.010M sodium buffer) which permits the crystallisation of HCY from the serum. Crystals obtained in 48 hrs were centrifuged (2,000 rpm, 5 min),

TABLE 2.1

H-2 haplotypes of the inbred mouse strains used in this study

Strain	H-2 Region									
	I-Subregion									
	K	N	A	B	J	E	C	S	U	D
CBA/H	k	k	k	k	k	k	k	k	k	k
B10BR	k	k	k	k	k	k	k	k	k	k
Balb/c	d	d	d	d	d	d	d	d	d	d
B10.D2	d	d	d	d	d	d	d	d	d	d
C3H.SW	b	b	b	b	b	b	b	b	b	b
B10	b	b	b	b	b	b	b	b	b	b
B10.A	k	k	k	k	k	k	d	d	d	d
B10.A(2R)	k	k	k	k	k	k	d	d	b	b
B10.A(3R)	b	b	b	b	b	k	d	d	d	d
B10.A(5R)	b	b	b	b	k	k	d	d	d	d
C3H.OL	d	d	d	d	d	d	d	k	k	k
A.TL	s	?	k	k	k	k	k	k	d	d
A.TH	s	s	s	s	s	s	s	s	s	d

washed in the buffer and subsequently dissolved in 1% w/v sodium bicarbonate solution. The HCY in this solution was recrystallised once more as described above, dissolved in 25 mls of 1% sodium bicarbonate and the protein content determined by spectrophotometer (OD_{620}) using coomassie dye method (Rylatt and Parish, 1982). The final concentration of HCY was 66mg/ml. HCY was stored in 10ml aliquots at $-20^{o}C$.

2.2.2 Dinitrophenylation of MON and HCY

MON at 10mg/ml and HCY at 20mg/ml were reacted with 20mg/ml of K_2CO_3 and 20mg/ml DNP-sulphonicacid at room temperature for about 24 hrs protected from light. This solution was dialysed for 24 hrs against normal saline with multiple changes of saline. The number of DNP groups per molecule of HCY or MON was estimated on spectrophotometer (OC_{360}) using formula as follows: (given that HCY has a molecular weight of 450,000 daltons, POL 40,000 daltons and DWP 17,600 daltons)

$$\text{DNP groups/HCY molecule} = \frac{OD_{360} \times \text{dilution}}{17,600} \Big/ \frac{\text{protein concentration (mg/ml)}}{450,000}$$

2.3 IMMUNIZATIONS

DNP-MON and DNP-HCY were made particulate by absorption to bentonite as described by Gallily and Garvey (1968). Mice were given one intraperitoneal injection of hapten-carrier coupled to bentonite every two weeks for a total of three injections. Each injection contained 200 µg of protein in 0.2ml of saline. Mice were used as spleen cell donors 10 or more days after the last injection.

Mice were primed to SRBC with one intravenous injection containing 10 µl of packed SRBC in 200 µl phosphate buffed saline (PBS). Spleens were used 7 or more days later.

NOTE: Spleen cells taken from mice primed to DNP-HCY in winter and summer months often showed no "memory", i.e. would not respond to DNP-HCY in culture. We finally overcame this problem by keeping these animals during and after the priming regime in a light, humidity and temperature (25°C) controlled room. Under these conditions, the majority of spleen cell preparations from the animals responded in culture and the animals remained primed for much longer periods.

2.4 TISSUE CULTURE MEDIA

Eagle's Minimal Essential Medium (EMEM) was prepared by dissolving 10g of medium powder (GIBCO, F-15) in 1 litre of double distilled deionized water (DDW) containing 42 mls of 5% sodium bicarbonate, 100u/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml neomycin.

Dulbecco's Modified Eagle's Medium (DMEM) was prepared by dissolving 10g of medium powder (GIBCO, H-16) in 1 litre of DDW containing 72mls of 5% sodium bicarbonate; 100u/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml neomycin. RPMI was prepared by dissolving 10g of medium powder (GIBCO) in 1 litre of DDW containing 45mls of 5% sodium bicarbonate and antibiotics as for EMEM.

EMEM, DMEM and RPMI were sterilised by filtration through a 0.22 µm membrane (Millipore Corporation) and were stored for less than three weeks.

2.4.1 Foetal Calf Serum (FCS)

FCS (Flow laboratories, Stanmore, Australia) was selected for its ability to maintain Tc cells in culture. When indicated, heat inactivated foetal calf serum (HIFCS) was prepared by incubating selected

selected serum batches at 56°C for 30 minutes. FCS used in maintenance assay (kindly donated by Dr. Lafferty) was screened for its lack of mitogenic activity in this assay.

2.4.2 Nucleic Acid Precursors (NAPs)

100mg of each cytosine, uridine, adenosine and guanosine (SIGMA) were dissolved in 100mls of DDW, autoclaved and stored at 4°C. For use in antibody cultures, 2mls of this stock were used per 100mls of EMEM, giving a final concentration of 20 µg/ml.

2.4.3 2-Mercaptoethanol (2-ME)

2-ME (SIGMA, type 1) stock solution was made up at 0.1M in DDW and stored at -20°C. The stock was diluted 1:1,000 to give a final concentration of 10^{-4}M for use in cultures.

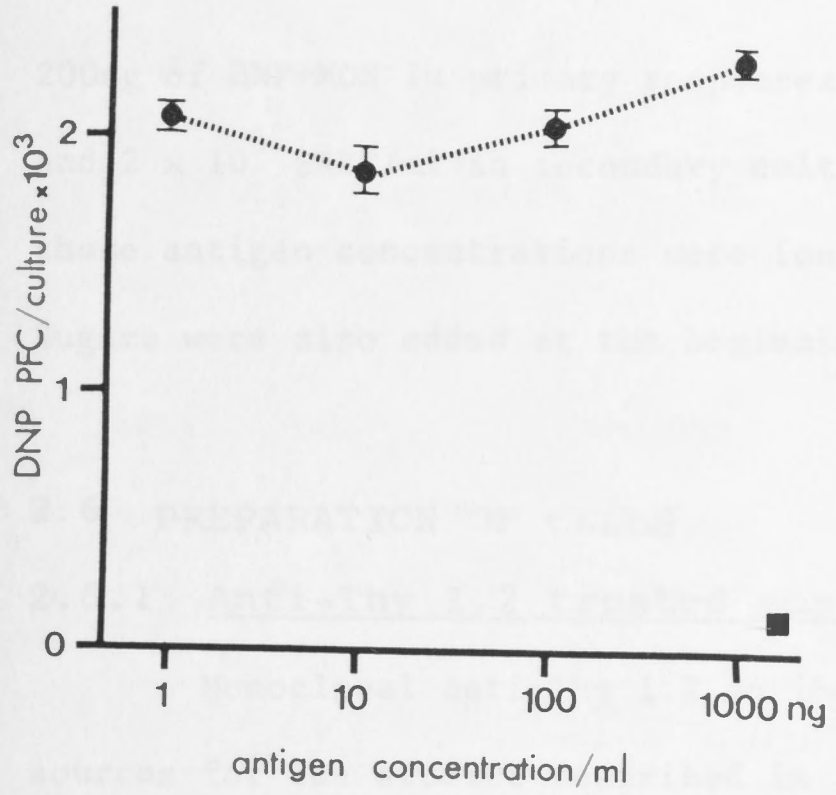
2.5 CELL CULTURES FOR ANTIBODY RESPONSES

Spleen cell suspensions were prepared by removing the organ aseptically and pressing spleen fragments through a fine stainless steel grid into culture medium containing EMEM, 5% FCS, 2-ME and NAPS. The resulting cell suspension was washed, declumped and resuppended in this culture medium at a concentration of 2×10^6 cells/ml.

Cell suspensions were cultured in 24-well Linbro trays (Hamden, Conn.) containing 2.5mls (5×10^6 cells) culture well. Each treatment was cultured in quadruplicate.

Primary antibody responses were assayed after 3 days of culture in a humidified 10% CO_2/7%O_2 atmosphere and secondary responses after 7 days of culture. Antigen was added at the beginning of the culture,

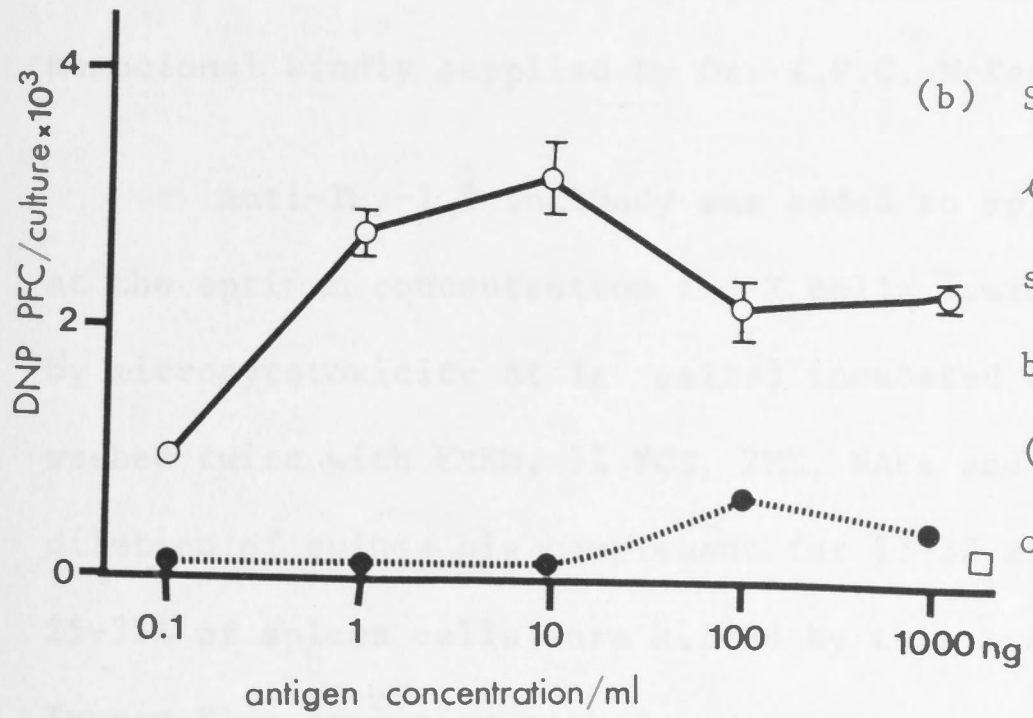
FIGURE 2.1 TITRATION OF ANTIGENS IN ANTIBODY RESPONSES



(a) Primary antibody response *in vitro* to DNP-MON

dotted line represents IgM PFC response, bars represent the standard errors of the means

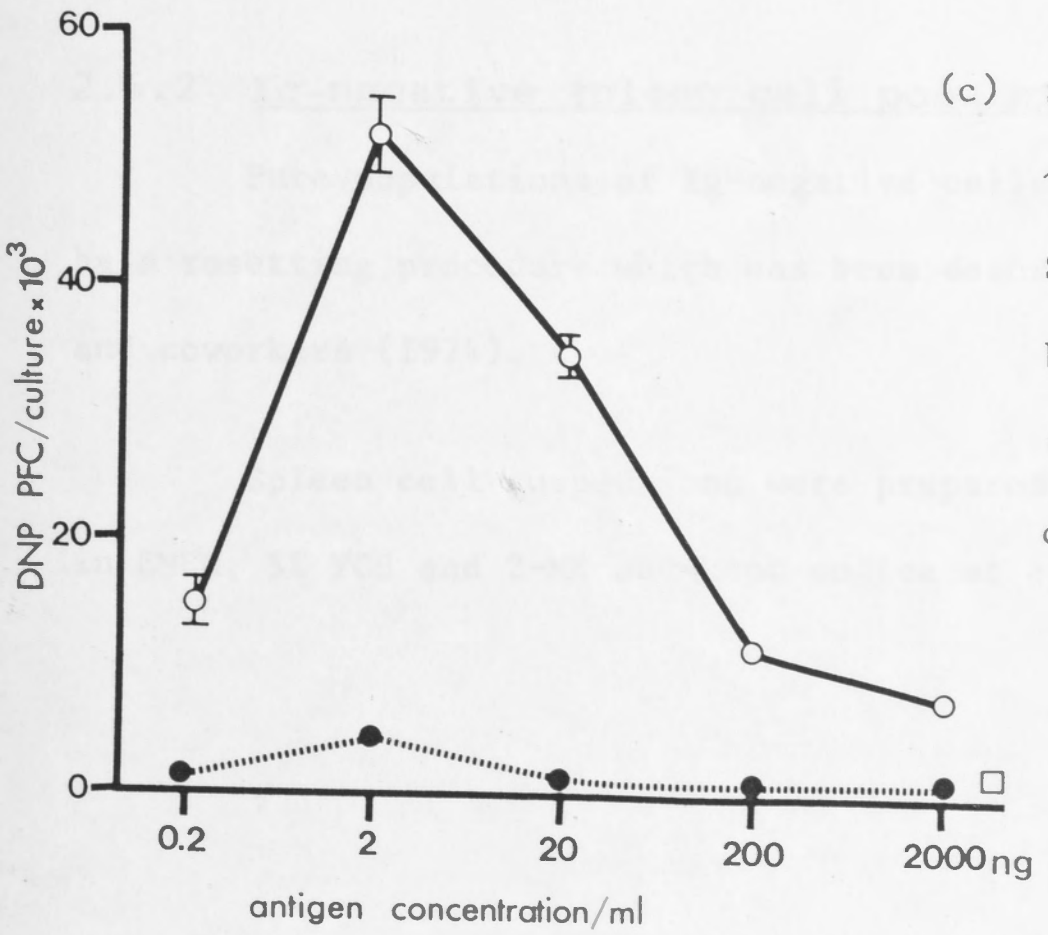
(■) is the response in absence of antigen.



(b) Secondary antibody response *in vitro* to DNP-MON

solid line is the IgG response, broken line IgM response.

(□) is the response in absence of antigen.



(c) Secondary antibody response *in vitro* to DNP-HCY

Solid line is the IgG response, broken line IgM response.

(□) is the response in absence of antigen.

200ng of DNP-MON in primary responses, 50ng/ml of DNP-MON, DNP-HCY and 2 x 10^6 SRBC/ml in secondary culture. In preliminary experiments, these antigen concentrations were found to be the optimum (see Figure 2.1). Sugars were also added at the beginning of the culture at 5mg/ml (~20mM).

2.6 PREPARATION OF CELLS

2.6.1 Anti-Thy 1.2 treated populations

Monoclonal anti-Thy 1.2 antibodies were obtained from three sources for the studies described in this thesis, namely clone F7D5 initially kindly supplied by Dr. P. Lake and subsequently purchased from Olac 1976, LTD, Brcester, Oxen., and clone 30H12, a rat anti-mouse monoclonal kindly supplied by Dr. I.F.C. McKenzie.

Anti-Thy-1.2 antibody was added to spleen cells (4 x 10^7 cells/ml) at the optimum concentration for T cells lysis (previously determined by microcytotoxicity of Ig^- cells) incubated on ice for 30-45 minutes, washed twice with EMEM, 5% FCS, 2ME, NAPs and incubated with a 1:2 dilution of guinea pig complement for 15-30 minutes at 37°C. On average, 25-35% of spleen cells were killed by this treatment as assessed by Trypan Blue exclusion method.

2.6.2 Ig-negative spleen cell populations

Pure populations of Ig-negative cells were obtained from spleen by a rosetting procedure which has been described previously by Parish and coworkers (1974).

Spleen cell suspensions were prepared as described in section 2.5 in EMEM, 5% FCS and 2-ME and kept on ice at 4 x 10^7 cells/ml.

SRBC were coated with sheep anti-mouse Ig as follows: 0.25mls of packed SRBC (washed 4 x with normal saline (N.S.)) was mixed with 4mls of N.S. and 20 µl of sheep anti-mouse Ig (125 mg/ml). While this was mixing on vortex mixer, 0.3mls of 0.1% $CrCl_3$ were added: this was left at room temperature for 5 minutes and the reaction stopped by the addition of 5mls of PBS. The SRBC coated with anti-mouse Ig were centrifuged, supernatant discarded and the procedure repeated to remove the last traces of free antibody. The anti-mouse Ig coated SRBC were resuspended in 2.25mls of culture medium (10% suspension) and were kept cold.

The cold spleen cells and anti-mouse Ig-SRBC were mixed, centrifuged (1,500 rpm, 5 min 4°C) to formed rosettes, gently resuspended and warmed up to 20°C. The suspension was layered on a cushion of Isopaque/Ficoll and centrifuged in MSE (4,000 rpm, 15 min, 20°C). The Ig^+ fraction moves to the bottom of the tube, the Ig^- fraction remains on interphase between medium and Isopaque/Ficoll. Ig^- fraction was harvested and washed 2 x to remove Isopaque/Ficoll. 2-3 x 10^7 Ig^- cells may be recovered per spleen. This Ig^- population was used in anti-Thy 1.2 titration and in MLR.

2.7 DETECTION OF ANTI-DNP PLAQUE-FORMING CELLS (PFC)

Cells secreting anti-DNP antibodies were estimated in glass slide chambers using the technique of Cunningham and Szenberg (1968). SRBC were coupled with dinitrophenylated rabbit anti-SRBC Fab (Strausbauch *et al.*, 1970) the preparation of which is outlined below. Indirect PFC were detected in the presence of sheep anti-mouse Ig developing serum (Wortis *et al.*, 1968).

In brief, cultured cells were harvested, pelleted by centrifugation and resuspended in 1ml of cold culture medium. 100 µl of cell suspension, 30 µl of 15% DNP-SRBC, 15 µl of guinea pig complement and/or 15 µl of developing serum were mixed together, placed into glass slide chambers, sealed with wax and incubated for 1 hour at 37°C. Cells secreting anti-DNP antibodies lyse DNP-coated SRBC around them, creating clear areas (plaques) which were enumerated under a dissecting microscope.

2.7.1 Preparation of anti-SRBC-Fab for anti-DNP PFC assay

Rabbits were injected intravenously twice a week for 3 weeks with 1ml of 25% washed SRBC in phosphate buffered saline (PBS). Seven days after the last challenge, the animals were bled and serum collected. Anti-SRBC antibody titres were assayed by a haemagglutination procedure (Langman, 1972) and were greater than 1:300.

Rabbit anti-sheep IgG in the serum was precipitated with 40% saturated ammonium sulphate $(NH_4)_2SO_4$ overnight at 4°C with constant stirring. The precipitated material was centrifuged (10,000 rpm, 10 min, 4°C), supernatant discarded, and the pellet washed with 40% $(NH_4)_2\ SO_4$. The protein was redisolved in 20mls of 0.1M phosphate buffer, pH 7.0, containing 0.01M cysteine and 0.002 M EDTA. The protein concentration measured at OD_{280} was ∿ 33mg/ml i.e. 660mg total.

The rabbit anti-sheep IgG was than digested with papain to obtain Fab and Fc fragments of IgG. Papain was added at 1% by weight i.e. 6.6mg/660mg of protein; incubated at 37°C for 6 hrs and dialysed against PBS for 24 hrs at 4°C. The content of the dialysis bag was centrifuged (10,000 rpm, 20 min, 4°C) to pellet crystallised Fc fragments, and filtered to remove low density precipitate which did not

sediment. The supernatant was concentrated to 4mls by pressure dialysis and centrifuged again to remove newly crystallised Fc fragments. The supernatant was applied onto G-200 column 48cm x 5 cm (Pharmacia) and eluted with PBS (5ml fractions, 10mls/hr). Fab-containing fractions were detected at OD_{280}, pooled, concentrated by pressure dialysis and the total protein concentration determined to be ∿ 2mg/ml (total protein ∿230mg). Rabbit anti-SRBC Fab in PBS were stored at -20°C.

2.7.2 Dinitrophenylation of Rabbit anti-SRBC Fab

DNP was coupled to Fab fragments as described by Strausbauch *et al.* (1970). 10mls of Fab was mixed with 100mg DNP-sulphonic acid and 100mg sodium carbonate (pH 9.3), left to react at room temperature for 24 hours away from light and subsequently dialysed for 48 hours against normal saline. Anti-SRBC DNP-Fab was stored at -20°C.

2.7.3 Dinitrophenylation of SRBC

SRBC were coated with DNP using dinitrophenylated rabbit anti-SRBC Fab fragments as follows: 1ml of packed SRBC (washed 4 times with saline) was mixed with 9mls of PBS and 200 µl of DNP-Fab for 1 hr at 37°C. DNP-SRBC were washed twice to remove free DNP-Fab and resuspended in 5mls of culture medium to be used in PFC assay.

2.7.4 Guinea Pig complement

Outbred guinea pigs were bled by heart puncture, blood clotted and serum collected by centrifugation (2,000 rpm, 4°C). The serum was then mixed with SRBC (washed previously with saline) and kept on ice for 30 min to remove any anti-SRBC antibodies. The SRBC were then pelleted by centrifugation, serum collected, aliquoted in 0.5ml aliquots and stored at -60°C.

2.8 SUGARS

The sugars used and their source were as follows: L - fucose, α - methyl glucoside, D - galactose, α- methyl galactoside and α - methyl mannoside from Calbiochem, San Diego, Ca; N - acetyl - D - glucosamine, N - acetyl - D - galactosamine and N - acetyl - D - mannosamine from Sigma, St. Louis, Mo; D - mannose from Fluka, A.G. Buchs, Switzerland and D - glucose from Univar, Ajax Chemicals, Sydney.

2.9 MIXED LYMPHOCYTE REACTIONS (MLR) *in vitro*

A MLR was generated between 5×10^5 Ig^- A.TH spleen responder cells and 5×10^5 2,000 X-irradiated A.TL stimulator cells in Linbro 24 well trays, containing RPMI, 5% HIFCS, 2-ME. Cultures were incubated in the presence of sugars at a concentration of 2mg/ml for 4 days in humidified $7\%O_2/10\%CO_2$ air (Woolnough *et al.*,1979).

The cultures were pulsed with ^{125}I UDR (0.5 Ci/well) for 18 hrs, harvested onto glass fibre filter paper discs (Whatman, Grade GF/A) using a Titertek cell harvester.

2.10 *In vitro* PRIMARY CYTOTOXIC T CELL RESPONSE

2.10.1 Generation of Tc cells

Tc cells were generated by mixing 5×10^6 CBA/H responder cells with 1.25×10^6 2,000R-irradiated (^{60}Co source) Balb/c stimulator spleen cells in 2.4ml medium in 24-well Linbro trays, each treatment in quadriplicate, as described previously (Woolnough *et al*, 1979). The cells were cultured in DMEM, 10%HIFCS, 2-ME for 5 days in $7\%O_2/10\%CO_2$ air mixture at 37^oC. Sugars were present throughout the culture of a concentration of 5mg/ml. The cells specific for $H\text{-}2^d$ antigens were assayed for killer activity against ^{51}Cr-labelled P815 mastocytom

($H\text{-}2^d$) target cells in a four-hour release assay (Lafferty *et al.*, 1976) which is described below.

2.10.2 Cytotoxic Assay

The cell populations to be assayed for cytotoxicity were pelleted by centrifugation (1,200g, 5 min) and resuspended in EMEM containing 10% HIFCS. 0.1ml of effect on cells were added to 0.1ml of labelled target cells in quadriplicate wells of flat-bottomed microtitre plates (Linbro, 76-003-05). Spontaneous lysis of the targets was determined by mixing 0.1ml of targets with 0.1ml of EMEM containing 10% HIFCS in quadriplicate. Total releasable chromium was determined by mixing 0.1ml of targets with 0.9ml of distilled water in plastic centrifuge tubes (water lysis). All cell mixtures were incubated for 4 hours at $37^{\circ}C$ in a humidified atmosphere of 10% CO_2 in air.

0.1ml of supernatant was removed from microtitre wells without disturbing the cells. Water lysis tubes were vortexed, centrifuged at 1,200 for 5 min, and 0.5ml of the supernatant was removed. Supernatants were counted for one minute in a Packard gamma scintillation spectrophotometer.

Cytotoxic activity (% specific lysis) is expressed as:

$$\frac{\text{test lysis - spontaneous lysis}}{\text{water lysis - spontaneous lysis}}$$

2.11 MAINTENANCE OF CELL LINES

2.11.1 P815 mastocytoma as Tc cell targets

P815 (strain origin DBA/2, Dunn and Potter, 1957) cell line was maintained by passage in tissue culture every 3 or 4 days about 5×10^4 viable cells were subcultured into $25cm^2$ tissue culture flasks

(Falcon, 3013) containing 5mls of DMEM and 10% HIFCS. Cells were incubated in a humidified atmosphere of 7% O_2/10% CO_2 air and were used as targets 24 hours after subculture.

2.11.2 PU5.18 macrophage cell line

PU5.18 cell line (Ralph and Nakionz, 1977) of Balb/c origin, was maintained by passage in tissue culture every 3-5 days. About 10^5 viable cells were removed from the monolayer by trypsin and subcultured into $25cm^2$ tissue culture flasks containing 5mls of DMEM, 10% HIFCS and 0.1m L-asparagine. When monolayers were confluent, 15 µl 1ml LPS W was added to the medium to induce IL-1 secretion.

2.11.3 Embryo fibroblasts

Primary cultures of embryo fibroblasts were prepared as described by Bellet and Younghusband (1979) from PVG rat and C57Bl/6 mouse embryos, and were grown in Eagle's medium "Autopow" (Flow Laboratories) supplemented with 10% FCS in $75cm^2$ plastic tissue culture flasks.

2.12 GENERATION OF TRF-CONTAINING SUPERNATANT

Spleen cells from mice primed to DNP-HCY were cultured as described in section 2.5 for 5 days with antigen. Cultures were harvested, centrifuged (2,000 rpm, 5 min), supernatant retained and centrifuged again to remove all spleen cells. This superantant was used directly in TRF assays.

Alternatively, primed spleen cells were cultured for 4 days as above, cells pelleted by centrifugation, supernatant discarded, pellet resuspended in EMEM containing 2-ME and antigen (no FCS), replaced in Linbro trays and cultured for 24 hrs more. This 24-hour

(day 4-5 of culture) supernatant was pelleted free of cells, and was concentrated 10-fold over a PM-10 membrane (Amicon, Lexington, Mass.) under N_2 at -15°C. The concentrated supernatant was sterilized by filtration through a 0.22 µm Millipore membrane and stored at -60°C. The rationale for the second part of TRF generation was to obtain TRF-containing supernatants which were free of FCS and hence could be concentrated with ease.

2.13 PREPARATION OF CONCANAVALIN-A ACTIVATED CULTURE SUPERNATANT (CON A CS)

Con A CS was made using a modification of the method described by Talmage *et al.*, (1977). 10^8 mouse spleen cells were cultured in 15mls of serum-free EMEM containing 10^{-4}M 2-ME and 5 µg/ml Concanavalin A (Con A; SIGMA, Grade IV). Cultures were set up in 75cm^2 tissue culture flasks (Falcon, 3024), gassed with 10% CO_2/7% O_2, 83% N_2 and incubated at 37°C for 2 hours. By this time most of the cells had adhered to the plastic substrate. The supernatant medium was discarded, and the cell monolayer was washed gently 3 times with 15mls of EMEM/2-ME to remove non-cell bound Con A. The monolayers were then flooded with 30mls of serum-free EMEM containing 10^{-4}M 2-ME. The flasks were regassed and incubated at 37°C for a further 16-20 hours.

The supernatant medium was harvested, centrifuged to remove cells and concentrated 10-fold over a PM-10 membrane (Amicon, Lexington, Mass.), filtered through a 0.22 µm Millipore membrane and stored at -20°C.

Con A CS prepared by this technique contained no detectable residual Con A, as determined by agglutination of sheep red blood cells (Lafferty *et al.*, 1981).

2.14 TRF ASSAY

Spleen cells from primed mice were treated with anti-Thy 1.2 antibody and complement as described in section 2.6.2, adjusted to $\sim 1.2 \times 10^6$ cells/ml and cultur-d with supernatants from antigen-activated or Con A-activated spleen cells in the presence of antigen. The ability of these supernatants to restore anti-DNP PFC responses was assayed 6 or 7 days later if added at culture initiation, or 4 days later if added on day 2 of the culture.

2.15 MAINTENANCE ASSAY

This assay measures the ability of IL-2 or T cell growth factor (TCGF) to support the continual blasting of activated T cells in cultures. The assay consists of two parts: generation of activated T cells and the assay of supernatants for maintenance activity.

2.15.1 Preparation of mouse T cell blasts

Spleen cells from C57Bl/6 ($H\text{-}2^b$) mice were washed, declumped and adjusted to a concentration of 10^6 cells/ml in EMEM, 10% HIFCS, 2-ME. Con A was added to cells at 1 µg/ml and the cell suspensions were plated in 24-well Linbro trays at 1 ml/well, gassed with humidified 7% O_2/10% CO_2 air and incubated for 3 days at 37°C.

2.15.2 The Assay

The activated T cells were washed and resuspended at 4×10^5 cells/ml in DMDM, 10% HIFCS and 2-ME. Lymphokine containing sample (50 µl of cell suspension was added to each well and trays incubated at 37°C in a humidified atmosphere of 7% O_2/10% CO_2 air. After 16-20 hours of culture, 1-2 µCi of ^{3}H-methyl thymidine (Amersham, specific activity 5Ci/mmol) were added to each well in 50 µl of EMEM and the cultures were incubated for a further 5 hours.

The cultures were harvested onto glass fibre filter paper discs (Whatman, Grade GF/A) using a Titertek cell harvester. The amount of thymidine incorporated by the cultures was determined by placing the discs in scintillation fluid and counting on a Packard liquid scintillation spectrophometer. Maintenance activity was expressed as counts/minute (cpm) and was considered significant if it was more than 3 standard deviations (SD) above the mean of 24 controls.

2.16 INTERLEUKIN - 1 (IL-1) ASSAY

IL-1 either in antigen-activated supernatants or in supernatants of LPS-stimulated PU-5-1.8 cell line was assayed on C3H.Hej thymocytes which are unreactive to LPS in the preparation.

Cell suspensions of C3H.Hej thymocytes were prepared as in section 2.5 and adjusted to 2×10^7 cells/ml in EMEM, 10% HIFCS, 2-ME. Con A at 1 µg/ml was added to the cell suspension. 50 µl of IL-1 containing supernatant was then placed in each well of a 96-well flat-bottomed tray in 2-fold dilutions out to 12 wells. 50 µl of cell suspension was added to each well and the plate incubated for 48 hours at $37^{o}C$ in humidified CO_2 air mixture. 24 control wells contained medium instead of supernatant.

48 hours later, 1-2 µCi of ^{3}H-menthyl thymidine was added to each well in 50 µl of EMEM and cultured for a further 5 hours. The cultures were harvested and thymidine incorporation assessed as for IL-2 in section 2.15.2.

2.17 STATISTICAL ANALYSIS OF RESULTS

The arithmetic mean and standard error (SE) or standard deviation (SD) of the mean were calculated by standard statistical methods. The Student's T-test was used to estimate the significance of the difference between the mean values of two groups. The difference between two groups was considered not significant (NS) if the ρ value was >0.05.

CHAPTER 3

Inhibition of secondary IgG responses by N-acetyl-D-galactosamine

3.1 INTRODUCTION

Evidence from a wide range of biological systems indicates that carbohydrates, usually expressed as gangliosides, can function as cell surface receptors for regulatory molecules. The best characterized receptor is the monosialoganglioside GM_1 which binds cholera toxin and subsequently results in adenylate cyclase activation (Van Heyningen, 1974; Fishman and Brady, 1976). Gangliosides have also been implicated in the cell membrane receptors for tetanus toxin (Van Heyningen, 1974), interferon (Vengris *et al.*, 1976), thyroid stimulatory hormone (Mullin *et al.*, 1978), and cell adhesion (Huang, 1978). At the immunological level, recent studies suggest that the receptor on macrophages for migration inhibition factor is also a ganglioside (Higgins *et al.*, 1978; Liu *et al.*, 1982).

Recently, a family if I region-associated (Ia) antigens expressing carbohydrate-defined antigenic determinants have been described both in mouse (Parish *et al.*, 1977) and man (Sandrin *et al.*, 1979). Since the carbohydrate-defined Ia antigens appear to be gangliosides, it seems likely that they may act as receptors for informational molecules between lymphoid cells. The observation that certain T cell-derived gangliosides can inhibit *in vitro* antibody responses (Esselman and Miller, 1977) is consistent with this idea.

Additionally, a large body of evidence suggests that Ia antigen specificities are associated with a range of immunoregulatory molecules or "factors" secreted by T cells. These factors mediate help (Armerding *et al.*, 1974; Munro and Taussig, 1975; Howie and Feldmann, 1977) and suppression (Tada *et al.*, 1976; Theze *et al.*, 1977) of antibody formation, contact hypersensitivity (Greene *et al.*, 1977) and mixed lymphocyte

reactions (MLR) (Rich *et al.*, 1977). At least some of these factors have been shown to carry carbohydrate-defined Ia specificities. Thus L-Glu^{60}-L-Ala^{30}-L-Tyr^{10} (GAT) and poly-L-(Tyr, Glu)-poly-DL-Ala--poly-L-Lys (T,G)A--L -specific helper factors appear to bear carbohydrate determinants (Howie *et al.*, 1979). Similarly, a T cell-derived suppressor factor of delayed-type hyper-sensitivity also appears to carry carbohydrate-defined Ia antigens (Liew *et al.*, 1980).

If carbohydrate-protein interactions (possibly I-region controlled) do indeed play a role in the communication between lymphocytes, one would predict that certain monosaccharides should selectively inhibit *in vitro* immune responses. This chapter describes studies which support this concept and suggests that N-acetyl-D-galactosamine (Gal NAC) inhibits secondary IgG responses by blocking the collaboration between T and B lymphocytes.

3.2 MATERIALS AND METHODS

3.2.1 Animals

CBA/H, A.TL, A.TH and Balb/c mice of either sex were used at 5-16 weeks of age. Monomeric flagellin (MON) was prepared from the flagella of *Salmonella typhimurium* SL 870, and haemocyanin (HCY) from *Jasus lalandii* crayfish as described in section 2.2. These proteins were dinitrophenylated with conjugation ratios of $DNP_{1.1}$MON and DNP_{42}HCY (see section 2.2). The sources of the monosaccharides are listed in section 2.8.

3.2.2 Immunizations

Mice were primed to DNP-MON and DNP-HCY as described in section 2.3. Briefly, the antigen was bound on bentonite and injected intra-

peritoneally three times at two-week intervals. Animals were used 2-6 weeks after last challenge.

3.2.3 *In vitro* Antibody responses

Spleen cells were cultured at a concentration of 2×10^6 cells/ml in EMEM, 5% FCS, 2-ME (10^{-4}M) in 24-well Linbro trays as described in Section 2.5. Primary antibody responses were assayed after 3 days of culture in a 10% CO_2/7% O_2 humidified atmosphere; secondary antibody responses after 7 days of culture. Sugars and antigen were added at the beginning of the culture.

3.2.4 MLR

A MLR was generated between 5×10^5 Ig^- A.TH spleen responder cells and 5×10^5 2,000 rads-irradiated A.TL stimulator spleen cells in the presence of sugars at a concentration of 2mg/ml. After 4 days of culture, each treatment was pulsed for 18 hours with 0.5 μCi/well of ^{125}I-labelled deoxyuridine. Further details of the MLR are described in section 2.9. Results were expressed as counts/minute incorporated.

3.2.5 *In vitro* Primary Tc cell responses

Tc cells were generated by culturing 5×10^6 CBA responder cells with 1.25×10^6 gamma-irradiated Balb/c stimulator cells (see section 2.10). Sugars were present throughout culture at a concentration of 5mg/ml. The cultures were harvested on the fifth day and cytotoxic activity assayed on ^{51}Cr-labelled P815 mastocytoma target cells. For details see section 2.10.2.

3.2.6 Assay of PFC

Antibody-secreting cells were enumerated using the technique of Cunningham and Szenberg (1968). To detect anti-DNP PFC, SRBC were coated with DNP. Indirect PFC were estimated in the presence of developing serum as described in section 2.7. Different classes of indirect PFC were assayed with the use of monospecific anti-IgG_1, IgG_{2a}, IgG_{2b} and IgA antisera (Litton Bionetics, Kensington, MD). Results were expressed as the mean of anti-DNP PFC/4 culture replicates.

3.2.6 Generation and assay of supernatant with T cell replacing helper activity

T cell replacing factor (TRF) was generated by culturing primed spleen cells (2×10^6/ml) for 5 days with antigen and then harvesting the culture supernatant. TRF activity was assayed by adding 2.5ml of supernatant to each well of primed spleen cells that had been cultured for 2 days with antigen and then T cell depleted by anti-Thy-1.2 and complement treatment (see section 2.6.1). The anti-DNP PFC response, assayed after an additional 5 days of culture, was a measure of helper activity.

3.3 RESULTS

3.3.1 Inhibition of *in vitro* immune responses by monosaccharides

A prediction of the hypothesis that carbohydrate-protein interactions are involved in lymphocyte communication is that monosaccharides should selectively inhibit immune responses. Thus, ten monosaccharides were screened for their effect on a range of *in vitro* immune responses.

The ten monosaccharides used in this study represent common sugars present in mammalian carbohydrates. It should be noted that amino sugars such as glucosamine could not be used in *in vitro* cultures as it was found that they were highly toxic for lymphocytes, killing or lysing all cells within 24 hours of culture.

3.3.2 Primary *in vitro* antibody responses

In these studies, the primary *in vitro* anti-DNP PFC response induced by DNP-MON was examined. This antigen induces only IgM PFC in a primary response, the PFC peaking after 3 days of culture. In the experiment shown in Table 3.1, at 5mg/ml (∿ 20mM), D-mannose was inhibitory, suppressing the anti-DNP PFC response to background levels without noticeably affecting cell viability of the culture. A similar inhibitory effect with this sugar was observed in a number of experiments.

3.3.3 Secondary *in vitro* antibody responses

In vitro secondary anti-DNP antibody responses were examined using spleen cells from mice that had been primed with either DNP-MON or DNP-HCY, the levels of anti-DNP PFC being assayed after 7 days of culture. The secondary PFC responses consisted of both IgM and IgG-secreting cells. However, with spleen cells from CBA mice, IgM peaked after 3 days of culture and declined to very low levels by day 7, whereas IgG PFC peaked on day 6 or 7. Thus the negligible day 7 IgM PFC responses are not included in Tables 3.2 and 3.3.

As in primary antibody response, D-mannose completely abrogated the secondary PFC responses induced by both DNP-MON and DNP-HCY. However, in contrast with the primary response, Gal NAc

EFFECT OF MONOSACCHARIDES ON PRIMARY *IN VITRO*
ANTIBODY RESPONSE TO DNP-MON

TREATMENT	IgM DNP-PFC/CULTURE	% INHIBITION
NO ANTIGEN	23 ± 5	
NO SUGAR	2,160 ± 133	
L-FUCOSE	1,732 ± 65	
D-GLUCOSE	1,463 ± 395	
N-ACETYL-D-GLUCOSAMINE	1,763 ± 57	
αMETHYL GLUCOSIDE	1,770 ± 72	
D-GALACTOSE	1,513 ± 74	
N-ACETYL-D-GALACTOSAMINE	1,953 ± 101	
αMETHYL GALACTOSIDE	1,600 ± 198	
D-MANNOSE	18 ± 12	100 (P=0.001)
N-ACETYL-D-MANNOSAMINE	1,970 ± 65	
αMETHYL MANNOSIDE	1,953 ± 83	

TABLE 3.1

Ability of different monosaccharides to inhibit the *in vitro* primary anti-DNP-MON antibody response of CBA/H spleen cells.

Results are expressed as mean and standard error (n=4). Monosaccharide concentration = 5mg/ml DNP-PFC assayed after 3 days of culture.

EFFECT OF MONOSACCHARIDES ON SECONDARY *IN VITRO*
ANTIBODY RESPONSE TO DNP-MON

TREATMENT	IgG DNP-PFC/CULTURE	% INHIBITION
NO ANTIGEN	0	
NO SUGAR	8,825 ± 324	
L-FUCOSE	6,768 ± 383	
D-GLUCOSE	6,325 ± 388	
N-ACETYL-D-GLUCOSAMINE	7,255 ± 222	
αMETHYL GLUCOSIDE	7,940 ± 386	
D-GALACTOSE	6,770 ± 851	
N-ACETYL-D-GALACTOSAMINE	849 ± 19	90 (P=0.001)
αMETHYL GALACTOSIDE	7,458 ± 814	
D-MANNOSE	10 ± 7	100 (P=0.001)
N-ACETYL-D-MANNOSAMINE	6,528 ± 310	
αMETHYL MANNOSIDE	7,590 ± 279	

TABLE 3.2

Ability of different monosaccharides to inhibit the *in vitro* secondary anti-DNP-MON antibody response of CBA/H spleen cells.

The results are expressed as means and atandard errors (n=4). Monosaccharide concentration = 5mg/ml; DNP-PFC assayed after 7 days of culture.

EFFECT OF MONOSACCHARIDES ON SECONDARY *IN VITRO* ANTIBODY RESPONSE TO DNP-HCY

TREATMENT	IgG DNP-PFC/CULTURE	% INHIBITION
NO ANTIGEN	5 ± 3	
NO SUGAR	14,450 ± 2,147	
L-FUCOSE	4,705 ± 1,425	67 (P=0.01)
D-GLUCOSE	12,088 ± 1,803	
N-ACETYL-D-GLUCOSAMINE	6,330 ± 615	56 (P=0.01)
αMETHYL GLUCOSIDE	14,723 ± 528	
D-GALACTOSE	13,195 ± 517	
N-ACETYL-D-GALACTOSAMINE	1,858 ± 86	87 (P=0.001)
αMETHYL GALACTOSIDE	15,200 ± 966	
D-MANNOSE	3 ± 3	100 (P=0.001)
N-ACETYL-D-MANNOSAMINE	11,275 ± 284	
αMETHYL MANNOSIDE	11,225 ± 632	

TABLE 3.3

Ability of different monosaccharides to inhibit the *in vitro* secondary anti-DNP-HCY antibody response of CBA/H spleen cells.

Results are expressed as mean and standard error of our determinantions. Sugar concentration = 5mg/ml. PFC response was assayed after 7 days of culture.

inhibited about 90% of the IgG response to these two antigens (see Tables 3.2 and 3.3). Two other sugars consistently inhibited the response to DNP-HCY, although the blocking was not as striking as with Gal NAc. These sugars were N-acetyl-D-glucosamine (Glc NAc) and L-fucose (Fuc), inhibiting IgG levels by 56 and 67% respectively (Table 3.2). Comparable results with DNP-HCY primed cells were obtained in additional experiments. In DNP-MON-primed cultures, Glc NAc and Fuc exerted significant inhibition in 2 out of 5 experiments. However, as depicted in Table 3.2 inhibition by these sugars was not always observed with this antigen.

3.3.4 Responses to alloantigens *in vitro*

Two different responses to alloantigens were examined: a MLR against an inter - H-2 (I-region) difference and the Tc cells generated by complete incompatibility across the H-2 complex. As in both primary and secondary antibody responses *in vitro*, D-mannose completely inhibited both the MLR to I-region antigens (Table 3.4) and the generation of Tc cells (Table 3.5). However, Gal NAc exerted no inhibitory effect at all in these responses, nor did Glc NAc and Fuc, thus highlighting the specificity of these sugars for secondary antibody responses.

3.3.5 Mode of inhibition by Gal NAc

The data presented above indicates that Gal NAc clearly selectively inhibits secondary antibody responses. A more detailed analysis of the mode of inhibition by this sugar was performed. The inhibitory effect of this sugar was concentration-dependent. As can be seen in Figure 3.1, significant inhibition was still seen at a concentration

INHIBITION OF MIXED LYMPHOCYTE REACTION BY VARIOUS SUGARS

RESPONDERS (Ig^- SPLEEN)	STIMULATORS (2,000R X-IRRAD. SPLEEN)	SUGAR 2MG/ML	C.P.M. (^{125}I UDR)
$5x10^5$ A.TH	$5x10^5$ A.TH	-	10,965 ± 840
"	$5x10^5$ A.TL	-	47,759 ± 1,037
"	"	L-FUCOSE	39,250 ± 5,932
"	"	L-RHAMNOSE	47,271 ± 1,315
"	"	D-GALACTOSE	41,817 ± 896
"	"	N-ACETYL-D-GALACTOSAMINE	45,599 ± 5,911
"	"	D-GLUCOSE	47,306 ± 2,153
"	"	N-ACETYL-D-GLUCOSAMINE	47,112 ± 1,403
"	"	D-MANNOSE	14,009 ± 1,195
"	"	N-ACETYL-D-MANNOSAMINE	40,727 ± 2,846

TABLE 3.4

Inhibition of MLR by different sugars.

5×10^5 responder cells were mixed with 5×10^5 2,000rd X-irradiated stimulator spleen cells in the presence of 2mg/ml of monosaccharide. The extent of stimulation was assayed after 4 days of culture.

Mean (n=3) standard error.

EFFECT OF SUGARS ON THE GENERATION OF CYTOTOXIC T CELLS[a]

SUGARS (5 MG/ML)	SPECIFIC LYSIS %[b]
-	37.4 ± 1.5
L-FUCOSE	44.8 ± 1.1
D-GLUCOSE	44.8 ± 1.6
Glc NAc	30.6 ± 2.8
METHYL-α-D-GLUCOSIDE	44.1 ± 4.7
D-GALACTOSE	48.2 ± 2.3
Gal NAc	42.7 ± 2.3
METHYL-α-D-GALACTOSIDE	35.9 ± 0.7
D-MANNOSE	0

a) CYTOTOXIC T CELLS WERE GENERATED BY INCUBATING 1.25×10^6 IRRADIATED BALB/C SPLEEN CELLS WITH 5×10^6 CBA/H SPLEEN CELLS

b) MEAN (n = 4) ± STANDARD ERROR

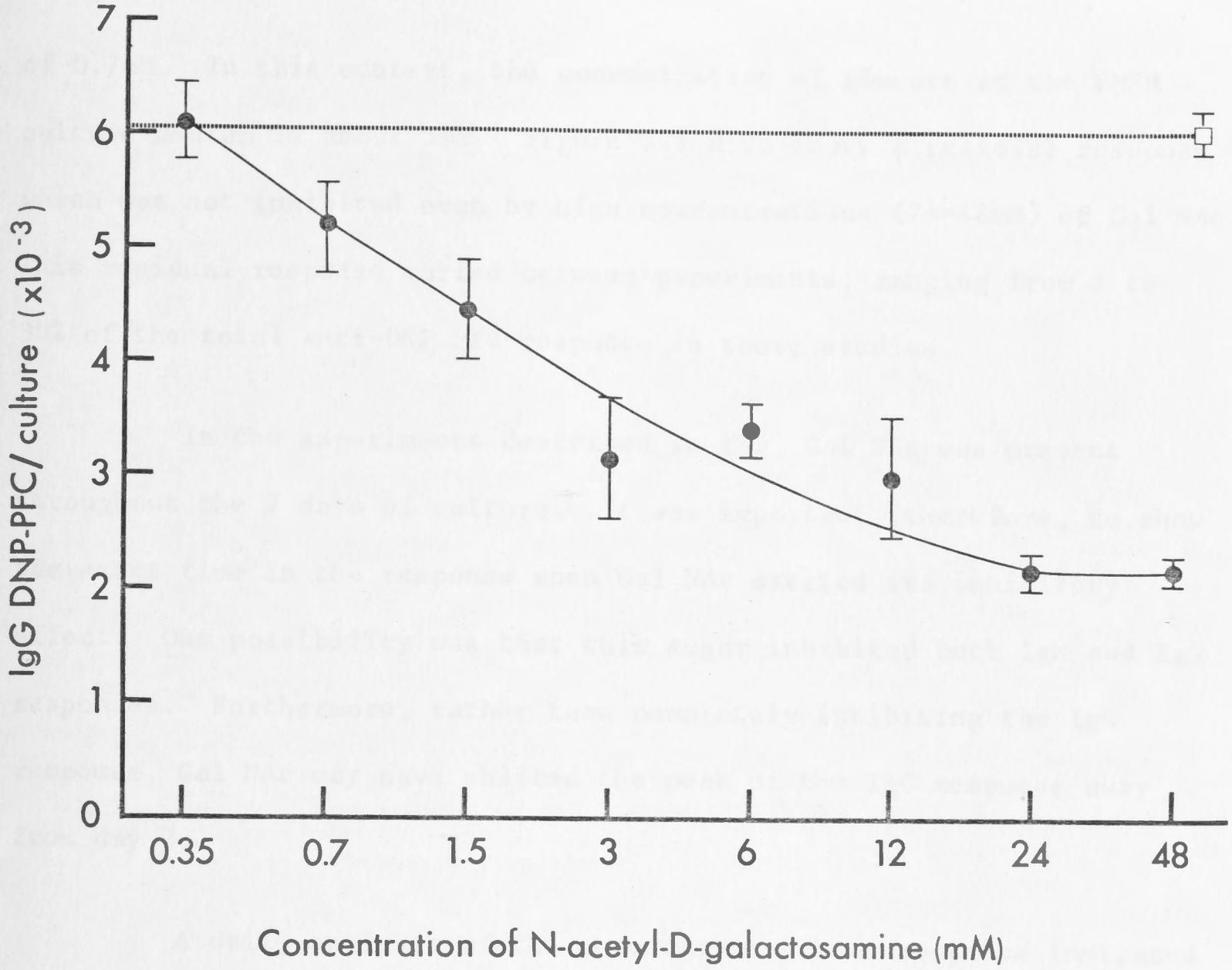


FIGURE 3.1

Ability of different concentrations of Gal NAc to inhibit the secondary anti-DNP-HCY antibody response *in vitro*. Dotted Line (·····) represents response in the absence of sugar. Results are means of the day 7 PFC responses. Vertical bars are standard errors of means (n=4).

of 0.7mM. In this context, the concentration of glucose in the EMEM culture medium is about 5mM. Figure 3.1 also shows a residual response which was not inhibited even by high concentrations (24-48mM) of Gal NAc. This residual response varied between experiments, ranging from 5 to 30% of the total anti-DNP PFC response in these studies.

In the experiments described so far, Gal NAc was present throughout the 7 days of culture. It was important, therefore, to show the exact time in the response when Gal NAc exerted its inhibitory effect. One possibility was that this sugar inhibited both IgM and IgG responses. Furthermore, rather than completely inhibiting the IgG response, Gal NAc may have shifted the peak of the IgG response away from day 7.

A daily analysis of the secondary antibody response indicated that IgM peaked on day 3 and IgG on day 7 (Figure 3.2). Similar peaks were obtained for DNP-HCY and DNP-MON primed cultures, although the IgM peak was higher with DNP-MON primed cells.

A daily analysis of Gal NAc inhibition is depicted in Figure 3.2 and highlights the inability of this sugar to affect the IgM response but its very strong blocking of IgG production. It should be noted that in the presence of sugar, there appears to be no shift in the peak of the IgG response: in fact, very few IgG-producing cells were detected after 10 days of culture. A similar analysis of the secondary antibody response to DNP-MON revealed that Gal NAc had no effect on IgM PFC but inhibited the IgG production throughout a 10-day culture period. When plaques were developed with monospecific antisera, it was seen that the IgG response consisted of approximately 90% IgG_1 and 10% IgG_{2a}

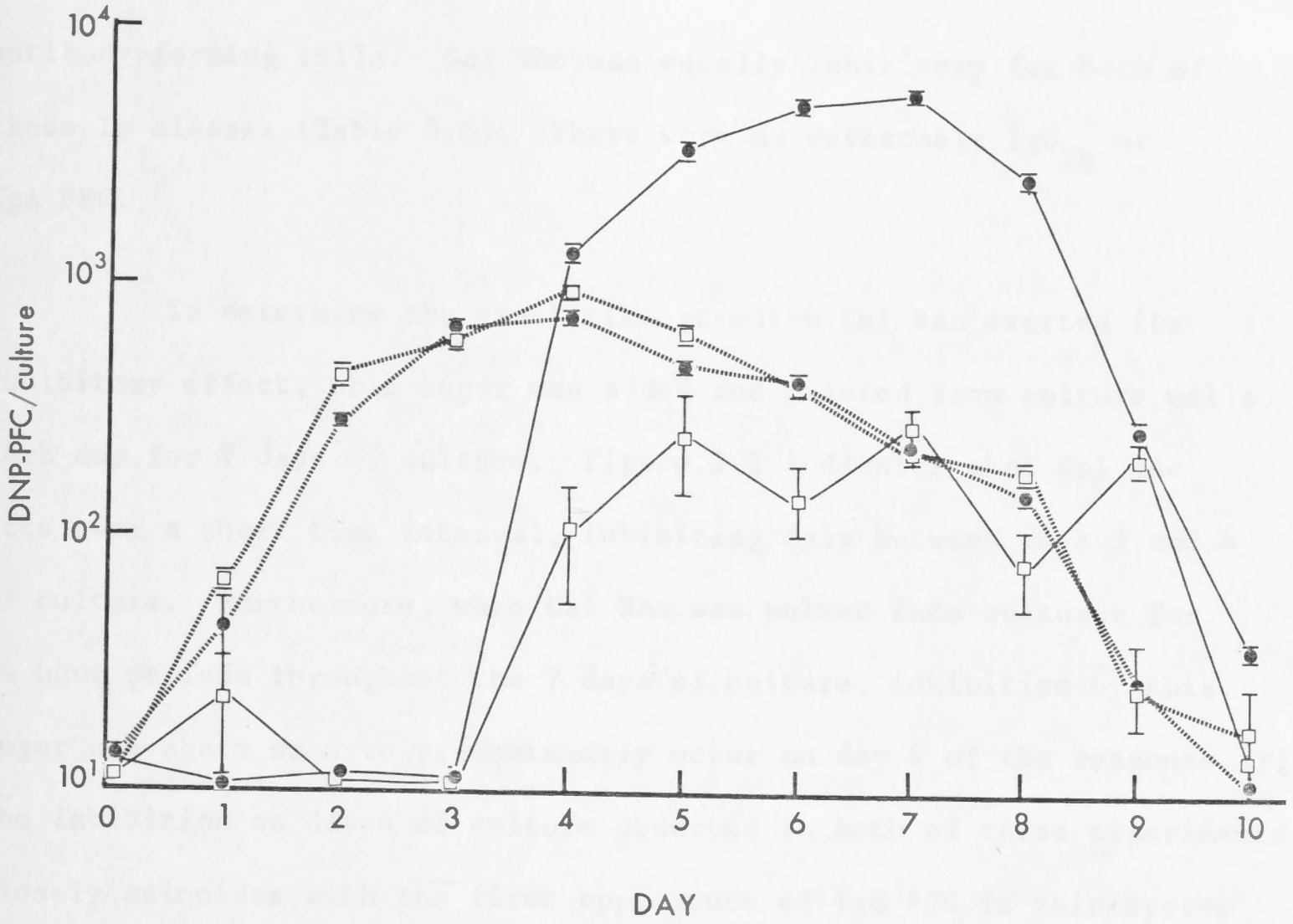


FIGURE 3.2

Kinetics of secondary anti-DNP-HCY antibody response *in vitro* in the absence (●) and in the presence (□) of Gal NAc. Solid line represents IgG PFC levels, broken line IgM PFC levels. Each point represents the mean of 4 determinations. Vertical bars are standard errors of means.

antibody-forming cells. Gal NAc was equally inhibitory for both of these Ig classes (Table 3.6). There were no detectable IgG_{2b} or IgA PFC.

To determine the exact time at which Gal NAc exerted its inhibitory effect, this sugar was added and deleted from culture wells each day for 7 days of culture. Figure 3.3 indicates that Gal NAc acts over a short time interval, inhibiting only between days 3 and 4 of culture. Furthermore, when Gal NAc was pulsed into cultures for 24 hour periods throughout the 7 days of culture, inhibition by this sugar was again seen to predominantly occur on day 4 of the response (Fig 3.4). The inhibition on day 4 of culture observed in both of these experiments closely coincides with the first appearance of IgG PFC in this system (Figure 3.2).

3.3.6 Mode of inhibition by L-fucose

Data presented in Table 3.3 shows that Fuc selectively inhibits secondary antibody responses to DNP-HCY. As with Gal NAc, a more detailed analysis of the mode of inhibition by Fuc was performed. Fuc did not inhibit IgG responses as acutely as Gal NAc. However, as can be seen in Figure 3.5, a daily analysis of Fuc-inhibition showed that this sugar did not shift the peaks of the antibody response and that IgG production was blocked significantly.

As with Gal NAc, when Fuc was added and deleted from culture wells each day for 6 days of culture, this sugar exerted its inhibitory effect between days 2 and 4 of the culture (Figure 3.5).

TABLE 3.6

Isotype composition and Gal NAc inhibition of secondary IgG response to DNP-MON in CBA/H cultures

(a) Isotype composition

Isotype	day 7 PFC/culture*
IgG1	1,650 ± 33
IgG2a	245 ± 19
IgG2b	95 ± 10
IgA	53 ± 18

(b) Gal NAc inhibition of secondary IgG1 and IgG2 response to DNP-MON in CBA/H cultures

Treatment	day 1 PFC/culture*	
	IgG1	IgG2
Antigen only	10,875 ± 489	1,790 ± 258
Gal NAc	4,068 ± 528	1,095 ± 88

Plaques were developed with monospecific antisera on day 7 of culture. Gal NAc was present at 5mg/ml

* mean and standard error of four determinations

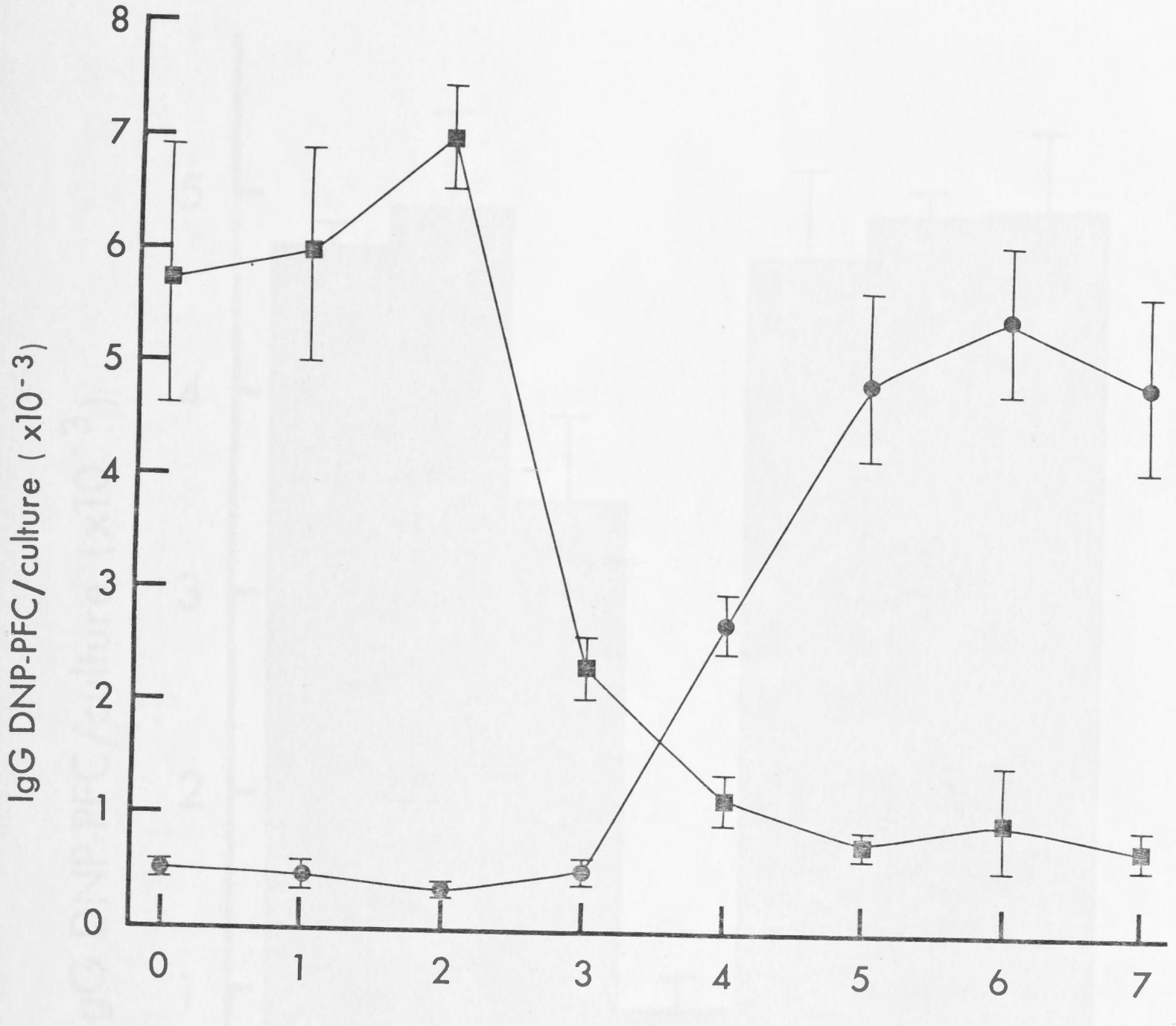


FIGURE 3.3

Influence of the time of addition and deletion of Gal NAc on a secondary anti-DNP-MON antibody response *in vitro*. The sugar with either added (●) or deleted (■) on each day of culture and all cultures assayed for anti-DNP PFC on day 7. Vertical bars are standard errors of means (n=4).

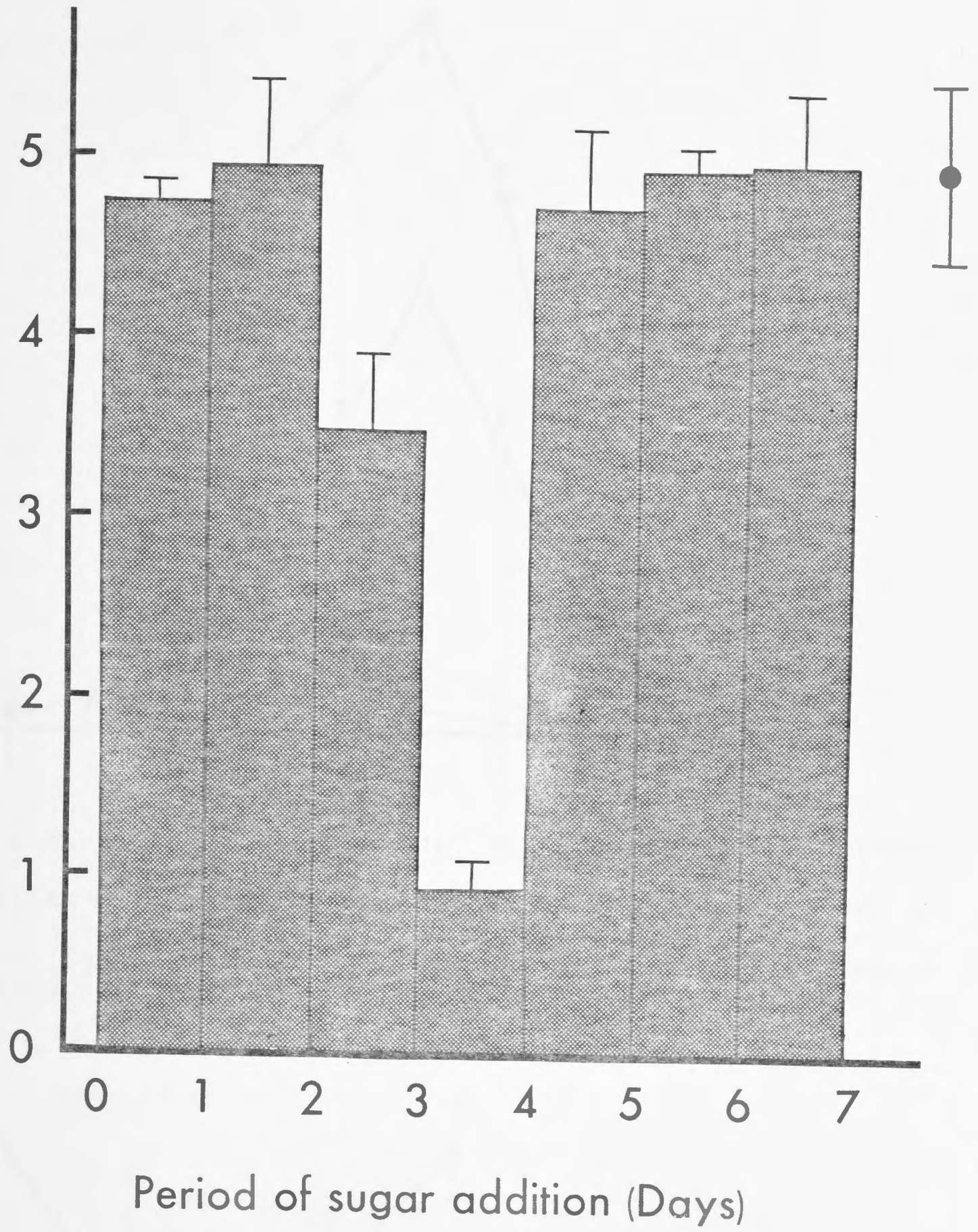
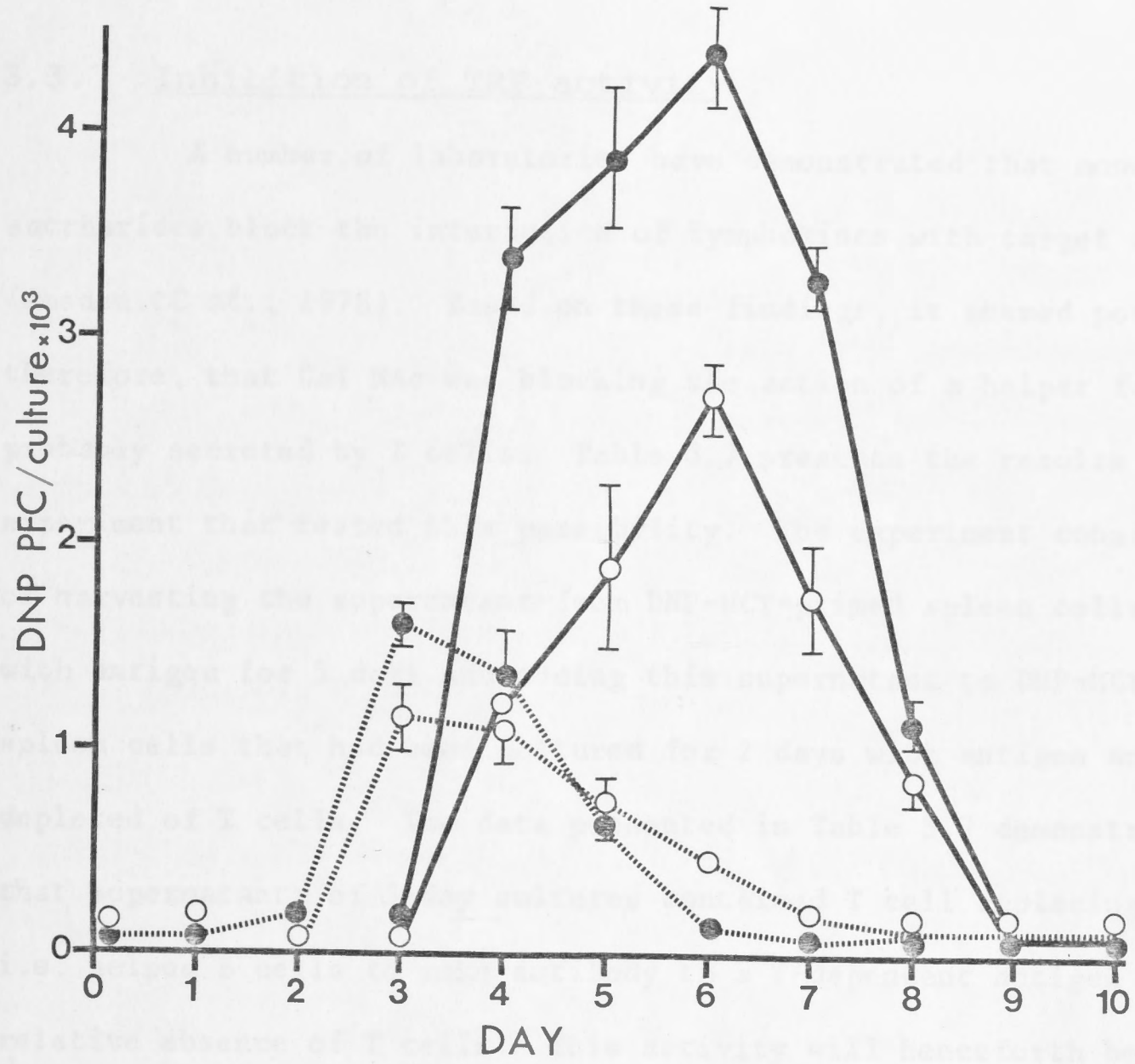


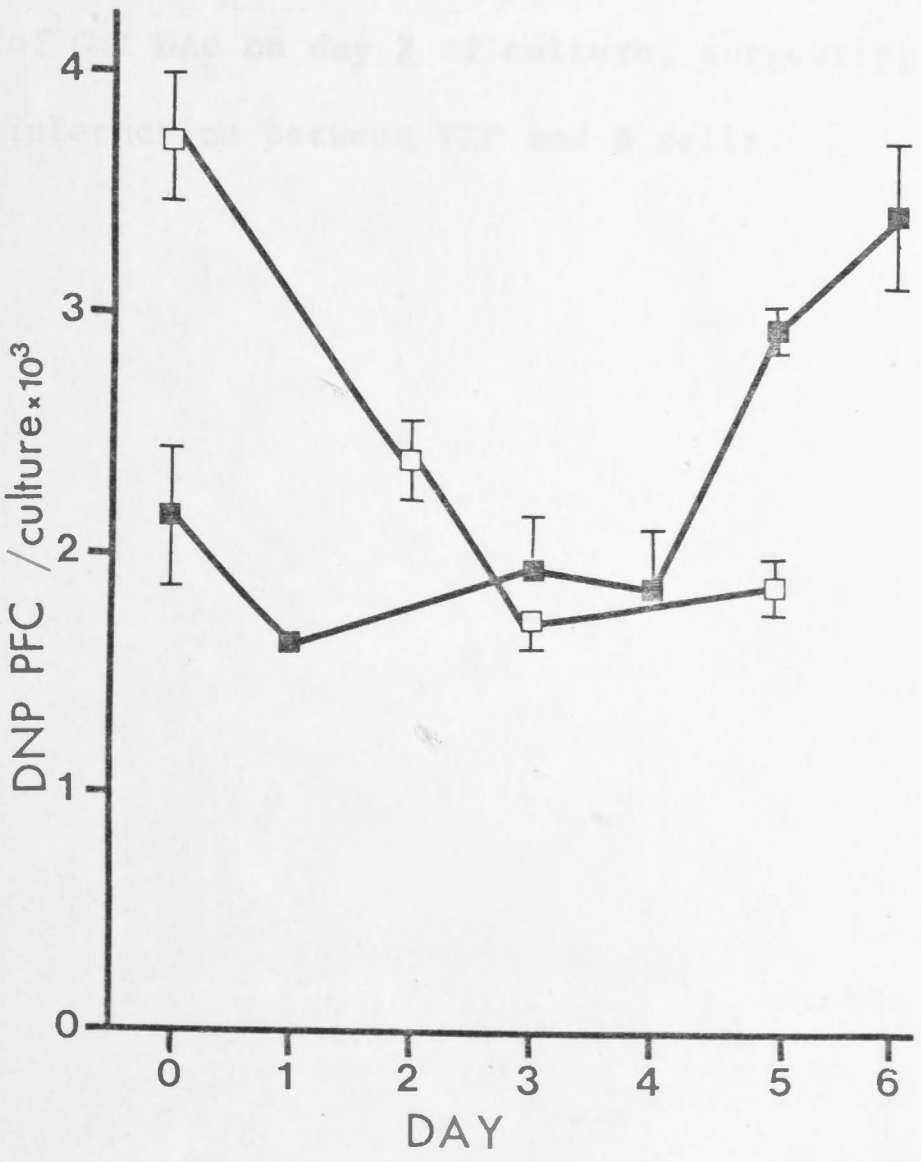
FIGURE 3.4

Effect of pulsing cultures for 24-hour periods with Gal NAc on a secondary *in vitro* anti-DNP-MON antibody response. All cultures were assayed for anti-DNP-PFC on day 7. Each column represents the mean and standard error of 4 determinations. The bar on the right represents the response in absence of sugar.

FIGURE 3.5



L-fucose inhibition of secondary in vitro anti-DNP-HCY antibody response:
(a) Kinetics of the antibody response in absence ( ) and in presence ( ) of Fuc. Solid line represents IgG PFC levels, brokin line IgM PFC levels. Each point represents the mean of 4 determinations. Vertical bars are standard errors of mean.



(b) Influence of the time of addition and deletion of Fuc on the antibody response. Fuc was either added ( ) or deleted ( ) on each day of culture and all cultures assayed for anti-DNP PFC on day 6. Vertical bars are standard errors of mean (n=4)

3.3.7 Inhibition of TRF activity

A number of laboratories have demonstrated that monosaccharides block the interaction of lymphokines with target cells (Amsden *et al.*, 1978). Based on these findings, it seemed possible, therefore, that Gal NAc was blocking the action of a helper factor, probably secreted by T cells. Table 3.7 presents the results of an experiment that tested this possibility. The experiment consisted of harvesting the supernatant from DNP-HCY-primed spleen cells cultured with antigen for 5 days and adding this supernatant to DNP-HCY primed spleen cells that had been cultured for 2 days with antigen and then depleted of T cells. The data presented in Table 3.7 demonstrates that supernatants of 5-day cultures contained T cell replacing activity i.e. helped B cells to make antibody to a T-dependent antigen in the relative absence of T cells. This activity will henceforth be referred to as T cell replacing factor (TRF). It should be noted that supernatants from cultures without antigen stimulation lacked TRF activity. The TRF activity of this supernatant was completely abrogated by the addition of Gal NAc on day 2 of culture, suggesting that Gal NAc prevents an interaction between TRF and B cells.

TABLE 3.7

INHIBITION OF T-CELL REPLACING ACTIVITY BY N-ACETYL-D-GALACTOSAMINE

TREATMENT	IgG DNP-PFC/CULTURE*
NO TRF	532 ± 59
TRF	11,983 ± 596
TRF + GAL NAC†	336 ± 119

SPLEEN CELLS FROM DNP-HCY PRIMED MICE, CULTURED FOR 2 DAYS WERE TREATED WITH ANTI-THY-1.2 AND COMPLEMENT; AND RECONSTITUTED WITH DAY 5 CULTURE SUPERNATANT (TRF). TRF ABILITY WAS MEASURED AFTER ADDITIONAL 5 DAYS OF CULTURE.

* MEAN AND STANDARD ERROR (N=4)

† SUGAR AT 5 MG/ML

3.4 DISCUSSION

In this chapter, a range of monosaccharides were tested for their ability to inhibit a variety of *in vitro* immune responses. It was found that Gal NAc strongly inhibited the secondary IgG antibody response to both DNP-MON and DNP-HCY but had no effect on a primary antibody response, generation of Tc cells to alloantigens and MLR. This selective inhibition by Gal NAc had some additional unique features:

(a) The sugar inhibited only IgG production and had no effect on either primary or secondary IgM levels. The inhibition studies depicted in Figure 2 also suggest that the initiation of IgG synthesis is independent of IgM shutdown. In addition, both classes of IgG detected in the *in vitro* responses, namely IgG_1 and IgG_{2a} were equally inhibited by Gal NAc.

(b) The secondary IgG responses could be blocked by the presence of Gal NAc over a very short time period. In fact, addition and deletion experiments (Figure 3), as well as the pulsing (Figure 4), show that Gal NAc needs to be present only on day 4 of the response to exert its inhibitory effect. Furthermore, the kinetic studies (Figure 2) indicate that this day coincides with the first appearance of IgG PFC in this system.

(c) Presence of Gal NAc in the T cell-depleted cultures prevented the restoration of a normal IgG response by a culture-derived TRF.

Collectively, the data imply that Gal NAc inhibits the initiation of IgG synthesis. The TRF experiment (Table 3.7) indicates that Gal NAc has no effect on the induction of T helper cells but probably blocks the interaction of TRF for antibody synthesis with its target cell, probably a B cell. However, although unlikely, these experiments do not exclude the possibility that Gal NAc acts directly on the precursors of IgG PFC rather than blocking the interaction of TRF with B cells.

Blocking of interaction between a factor and its target cell is by no means a new concept. The action of migration inhibition factor, macrophage chemotatic factor and neutrophil chemotactic factor can be inhibited by L-fucose and L-rhamnose (Higgins *et al.*, 1978; Amsden *et al.*, 1978). Sugars have also been reported to be able to inhibit the ability of lymphokines to induce skin reactions to an antigen (Baba *et al.*, 1979).

In this report, 3 other sugars were shown to have an effect on *in vitro* immune responses. D-mannose completely inhibited all responses tested and hence was nonspecific in its action. It should be pointed out that this sugar was not toxic for cultured cells but appeared to inhibit their differentiation in response to antigen, (i.e. the number of viable cells in cultures containing D-mannose was the same as in cultures without added antigen). Studies in Chapter 6 suggest that D-mannose affects lymphocyte metabolism.

Fuc and Glc NAc selectively inhibited secondary *in vitro* antibody responses to DNP-HCY. Their occasional inability to inhibit DNP-MON primed cultures is difficult to explain, but may be due to the fact

that mice are frequently preprimed to MON by the bacterial flora of the gut or by natural pathogens such as *Proteus* (Venning, 1974). Therefore it is possible that more primed T_H cells and consequently more TRF is present in these responses and hence the difficulty in inhibiting the responses with Fuc and Glc NAc. Another possibility if that these two sugars are "poorer fit" i.e. they may be of secondary importance in the structure these sugars are mimicking and hence do not always successfully block the binding of TRF to its target cell. In this context it should be noted that these two sugars were usually less effective inhibitors than Gal NAc.

Glc NAc is a close analogue of Gal NAc and hence it is possible that it mimics the action of Gal NAc. However in subsequent experiments Glc NAc proved to be an inconsistent inhibitor i.e. would be significantly inhibitory in only about half of the experiments and hence it was not used further.

Fuc, on the other hand, is structurally quite different from Gal NAc and therefore may have a different mechanism of inhibition. However, daily analysis of antibody inhibition by Fuc (Figure 3.5) indicates that this sugar inhibits secondary antibody response *in vitro* in a manner similar to that exerted by Gal NAc.

Finally, it could be argued that the inhibitory sugars exert nonspecific or osmotic effects on cell cultures. A number of observations make this supposition unlikely:

- the inhibitory sugars, Gal NAc, Glc NAc and Fuc showed specificity for secondary IgG responses *in vitro* only and no other response tested was affected by these 3 sugars

- the noninhibitory sugars were present in cultures at the same concentration as Gal NAc, yet had no apparent osmotic effects.

- Gal NAc exerts its inhibitory effect over a very short period of time, between day 3 and 4 of culture

- genetic analyses of IgG inhibition presented in Chapter 4 demonstrate that the inhibition is under I region control and varies between mouse strains.

Data presented in this chapter are consistent with the hypothesis that carbohydrate-determinants play a role in communication between lymphocytes, a point which will be discussed further in later chapters.

3.5 SUMMARY

Evidence is presented that a number of monosaccharides inhibit secondary IgG responses *in vitro*, but have no effect on primary *in vitro* antibody responses, MLR or the generation of Tc cells. The most striking specific inhibition was produced by Gal NAc. This sugar exerted its inhibitory effect on day 4 of the culture, the day when IgG PFC first appeared. Furthermore, Gal NAc could override the action of a TRF in T-cell-depleted cultures. Collectively, the data indicates that Gal NAc inhibits the initiation of IgG synthesis, possibly by blocking the interaction of a helper factor for IgG synthesis with its target cell.

CHAPTER 4

Inhibition of secondary IgG responses by monosaccharides: Evidence for I-region control

4.1 INTRODUCTION

It is generally accepted that the murine MHC controls glycoproteins whose antigenic determinants reside in their protein portion. Recently, a second series of polymorphic antigens under I-region control have been described that are carbohydrate in nature. The role of Ia antigens has been implicated in many immunological phenomena such as T cell-macrophage interactions (Schwartz *et al.*, 1978; Erb *et al.*, 1980), T cell help (Hodes *et al.*, 1980; Tada and Okumura, 1979) and T cell suppression (Tada and Okumura, 1979; Theze *et al.*, 1977). However, the relative importance of the protein-defined and carbohydrate-defined Ia antigen in these phenomena is unknown.

In Chapter 3 data was presented which showed that some simple sugars selectively inhibit the secondary *in vitro* IgG responses of CBA/H spleen cells, probably by blocking the interaction of a T cell replacing helper factor (TRF) with a target cell, probably a B cell. These results suggest that carbohydrate recognition is involved in initiation of IgG synthesis, simple sugars blocking this interaction by mimicking the carbohydrate structure(s) involved. If carbohydrate-defined Ia antigens are the structures recognised, then, due to the great polymorphism of these antigens (Parish *et al.*, 1982), it might be expected that the IgG response of mouse strains of different I-region haplotype would be inhibited by different sugars.

The experiments presented in this chapter examined sugar inhibition of secondary IgG responses in a range of mouse strains. The inhibitory sugars differed between mouse strains as predicted and these differences were found to map within the I-region of the H-2 complex, specifically to the I-J and I-C subregions. These findings support the

concept that carbohydrate Ia antigens play a role in I-region controlled cell-cell interactions.

4.2 MATERIALS AND METHODS

4.2.1 Animals

All mouse strains used and their H-2 haplotypes are presented in Table 2.1. Only females were used at 6-14 weeks of age.

4.2.2 Antigens

Haemocyanin (HCY) was recrystallized from the haemolymph of crayfish and dinitrophenylated as described in Section 2.2. The conjugation ratio used was $DNP_{26}HCY$.

4.2.3 Sugars

The source of monosaccharides is listed in section 2.8. All sugars were added at the beginning of culture and were used at a final concentration of 5mg/ml in culture medium (∿20mM).

4.2.4 Immunizations

DNP-HCY was made particulate by adsorption to bentonite as described in section 2.3. Mice were given one i.p. injection of this antigen every two weeks for a total of three injections. Each dose contained 200 µg of protein in 0.2ml of phosphage-buffered saline. Animals were used ten or more days after the last injection.

The priming of animals proved difficult at this stage. During the summer and winter months (in 2 successive years), very few animals responded in *in vitro* cultures, despite the three doses of DNP-HCY given *in vivo*. Only 1 out of 3 or 1 in 4 "batches" of animals were primed

and these gave responses only if taken within a fortnight of the last challenge *in vivo*. In spring and autumn, mice were primed more consistently. Administration of antibiotics proved unsuccessful as did the reduction of animals per cage.

We finally solved the problem of priming by keeping and priming the mice in a temperature (25°C), light and humidity-controlled environment, but otherwise treated as described in section 2.3. Under these conditions, the majority of animals gave good responses consistently for up to 3 months after the last challenge (the longest interval tested).

4.2.5 Cell Cultures

Spleen cells were cultured in Linbro 24-well trays for 7 days in EMEM, 5% FCS, 10^{-4}M 2-ME as described in section 2.5. Each well contained 5×10^6 cells in 2.5ml medium and each treatment was cultured in quadriplicate.

4.2.6 Assay of anti-DNP PFC response

Cells secreting anti-DNP antibodies were estimated at the peak of the IgG response (day 7), SRBC were coated with dinitrophenylated anti-SRBC Fab and indirect plaque-forming cells were estimated in the presence of developing serum. For more details of the assay, see section 2.7.

4.3 RESULTS

4.3.1 Analysis of sugar inhibition in different mouse strains

Eight monosaccharides were tested for their ability to inhibit the secondary *in vitro* anti-DNP-HCY IgG responses of a number of different

mouse strains. Table 4.1 presents typical sugar inhibition data for six different mouse strains. Each strain was tested on at least two separate occasions with comparable inhibition data being obtained in each experiment.

It can be seen in Table 4.1 that Gal NAc inhibited all strains tested. However, some sugars exhibited strain-specific inhibition. These differences appeared to be H-2 controlled, since the inhibition patterns were dependent upon the H-2 haplotypes rather than the non-H-2 background of the mouse strains used. For example, B10.BR and B10.D2 cells that share the B10 background but are H-2 incompatible were inhibited by different sugars. Three monosaccharides emerged as H-2 haplotype-specific inhibitors. L-fucose (Fuc) only inhibited the k haplotype (CBA/H, B10.BR) but not the d (Balb/c, B10.D2) or b (C3H.SW, B10) haplotypes, whereas D-galactose (Gal) and N-acetyl-D-mannosamine (Man NAc) inhibited d and b haplotypes but had no inhibitory effect on the k haplotype. $H\text{-}2^{s}$ and $H\text{-}2^{q}$ haplotypes also showed strain specific sugar inhibition pattern identical to the d and b haplotype but since we had no appropriate recombinants within these haplotypes, they were not mapped further.

4.3.2 Sugar inhibition in H-2 recombinant mouse strains

The next series of experiments attempted to map the region(s) in the H-2 complex controlling the differences in sugar inhibition by testing a range of k, b and d haplotype recombinants. The results of these experiments are collated in Table 4.2 and summarized schematically in Figure 4.1.

TABLE 4.1 Ability of Different Monosaccharides to Inhibit the Secondary IgG Response of Different Mouse Strains

Treatment	Mouse Strain					
	CBA/H H-2^k	B10.BR H-2^k	BALB/c H-2^d	B10.D2 H-2^d	C3H.SW H-2^b	B10 H-2^b
Background (no antigen)	5 ± 3	15 ± 12	960 ± 143	0	0	0
Antigen only	14,450 ± 2,147	4,218 ± 295	55,050 ± 4,490	2,165 ± 243	13,390 ± 422	6,375 ± 823
L-fucose	4,705 ± 1,425*	282 ± 53**	52,470 ± 2,152	2,438 ± 126	13,933 ± 1,471	5,525 ± 1,154
D-glucose	12,088 ± 1,803	4,470 ± 580	53,350 ± 3,654	2,378 ± 169	12,588 ± 860	5,075 ± 175
α-methyl-D-glucoside	14,725 ± 528	n.d.	52,960 ± 4,101	2,757 ± 177	15,687 ± 1,504	4,975 ± 396
D-galactose	13,195 ± 517	4,488 ± 491	30,340 ± 2,797*	638 ± 182*	5,133 ± 486**	2,450 ± 589**
N-acetyl-β-D-galactosamine	1,858 ± 86**	418 ± 169**	15,550 ± 955**	278 ± 73**	845 ± 300**	1,725 ± 111**
α-methyl-D-galactoside	15,200 ± 966	3,625 ± 350	48,970 ± 1,744	2,235 ± 71	13,010 ± 1,100	8,200 ± 549
N-acetyl-β-D-mannosamine	11,275 ± 284	3,847 ± 319	30,660 ± 1,712*	340 ± 174*	1,165 ± 596**	2,325 ± 390*
α-methyl-D-mannoside	11,225 ± 632	4,018 ± 167	53,600 ± 2,058	n.d.	10,890 ± 1,417	4,900 ± 613

Results expressed as day 7 anti-DNP IgG PFC/culture ± standard error of four determinations. Significant inhibition indicated by: * ($P < 0.01 > 0.001$) and ** ($P < 0.001$).

TABLE 4.2 Ability of Different Monosaccharides to Inhibit the Secondary IgG Response of H-2 Recombinant Strains of Mice

Treatment	H-2 Recombinant Strain					
	B10.A	B10.A(2R)	B10.A(4R)	C3H.OL	B10.A(3R)	B10.A(5R)
Background (no antigen)	10 ± 13	n.d.	50 ± 29	85 ± 26	15 ± 15	5 ± 3
Antigen only	6,625 ± 235	3,100 ± 248	12,125 ± 1,327	4,826 ± 404	10,800 ± 828	7,100 ± 252
L-fucose	3,650 ± 611*	1,000 ± 235**	13,500 ± 919	5,800 ± 266	11,750 ± 961	3,263 ± 624*
D-glucose	6,663 ± 306	2,875 ± 85	13,175 ± 1,355	5,083 ± 317	10,700 ± 762	7,563 ± 434
α-methyl-D-glucoside	6,300 ± 438	2,700 ± 394	14,075 ± 1,448	4,995 ± 500	11,000 ± 286	7,775 ± 726
D-galactose	3,567 ± 120**	1,575 ± 217*	2,225 ± 293**	2,710 ± 247*	6,125 ± 466**	2,625 ± 583**
N-acetyl-β-D-galactosamine	1,350 ± 1,031**	100 ± 71**	1,975 ± 421**	2,150 ± 224*	1,975 ± 335**	1,650 ± 325**
α-methyl-D-galactoside	7,700 ± 593	3,200 ± 268	10,600 ± 631	4,555 ± 191	n.d.	6,925 ± 518
N-acetyl-β-D-mannosamine	3,400 ± 657*	800 ± 82**	5,175 ± 634*	2,760 ± 115*	3,275 ± 392**	2,150 ± 985**
α-methyl-D-mannoside	7,300 ± 1,108	2,650 ± 166	15,250 ± 1,723	4,840 ± 373	11,100 ± 954	7,113 ± 659

Footnotes as in Table 1

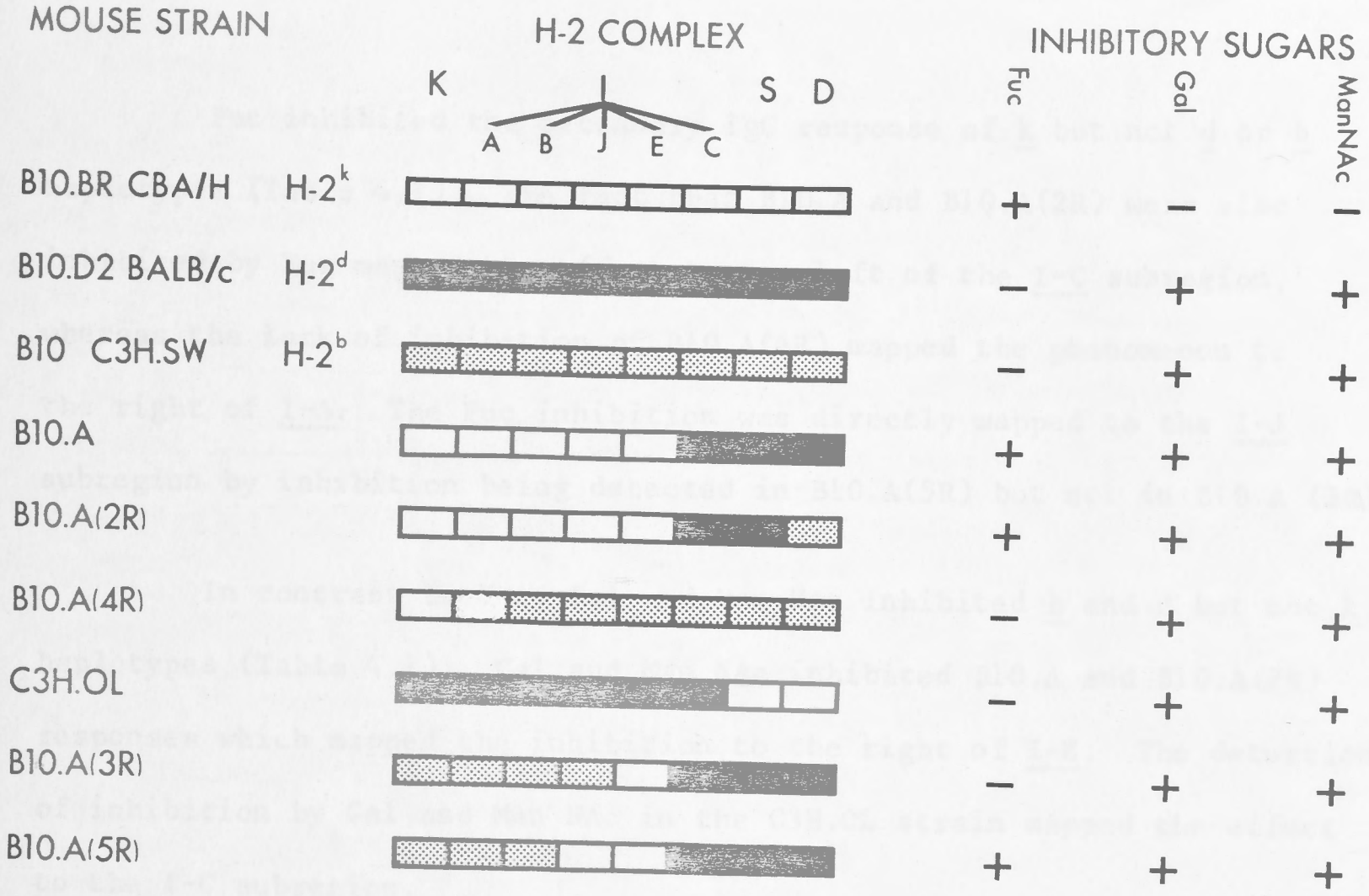


FIGURE 4.1

Summary of the ability of different sugars to inhibit the secondary IgG antibody response in a range of mouse strains

The genetic makeup of the mouse strains is represented by:

□ for the k haplotype

■ for the d haplotype

▩ for the b haplotype

− no inhibition by sugars

+ significant inhibition

Fuc inhibited the secondary IgG response of k but not d or b haplotypes (Table 4.1). The fact that B10.A and B10.A(2R) were also inhibited by Fuc mapped the effect to the left of the I-C subregion, whereas the lack of inhibition of B10.A(4R) mapped the phenomenon to the right of I-A. The Fuc inhibition was directly mapped to the I-J subregion by inhibition being detected in B10.A(5R) but not in B10.A (3R).

In contrast to Fuc, Gal and Man NAc inhibited b and d but not k haplotypes (Table 4.1). Gal and Man NAc inhibited B10.A and B10.A(2R) responses which mapped the inhibition to the right of I-E. The detection of inhibition by Gal and Man NAc in the C3H.OL strain mapped the effect to the I-C subregion.

4.4 DISCUSSION

The sugar inhibition of secondary IgG responses shown in Chapter 3 was further analysed in this chapter and the data presented here shows that the effect is linked to the H-2 complex and maps within the I-region.

Thus Fuc only inhibited responses in cell cultures of k haplotype and this inhibition mapped to the I-J subregion, whereas two other sugars, Man NAc and Gal inhibited d and b haplotype and this effect was shown to be under I-C subregion control.

Gal NAc inhibited the secondary IgG response of all mouse strains tested and hence the genetic region controlling this inhibition could not be mapped. However, there is a close structural similarity between Gal NAc and Man Nac in that they share the N-acetyl-β-D-structure of the sugar while Gal shares the D-galactose portion of Gal NAc. It seems

likely, therefore, that inhibition by Gal NAc, like Man NAc and Gal, is controlled by the I-C subregion.

It appears then, that the I-C subregion controls a carbohydrate structure involved in the interaction of TRF with target cells whose dominant sugar is Gal NAc, since this sugar was the most potent inhibitor of all monosaccharides tested (see Tables 4.1 and 4.2). The different patterns of inhibition seen in d and k haplotypes suggests that the linkage of Gal NAc in the carbohydrate structure differs between mouse strains.

On the other hand, inhibition by Fuc was controlled by a completely different genetic region, namely the I-J subregion. It is possible that the I-J subregion in the d and b haplotypes also controls carbohydrate structures, but presumably inappropriate sugars were used in our experiments to demonstrate this point.

Collectively, these data suggest that the I-J and I-C subregions of the murine MHC control carbohydrate structures involved in the delivery of help for secondary IgG responses. Whether the I-J and I-C subregions control products (possibly glycosyltransferases) which make up a single carbohydrate structure akin to the complementarity described for protein Ia antigens (Jones *et al.*, 1978; Lafuse *et al.*, 1980), or whether these subregions control two separate carbohydrate structures involved in the activation of IgG synthesis requires further investigation.

This is the first time to my knowledge that a helper function has been ascribed to the I-C subregion. The I-J subregion, on the other hand, has been shown to control antigens expressed on helper T cells (Tada *et al.*, 1978) and on antigen-specific helper factors (Howie *et al.*,

1979). More significantly, there is some evidence that both the I-J and I-C subregions control carbohydrate Ia antigens. Howie *et al.* (1979) raised the possibility that the I-J subregion determinants on an antigen-specific helper factor were carbohydrate in nature, whereas Sandrin *et al.*(1981) recently detected I-C subregion controlled carbohydrate antigens on murine B lymphocytes.

4.5 SUMMARY

Certain monosaccharides selectively inhibit secondary IgG responses *in vitro*. Genetic analyses described in this chapter revealed that the inhibitory sugars differed between mouse strains and these differences mapped to the I-J and I-C subregions of the murine MHC. These results imply that interaction between T and B lymphocytes can involve the recognition of I-region controlled carbohydrate structures.

CHAPTER 5

"Further characterisation of a monosaccharide - inhibitable T cell replacing factor"

5.1 INTRODUCTION

In chapter 3, evidence was presented that supernatants from antigen-stimulated primed spleen cells contained T cell-replacing (TRF) activity which was abrogated by the addition of Gal NAc to the cultures. The active component(s) in these supernatants was tentatively assigned the acronym TRF because it was able to restore the antibody response of T cell depleted, DNP-HCY primed, spleen cells when added on the second day of a 6-day incubation period.

This chapter represents an attempt to further characterise some functional and physical properties of this soluble TRF and to elucidate the inhibition of its activity by simple sugars.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Mice were bred and maintained at the J.C.S.M.R. and were used at 5-16 weeks of age.

5.2.2 Antigens

Dinitrophenylated haemocyanin from *Jasus lalandii* and MON from *Salmonella typhimurium* were prepared as described in section 2.2. SRBC were obtained from the same sheep for all experiments and were washed four times with normal saline before use.

5.2.3 Immunizations

Mice were primed to DNP-HCY as described in section 2.3. Briefly, bentonite-bound antigen was injected intraperitoneally three times at two-weekly intervals. Mice were kept in a light, temperature and humidity-controlled room and were used 2-12 weeks

after the last challenge. Mice primed to SRBC were given one dose of 10^8 SRBC (10 µl packed SRBC in 200 µl PBS) intravenously and were used 7-10 days later.

5.2.4 Sugars

The source of sugars is listed in section 2.8. All sugars were added at the beginning of culture unless otherwise indicated, and were used at a final concentration of 5 mg/ml in culture medium (approximately 20mM).

5.2.5 Cell cultures

Spleen cells were cultured in Linbro 24-well trays for 6 or 7 days in EMEM, 5% FCS, 2-ME and NAPs as described in section 2.5. Antigen concentrations were 2 x 10^6 cells/ml for SRBC and 50 ng/ml for DNP-HCY, DNP-MON, HCY and MON.

5.2.6 Preparation of cells

Spleen cells were separated into Ig^- and Ig^+ subpopulations by rosetting the Ig^+ cells and fractionating the rosetting and non-rosetting cells on an Isopaque/Ficoll gradient as described in section 2.6. The Ig^- cells were then cultured as usual.

$Thy\text{-}1^-$ spleen cell populations were generated by treating the spleen cells with monoclonal anti-Thy 1.2 and complement as described in section 2.6. The spleen cells were either treated at the beginning or after two days of culture. The Thy 1.2^- population was subsequently cultured as usual.

Serious problems were encountered with the anti-Thy 1.2 and complement (C') treatment of spleen cell populations, these treated cells at times giving substantial PFC responses in the absence of TRF. In fact, at times these "controls" gave larger PFC responses than complement-treated or untreated cell cultures suggesting a preferential elimination of suppressor T cells. These high background PFC levels occurred despite the fact that anti-Thy-1 treatment reproducibly killed 30-40% of spleen cells. Furthermore, when primed cells from a number of different strains were treated concurrently in an identical manner, some cell cultures had very low background PFC responses whereas others exhibited a high level of activity. Increasing the amount of anti-Thy 1.2 antibodies or the incubation time with antibody and complement had no effect. These differences could be due to a range of factors such as low Thy-1 density on highly primed T cells and bacterial infections contaminating spleen cells with LPS. However, the high background PFC levels often made modest increases in PFC responses due to TRF addition difficult to evaluate.

5.2.7 Detection of PFC

Cells secreting anti-DNP antibodies were assayed after 6 or 7 days of culture on DNP-SRBC monolayers in the presence of complement and/or developing serum. Cells secreting anti-SRBC antibodies were assays on SRBC monolayer. For more details see section 2.7.

5.2.8 Generation of TRF

Generation of TRF either by antigen or Con A-stimulation is described in sections 2.12 and 2.13. Supernatants containing TRF

generated by culturing DNP-HCY-primed cells with antigen were either used immediately or were concentrated ten fold and frozen. Con A generated supernatants (CS) were always concentrated ten fold and frozen. TRF containing supernatants were assayed for activity by adding them to Thy-1$^-$ spleen cells either at the beginning of culture or after 2 days of cultures.

The major problem with TRF generated from antigen-stimulated cultures was its lability. Whereas the day 5 supernatant always contained TRF activity (providing mice were primed), the majority of preparations lost their TRF activity when concentrated and frozen at -60^{0}C. Procedures such as concentration by pressure dialysis rather than over Amicon membranes, freeze drying, freezing unconcentrated or adding FCS to preparations were all unsuccessful in retaining the activity of the supernatants.

5.2.9 Assay for IL-1 and IL-2

An IL-1 containing supernatant was made by stimulating the macrophage cell line PU-5-1.8 with LPSW (section 2.11.2). Supernatants were concentrated 10-fold and stored at -20^{o}C. The supernatants were assayed for their ability to initiate C3H.Hej thymocyte proliferation as described in section 2.16.

Supernatants were assayed for their content of IL-2 on activated T cells in a maintenance assay as described in section 2.15.

5.2.11 gel Filtration of TRF activity

Sephacryl 200 ( ) column containing 270 mls of gel was packed at room temperature at 40 ml/hour with EMEM and 10^{-4}M 2-ME for 24 hours. 3 ml sample of 10-fold concentrated superantant containing TRF activity was applied to the column and filtered with EMEM, 2-ME at 4^{o}C using peristaltic pump at a rate of 40 mls/hour. 100 fractions (50 drops or 3.25 mls/fraction) were collected on fraction collector, individually sterilised with millipore filters and assayed for protein content by coomassie blue method (Rylatt and Parish, 1982) and for TRF activity on T cell depleted primed spleen cells.

5.3 RESULTS

5.3.1 Detection of TRF activity in culture supernatants

Table 3.7 presented in chapter 3 demonstrated that the supernatants from secondary DNP-HCY cultures could restore the anti-DNP PFC response of anti-Thy 1.2 + C' treated, DNP-HCY primed, spleen cells. In the next series of experiments this culture supernatant was concentrated ten-fold by Amicon filtration and then a range of dilutions tested for TRF activity. The two experiments in Table 5.1 demonstrate that the supernatant contained TRF activity over a one hundred-fold concentration range, exhibiting plateau-like activity. It should be noted, however, that the PFC response of TRF containing cultures was at times not as high as intact (i.e. non-anti-Thy 1.2-treated) spleen, e.g. experiment 1 in Table 5.1. Furthermore, many supernatants from DNP-HCY-primed cultures lacked TRF activity after concentration and freezing.

In an attempt to overcome this variability, three different types of culture supernatants were assayed for TRF activity, namely

(i) culture supernatants derived from the fourth and fifth day of incubation from antigen-activated DNP-HCY primed spleen cells, the supernatant being tested for TRF activity immediately after harvesting;

(ii) the above supernatant which was concentrated 10-fold over an Amicon PM10 membrane and stored at -60^{o}C, thawing once only and

(iii) culture supernatants of Con A activated unprimed spleen cells which were 10 x concentrated and stored at -20^{o}C.

Table5.1 Titration of TRF activity in culture supernatants of DNP-HCY primed spleen cells

Treatment of Spleen Cells	TRF (%)	DNP PFC/Culture ± SE	
		IgM	IgG
Experiment 1			
Complement (C') alone	-	1,425±240	37,325±1,848
Anti-Thy 1.2 + C'	-	75±18	550±128
	12.5	43±8	4,345±546
	10	65±25	3,907±336
	5	83±34	4,383±510
	2.5	135±39	5,903±773
Experiment 2			
Complement (C') alone	-	103±22	9,707±276
Anti-Thy 1.2 + C'	-	650±66	4,928±346
	15	115±29	11,746±913
	5	205±67	12,235±2,943
	1.5	393±106	19,564±2,943
	0.15	480±42	14,569±1,643

Anti-Thy 1.2 and complement-treated CBA/H spleen cells were cultured for 7 days with DNP-HCY and varying doses of DNP-HCY-generated, 10x concentrated CBA/H TRF. Results are expressed as means and standard errors of four determinations.

As can be seen from Table 5.2 all three supernatants could reconstitute a PFC response in DNP-HCY primed anti-Thy 1.2 + c' treated spleen cell cultures and, moreover, all three contained TRF activity which could be inhibited by Gal NAc. Interestingly, Con A generated supernatants were consistently more active and retained TRF activity during storage better than antigen-stimulated supernatants.

Additional experiments revealed that supernatants from DNP-HCY primed and stimulated Ig^- spleen cells contained TRF activity (Table 5.3), a result which suggested that the TRF was T cell derived. Furthermore, TRF-containing supernatants could be generated by stimulating DNP-HCY primed spleen cells with HCY alone (Table 5.4).

5.3.2 Properties of TRF

Chapter 4 of this thesis presented evidence that the sugar inhibition of secondary IgG responses to T-dependent antigens was strain-specific and controlled by I-region genes. Thus the response of the $\underline{H\text{-}2}^k$ haplotype was inhibited by Fuc but not by Gal or Man NAc, whereas the response of $\underline{H\text{-}2}^b$ and $\underline{H\text{-}2}^d$ haplotypes was inhibited by Gal and Man NAc but not by Fuc.

Since Gal NAc inhibited TRF activity (Table 5.2) it seemed likely that the sugar inhibition of TRF activity would also be strain specific. As can be seen from Table 5.5, CBA ($\underline{H\text{-}2}^k$)-derived TRF was inhibited by Fuc but not by Gal and Man NAc in CBA spleen cell cultures, whereas C57Bl/6 ($\underline{H\text{-}2}^b$)-derived TRF activity was inhibited by Gal and Man NAc but not by Fuc in C57Bl/6 cultures. Gal NAc inhibited both TRF systems whereas D-glucose had no effect on the PFC responses. Hence the inhibition of TRF activity is analogous

Table 52 Assessment of Different Culture Supernatants for TRF Activity

Expt.	Source of TRF	TRF	IgG DNP PFC/Culture	
			No Sugar	GalNAc
1.	None added	-	532±59	
	DNP-HCY stimulated secondary culture	20% unconcentrated	11,923±596	336±119
2.	None added	-	5,925±486	
	DNP-HCY stimulated secondary cultures	0.5% 10x concentrated	14,000±1,087	5,875±553
3.	None added	-	55±38	
	ConA-activated spleen cells	0.5% 10x concentrated	1,930±332	585±143

Supernatants containing TRF activity were added to anti-Thy 1.2 + C' treated DNP-HCY primed CBA/H splee cells at the beginning of the culture. GalNAc was added on day 0 at 5 mg/ml. DNP PFC responses were assayed after 7 days of culture and expressed as means and standard errors of four determinations.

Table5.3 Evidence that DNP-HCY primed Ig^- spleen cells produce TRF when cultured with antigen

Treatment	IgG DNP PFC/culture
No TRF added	55 ± 38
Ig^- spleen cell derived CBA TRF (0.5%)	500 ± 127
Ig^- spleen cell derived C57Bl/10 TRF (0.6%)	1,210 ± 250

TRF-containing supernatant was generated by culturing Ig^- CBA or C57Bl/10 spleen cells with DNP-HCY for 5 days and 10x concentrated. TRF was added to anti-Thy 1.2 + C' treated DNP-HCY primed BALB/c spleen cells at the beginning of culture. Results are expressed as means and standard errors of day 6 PFC response.

Table5.4 Evidence that HCY alone can induce DNP-HCY primed spleen cells to produce TRF

Concentration of HCY generated TRF added	IgG DNP PFC/Culture
None	173 ± 94
60	1,970 ± 101
30	1,455 ± 192
15	638 ± 82

TRF was generated by culturing DNP-HCY-primed spleen cells with 50 ng/ml of HCY for 5 days. Unconcentrated supernatant was added to anti-Thy 1.2 + C' treated DNP-HCY primed BALB/c spleen cells and cultured for 7 days.

Table 5.5 Ability of different monosaccharides to inhibit the activity of C57Bl/10 (H-2^b) and CBA (H-2^k) derived TRF

Treatment	Sugar (5mg/ml)	IgG DNP-PFC/Culture C57Bl/10 (H-2^b)	CBA (H-2^k)	CBA (H-2^k)
No TRF	-	1,680±346	155±66	675±75
TRF (0.5%)	-	4,087±667	2,760±81	18,210±1,215
"	D-glucose	5,212±301	3,373±291	N.D.
"	L-fucose	4,027±238	1,195±187 (57)	11,110±576 (40)
"	D-galactose	2,053±343 (50)	N.D.	19,200±781
"	GalNAc	1,700±345 (59)	1,187±89 (57)	4,470±273 (75)
"	ManNAc	1,985±174 (52)	2,838±132	17,708±448

Antigen-generated, 10x concentrated supernatant from CBA cultures was added to anti-Thy 1.2 + C'-treated CBA spleen cells and TRF from C57Bl/10 cultures was added to anti-Thy 1.2 + C' treated C57Bl/10 spleen cells at the beginning of the culture. Tesults are expressed as the means and standard errors of day 7 PFC response (n=4). Values in brackets represent % inhibition and PFC responses which represent significant inhibition are enclosed.

to the strain-specific inhibition of IgG responses by sugars in unfractionated spleen cell cultures seen in chapter 4.

Although the sugar inhibition of TRF activity was H-2 haplotype-specific, the delivery of help by TRF was not strain specific. Thus it was found (Table 5.6) that TRF preparations from CBA/H ($H\text{-}2^k$), BALB/C ($H\text{-}2^d$) and C57Bl/6 ($H\text{-}2^b$) mice could readily help CBA/H and BALB/C primed spleen cells to respond to DNP-HCY, indicating that the TRF can act across H-2 differences.

Preliminary experiments also suggest that the TRF lacks antigen specificity. Culture supernatants containing TRF from DNP-HCY primed spleen could help anti-Thy 1.2 + c' treated spleen cells primed to SRBC (Table 5.7) to respond to SRBC, indicating that TRF is not antigen specific, a notion supported by the fact that supernatants from Con-A activated unprimed spleen cells contain TRF activity for anti-DNP-HCY responses (Table 5.2).

Absorbtion experiments also revealed that the TRF activity in the supernatant of DNP-HCY cultures could be absorbed by normal spleen cells at $0^{\circ}C$, but not at $37^{\circ}C$ (Table 5.8), suggesting that the TRF interacts with cell surface structures.

Finally, in an attempt to semi-purify TRF, Con-A generated supernatant was fractionated by gel filtration on a Sephacryl 200 column. The peak of TRF activity, as assayed by anti-DNP PFC responsiveness, was eluted in the void volume of the column indicating that the biologically active TRF molecules have a molecular weight of

Table5.6 Lack of strain specificity in TRF action

TRF source	IgG DNP-PFC/Culture	
	CBA (H-2^k) cells	BALB/c (H-2^d) cells
No TRF added	1,228 ± 253	1,603 ± 220
CBA (H-2^k)	3,595 ± 337	3,285 ± 512
BALB/c (H-2^d)	5,528 ± 198	3,673 ± 263
C57B1/6 (H-2^b)	4,500 ± 551	2,700 ± 07

10x concentrated TRF from DNP-HCY stimulated cultures of 3 mouse strains were added to anti-Thy 1.2 + C' treated, DNP-HCY-primed spleen cells at the beginning of culture. Results are expressed as the means and standard errors of day 6 PFC responses (n=4).

Table5.7 Lack of antigen specificity of TRF

Source of TRF	Anti-SRBC IgG PFC/Culture
No TRF added	5,858 ± 638
CBA (H-2^k)	10,958 ± 873
C57B1/10 (H-2^b)	8,700 ± 564

TRF from DNP-HCY stimulated cultures was added at the beginning of culture with antigen (SRBC) to anti-Thy 1.2 + C' treated CBA/H spleen cells primed to SRBC. Results are expressed as the means and standard errors of day 6 anti SRBC PFC response (n=4).

Table 5.8 Ability of spleen cells to absorb TRF activity

Treatment	IgG DNP-PFC/Culture
No TRF	4,928 ± 346
TRF (0.5%) unabsorbed	15,235 ± 1,083
TRF absorbed at $0^{o}C$	2,664 ± 357
TRF absorbed at $37^{o}C$	16,983 ± 453

TRF-containing supernatant was generated by culturing DNP-HCY primed CBA/H spleen cells with DNP-HCY for 5 days. The 10x concentrated supernatant was added to anti-Thy 1.2 + C' treated DNP-HCY primed CBA/H spleen cells and pfc assayed 7 days later. TRF was absorbed by incubating TRF with 10^{8} normal CBA/H spleen cells for 90 min either on ice or at $37^{o}C$.

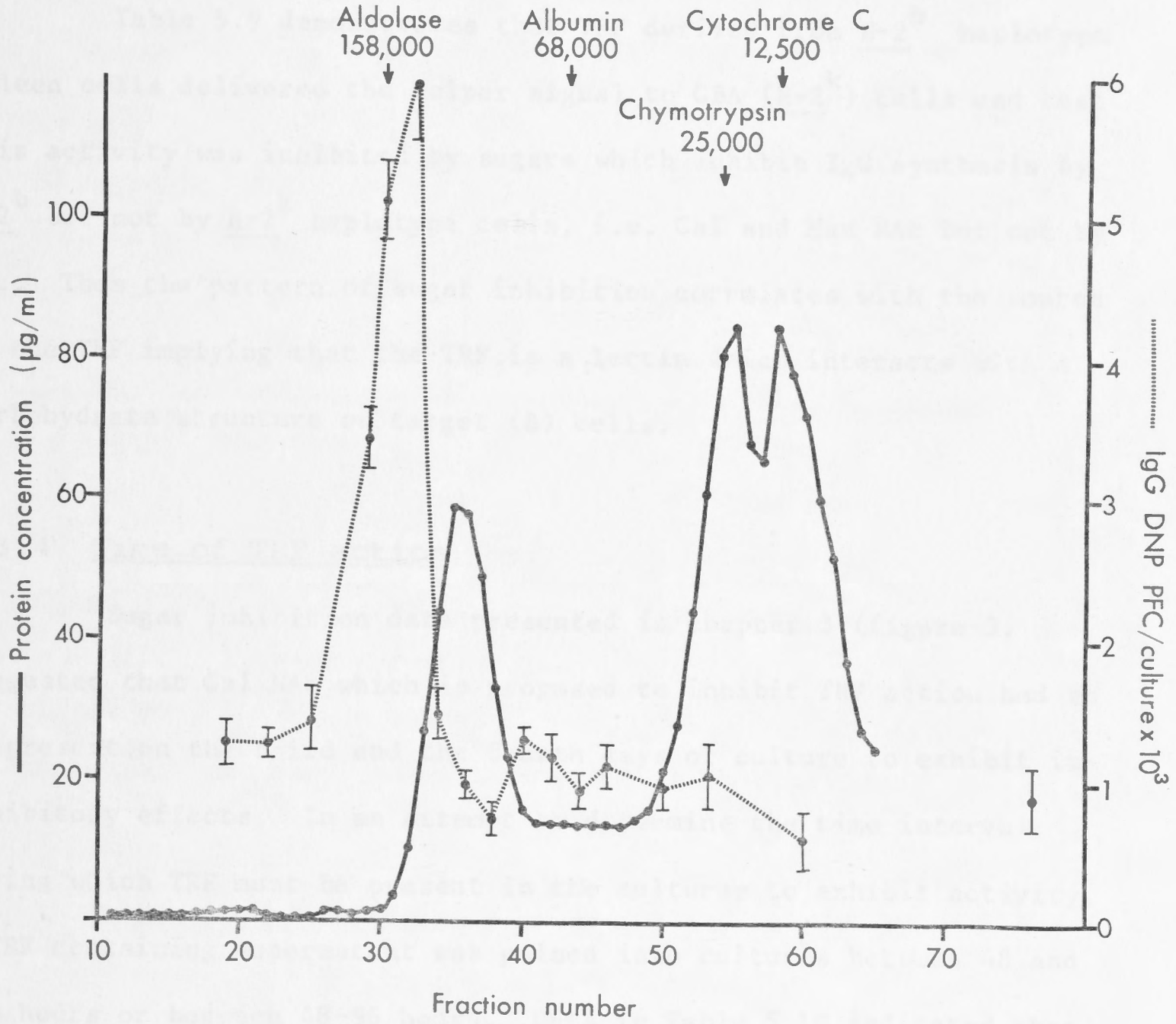
more than 100,000 daltons. In an attempt to avoid aggregation, the column was run in the presence of 2-ME (Figure 5.1). The size of antigen -nonspecific, H-2 unrestricted TRF is reported to be about 30,000 daltons (Hubner *et al*., 1978) and hence is inconsistent with the above result. The differences may be due to aggregation of molecules as a result of the concentration procedure or the use of Sephacryl 200 since it seems unlikely that TRF described here and in the literature are different molecules.

5.3.3 Mode of interaction of TRF with target cells

A hypothesis presented earlier in this thesis proposed that the interaction of TRF with its target cells involved carbohydrate recognition, analogous to a lectin binding an oligosaccharide. Thus, in the presence of the appropriate sugar, the binding of TRF to target cells is blocked and consequently the helper signal aborted.

Since the sugar inhibition of TRF is strain specific (Table 5.5) but the delivery of TRF activity is unrestricted by mouse haplotype (Table 5.6), it is possible to determine whether the TRF molecule is a lectin recognising carbohydrate structures on target cells or whether a lectin on target cells interacts with a carbohydrate moiety on TRF. The experimental protocol was to take H-2^b haplotype TRF and establish which sugars inhibited its activity on H-2^k haplotype cells. If the sugar inhibition correlates with the source of the target cell (i.e. H-2^k haplotype), then the lectin is on the target cells and TRF carries the carbohydrate moiety, whereas if the sugar inhibition correlates with the source of the TRF (i.e. H-2^b haplotype)

FIGURE 5.1



Gel filtration of ConA stimulated supernatant on Sephacryl 200.Fractions were assayed for the total protein content (——) and for TRF activity represented as day 7 anti-DNP IgG PFC response (.....). The migration distance of protein standards are indicated by arrows.The bar on the right represents response in absence of antigen. PFC response is represented as means and standard errors (n=4).

then the TRF is a lectin and the carbohydrate receptor is on the target cells.

Table 5.9 demonstrates that TRF derived from H-2^b haplotype spleen cells delivered the helper signal to CBA (H-2^k) cells and that this activity was inhibited by sugars which inhibit IgG synthesis by H-2^b but not by H-2^k haplotype cells, i.e. Gal and Man NAc but not by Fuc. Thus the pattern of sugar inhibition correlates with the source of the TRF implying that the TRF is a lectin which interacts with a carbohydrate structure on target (B) cells.

5.3.4 Time of TRF action

Sugar inhibition data presented in chapter 3 (figure 3. suggested that Gal NAc which is proposed to inhibit TRF action had to be present on the third and the fourth days of culture to exhibit its inhibitory effects. In an attempt to determine the time interval during which TRF must be present in the cultures to exhibit activity, a TRF containing supernatant was pulsed into cultures between 48 and 144 hours or between 48-96 hours. Data in Table 5.10 indicated that TRF only had to be present during the 48 hour period between the third and the fourth day of the culture to exert substantial helper activity in spleen cell cultures.

A number of observations in the literature also indicate that TRF is more effective when added to cultures on the second day of the culture period which presumably would not allow sufficient time for the TRF-containing supernatants to act indirectly via the activation of T cells. In an attempt to explore this point, primed

Table 5.9 Sugar inhibition of TRF activity is related to source of TRF rather than nature of target cells

Treatment	IgG DNP PFC/Culture	
	B10 TRF (%)	C57B1/6 TRF (0.5%)
None	1,480 ± 114	465 ± 91
TRF only	4,373 ± 406	1,670 ± 219
TRF + D-glucose	4,033 ± 178	1,745 ± 128
TRF + L-fucose	4,273 ± 399	1,527 ± 58
TRF + D-galactose	1,867 ± 345 (57)	855 ± 202 (49)
TRF + GalNAc	1,680 ± 106 (62)	680 ± 85 (60)

B10 TRF was generated by culturing DNP-HCY primed C57B1/10 spleen cells with DNP-HCY for 5 days and concentrating 10-fold. C57B1/6 TRF was a 10x concentrated ConA-generated supernatant from normal C57b1/6 spleen cells. TRF was added at the beginning of culture to anti-Thy 1.2 + C' treated DNP-HCY primed CBA spleen cells. Results are expressed as means and standard errors of day 6 PFC response. Values which represent significant inhibition are enclosed.

Table 5.10 Effect of time of addition of TRF to cultures on antibody responsiveness

Treatment	IgG DNP-PFC/Culture
No TRF	173 ± 94
TRF (20%) days 2-6	1,463 ± 135
TRF (20%) days 2-4	975 ± 66

TRF was generated by culturing DNP-HCY primed CBA/H cells with antigen for 5 days. Unconcentrated supernatant was added to DNP-HCY primed CBA/H spleen cells treated with anti-Thy 1.2 + C' on day 2 of culture. The response, assayed on day 6, is expressed as means and standard errors (n=4).

spleen cells were treated with anti-Thy 1.2 + c' either on day 0 and/or day 2 of the culture and TRF added on day 0 or day 2. The results presented in Table 5.11 indicate that

(i) when T cells are removed at the beginning of the culture, TRF is more active if added on day 2 rather than on day 0 of the culture;

(ii) if T cells are removed on day 0 and then again on day 2, TRF will exert little activity in these cultures;

(iii) however, if T cells are removed after 2 days of culture, the addition of TRF will result in the largest PFC response.

This suggests that for optimal antibody formation to hapten-carrier conjugates, intact T cells are required early and TRF later in the response.

5.3.5 Relationship between IL-1, IL-2 and TRF

Recent reviews suggest that IL-1 and IL-2 (see section 1) are capable of acting as helper factor in antibody responses, probably indirectly by inducing recruitment and proliferation of T_H cells. Since the antigen-generated and Con A-activated supernatants contain both IL-1 and IL-2 besides TRF, it was important to determine whether these molecules could act as helper factors in T cell depleted spleen cell cultures and, more significantly, whether these activities could be inhibited by sugars.

It was shown in Table 5.2 that Con A activated spleen cell supernatants, which contain IL-2 activity, exhibited TRF activity in T cell depleted cultures and this activity was inhibited by Gal NAc. In a similar manner, IL-2 (derived from a macrophage cell line) was

Table 5.11 Effect of time of T cell depletion on TRF activity

Anti-Thy-1.2+C' treatment	TRF addition	DNP-PFC/Culture IgM	DNP-PFC/Culture IgG
day 0	Nil	23±14	38±14
	day 0	38±28	420±0
	day 2	23±14	840±102
day 0 and 2	Nil	8±8	68±39
	day 0	8±8	53±53
	day 2	38±38	128±51
day 2	Nil	520±95	795±192
	day 2	450±121	2,340±270

TRF was a 10x concentrated ConA generated supernatant (0.5%) from BALB/c spleen cells. BALB/c spleen cells primed to DNP-HCY were either treated with anti-Thy 1.2 + C' at the beginning of the culture and/or after 2 days of culture. PFC responses, determined after 7 days of culture, are expressed as means ± standard errors (n=4).

assayed for TRF activity in cultures of T cell depleted C3H.Hej (LPS unresponsive) spleen cells primed to SRBC. As can be seen from Table 5.12 the addition of the IL-1 containing macrophage supernatant could restore PFC responsiveness and the TRF activity was also inhibited by Gal NAc. However, both IL-1 and IL-2 may act by recruiting T cell precursors or activating low Thy- 1.2^{+} T cells and hence could indirectly affect TRF production.

On the basis of the above proposal it was important to determine whether the ability of IL-1 and IL-2 to induce and support T cell proliferation was inhibited by monosaccharides. Table 5.13 reveals that the activity of neither IL-1 or IL-2 was inhibited by any of the sugars tested.

5.4 DISCUSSION

In this chapter, the general properties of a TRF activity that reconstituted secondary IgG responses and was inhibited by simple sugars was examined. The major findings of the chapter can be summarised as follows:

(i) TRF activity which was inhibited by Gal NAc, was not only detected in the culture supernatants of *in vitro* secondary antibody responses but was also found in supernatants of Con-A activated normal spleen cells (Table 5.2)

(ii) the TRF showed the same strain-specific pattern of sugar inhibition as intact spleen cell cultures, namely Fuc inhibiting the action of $\underline{H\text{-}2}^{k}$ haplotype derived TRF in $\underline{H\text{-}2}^{k}$ spleen cell cultures and Gal and Man NAc inhibiting $\underline{H\text{-}2}^{b}$-derived TRF

Table 5.12 Assay for TRF activity in IL-1 preparations

Treatment	anti-SRBC PFC/Culture	
	IgM	IgG
Experiment 1		
No IL-1	53±33	15±15
IL-1 (2%)	1,463±163	5,205±285
Il-1 (2%+GalNAc)	405±108	765±228
Experiment 2		
No Il-1	2,250±468	3,885±586
IL-1 (2%)	5,680±812	12,000±467
IL-1 (2%+GalNAc)	5,005±903	6,610±378

10x concentrated Il-1 containing supernatant was obtained by LPS W stimulation of the macrophage cell line PU-5-1.8. IL-1 was added at the beginning of culture to SRBC-primed anti-Thy 1.2 + C' treated C3H/HeJ spleen cells and cultured for 6 days with SRBC. Results are expressed as means and standard errors of 4 determinations.

Table 5.13 Ability of different monosaccharides to inhibit IL-1 and IL-2 (TCGF) activities

Treatment	CPM	
	IL-1	IL-2
No interleukin added	3,833±279	935±49
No sugar	25,287±2,417	33,726±2,209
D-glucose	40,880±8,062	32,010±2,039
L-fucose	32,766±6,032	32,082±907
G/c NAc	22,049±4,259	31,065±1,288
G/c Met	26,131±1,755	30,512±1,989
D-galactose	27,767±1,640	27,138±3,287
GalNAc	27,747±5,142	34,942±2,443
GalMet	N.D.	33,608±2.014
ManNAc	33,594±3,682	31,962±3,682
ManMet	N.D.	31,718±863

A supernatant containing IL-1 activity was added to C3H.HeJ thymocytes in the presence of ConA and in the presence of sugar (5mg/ml). A supernatant from ConA activated spleen cell cultures (containing IL-2) was added to 3-day old Con A activated B/6 spleen cells together with sugar at 5mg/ml. Results are expressed as CPM of ^{3}H-thymidine incorporated. For further details of the assays see section 2.15 and 2.16.

activity in H-2^b spleen cell cultures (Table 5.5).

(iii) In contrast to the sugar inhibition results, the TRF was not H-2 restricted in its action (Table 5.6). Furthermore, the factor lacked antigen specificity (Table 5.7), was probably secreted by T cells (Tables 5.3 and 5.4) and could be absorbed by normal spleen cells (Table 5.8).

(iv) The TRF acted late in the IgG response, needing only to be present between the third and the fourth days of culture to induce IgG synthesis (Table 5.10). On the other hand, optimal antibody formation required intact T cells early in the response and the addition of TRF later (Table 5.11).

(v) The pattern of sugar inhibition of C57Bl/6 (H-2^b)-derived TRF activity in CBA/H H-2^k cultures demonstrated that the TRF is a lectin which interacts with carbohydrate structures on target cells (Table 5.9).

(vi) Finally, the inability of monosaccharides to inhibit the action of IL-1 and IL-2 on T cells discriminated the TRF from these activities (Table 5.13).

The observation that TRF is H-2 unrestricted in its action, but can be inhibited by sugars in a H-2-specific manner may at first glance be paradoxical. However, the ability of specific sugars to block TRF from different strains suggests that the TRF's (or lectins) differ in their fine specificity of sugar recognition. Thus, as demonstrated in Table 5.5, CBA derived TRF binds Gal NAc and Fuc whereas C57Bl/6 TRF lectin binds Gal NAc, Man NAc and Gal. It should be emphasised however, that Gal NAc was always a strong inhibitor of TRF action, and, therefore, it is likely that carbohydrate acceptor

sites on target cells are sufficiently alike for these lectins to bind in a cross-reactive manner, resulting in TRF activity being H-2 unrestricted. It is possible, however, that if enough mouse strains differing in H-2 were screened, some may have carbohydrate acceptor sites sufficiently different for certain TRF's to be unable to bind. Alternatively, the carbohydrate acceptor on target cell may be a relatively conserved structure and any TRF would be active in all mouse strains. One way to analyse the similarity between the carbohydrate acceptors on cells differing at H-2 would be to titrate TRF from one haplotype, e.g. $H\text{-}2^k$ for its relative ability to reconstitute the PFC responses of syngeneic and allogeneic cells. If the affinity for self-receptors is higher than for non-self, then the TRF should be active to a higher titre in syngeneic than allogeneic cell culture.

What is the nature of the target cell for the TRF described in this chapter? It is not possible to predict this from data presented here, although an absroption on purified B cells rather than on whole spleen (Table 5.8) may provide the answer. As discussed in chapter 1 the proliferating B cell is presumably the immediate target cell of TRF. This conclusion is supported by a number of observations including the fact that TRF preparations produced by a T cell line which secretes TRF but not IL-2 induce long-term cultures of B cells or the B cell reoplastic line BCL_1 to synthesise immunoglobulin (Howard *et al.*, 1981; Pure *et al.*, 1981).

At this point it should be reemphasised that data presented in chapter 4 indicate that the sugar inhibition of TRF is controlled

by genes in the I-region. These observations suggest that the TRF binding of carbohydrate receptors on B cells is I-region controlled and raises the possibility that the TRF molecule is also MHC encoded. These possibilities could be verified experimentally by measuring the ability of anti-Ia sera to either block the absorbtion of TRF to spleen cells or to remove TRF activity from culture supernatants.

Data presented in Tables 5.2 and 5.12 demonstrate that IL-2 and IL-1 containing supernatants can restore the PFC response of T cell depleted spleen cell cultures to DNP-HCY and that these activities, like that of TRF,are inhibited by Gal NAc. However, neither IL-1 activity in the thymocyte proliferation assay, nor IL-2 activity in the maintenance assay was inhibited by any of the sugars tested (Table 5.13). This suggests that IL-1 and IL-2 act by recruiting T_H cells which subsequently secrete TRF and thus indirectly serve as "helper" factors. Some evidence, discussed in chapter 1, suggests that B cells may be the direct target for IL-1 activity, although only in the early stages of activation. The bulk of the evidence indicates that T cells are the more likely target cells for IL-1 and IL-2 (reviewed by Farrar and Hilfiker, 1982).

The ability to inhibit the TRF activity by specific simple sugars and their apparent lack of effect on IL-1 and IL-2 may provide a useful tool for functional distinction between these factors. Moreover, it may be possible to selectively bind and elute TRF from Gal NAc coupled columns, although the recent availability of T cell lines which secrete TRF but not IL-2 (Takatsu *et al.*, 1980; Swain *et al.*, 1981) makes this method for TRF purification less attractive.

Finally, it should be noted that TRF-containing supernatants did not usually totally reconstitute the PFC response of T-cell-depleted spleen cell cultures to normal levels. This incomplete reconstitution is probably due to the requirement of intact T cells (or their antigen-specific products) for early B cell activation (Table 5.11), whereas TRF acts late in the response, supplying the final signal for differentiation of B cells into antibody forming cells. It could be argued that in instances where TRF completely reconstituted the PFC response (Table 5.1) either the supernatant contained sufficient IL-1 and/or IL-2 to recruit enough T cells for early B cell activation or a residue of T_H cells remained after anti-Thy 1.2 + c' treatment and supplied the early activation signals.

5.5 SUMMARY

This chapter describes an analysis of a TRF activity that reconstituted the secondary IgG response of T cell-depleted spleen cell cultures and whose activity was inhibited by Gal NAc. It was found that this TRF activity was present in Con A supernatants from normal spleen cells as well as in supernatants from antigen-activated spleen cell cultures. Although the TRF was not strain-specific in its action it showed the same strain specific pattern of sugar inhibition as intact spleen cell cultures. Furthermore, the TRF lacked antigen specificity, was probably secreted by T cells, could be absorbed by normal spleen cells and acted late in IgG induction.

Evidence is also presented which indicates that the TRF molecule is a lectin which interacts with a carbohydrate receptor on target cells. The inability of sugars to inhibit IL-1 and IL-2 activity also indicates that the TRF is distinct from these activities.

CHAPTER 6

Analysis of inhibition of immune responses by D-mannose

6.1 INTRODUCTION

In chapter 3 of this thesis data was presented which demonstrated that a number of *in vitro* immune responses could be completely inhibited by the addition of D-mannose to the cultures. Unlike Gal NAc and Fuc, D-mannose inhibited all *in vitro* immune responses examined, namely primary and secondary antibody response to T-dependent antigens, the generation of primary Tc cells to alloantigens and proliferative responses to alloantigens (MLR). This chapter further analyses the inhibition of immune responses by D-mannose and presents evidence that D-mannose inhibits lymphocyte proliferation, possibly by interfering with cellular metabolism.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Mice of both sexes were used at 6-16 weeks of age. The haplotypes of the various strains used are defined in Table 2.1.

6.2.2 Antigens

MON was prepared from the flagella of *Salmonella typhimurium* SL870, and HCY was recrystallised from the haemolymph of *Jasus lalandii*. The description of these preparative methods and the dinitrophenylation of these proteins is described in more detail in section 2.2. TNP was coupled to LPS (E. coli 055:135 Difco Lab.) according to the method described by Quintas and Lefkovits (1976). DNP was coupled to Ficoll (Pharmacia, Sweden) and polyacrylamide Bio-Gel P10 beads as described by Inman (1975).

6.2.3 Sugars

The sugars used and their source were as follows: D-mannose N-acetyl-D-mannosamine (ManNAc), D-mannitol and mannan from Sigma, St. Louis, Miss. and α-methyl-mannoside from Calbiochem, San Diego, Ca.

6.2.4 Immunisations

Mice were primed to DNP-MON and DNP-HCY as described in section 2.5. Briefly, the antigen was bound onto bentonite and injected i.p. three times at 2-weekly intervals. Animals were used 2-10 weeks after the last challenge.

6.2.5 *In vitro* antibody responses

The *in vitro* culturing procedures are described in section 2.5. Briefly, spleen cells were cultured with EMEM, 5% FCS, 2-ME (10^{-4}M) and NAPs at a concentration of 2×10^6 cells/ml in Linbro trays in a 10% CO_2, 7% O_2, air mixture. Primary antibody responses were cultured for 3 days and secondary responses for 7 days. Anti-DNP-PFC were assayed using SRBC coated with DNP (see section 2.7).

6.2.6 *In vitro* primary cytotoxic T cell responses

Tc cells were generated by mixing 5×10^6 CBA responder spleen cells with 1.25×10^6 gamma-irradiated Balb/c stimulator spleen cells. The cultures were harvested after 5 days and Tc cells specific for $H\text{-}2^d$ antigens assayed against ^{51}Cr-labelled P815 mastocytoma target cells. Results are expressed as % ^{51}Cr release (for details see section 2.10).

6.2.7 Cell lines

The fibroblasts either from a rat or a mouse were cultured in Autopow supplemented with L-glutamine and buffered with IM tris as described in section 2.11.3. P815, EL-4, NS-1 and WEHI-3 cell lines were grown in Falcon flasks in DMEM and 10% HIFCS in a 10% CO_2, 7% O_2 atmosphere. These cell lines were maintained in the John Curtin School of Medical Research, either in the Department of Microbiology or the Department of Immunology.

6.3 RESULTS

6.3.1 Ability of D-mannose to inhibit a range of *in vitro* immune responses

Amongst the data presented in chapter 3 was the observation that D-mannose inhibited *in vitro* primary and secondary antibody responses to the hapten-carrier conjugates DNP-MON and DNP-HCY, *in vitro* proliferative responses to alloantigens and the *in vitro* generation of primary Tc cells to alloantigens. In addition, data presented in Table 6.1 demonstrates that D-mannose inhibited responses of unprimed spleen cells to three different T-cell-independent antigens, namely DNP-Ficoll, DNP-polyacrylamide beads and TNP-LPS. However, while D-mannose inhibited antibody responses to T-cell dependent antigens and responses to alloantigens to background levels (see chapter 3), the responses to T-independent antigens were inhibited only partially (50-80%) despite the use of the same sugar concentration (Table 6.1).

Additional experiments revealed that the inhibitory effect of D-mannose was concentration-dependent. As can be seen in Figure 6.1 significant inhibition of a primary IgM response and the generation of Tc cells to alloantigens was still seen at a concentration of

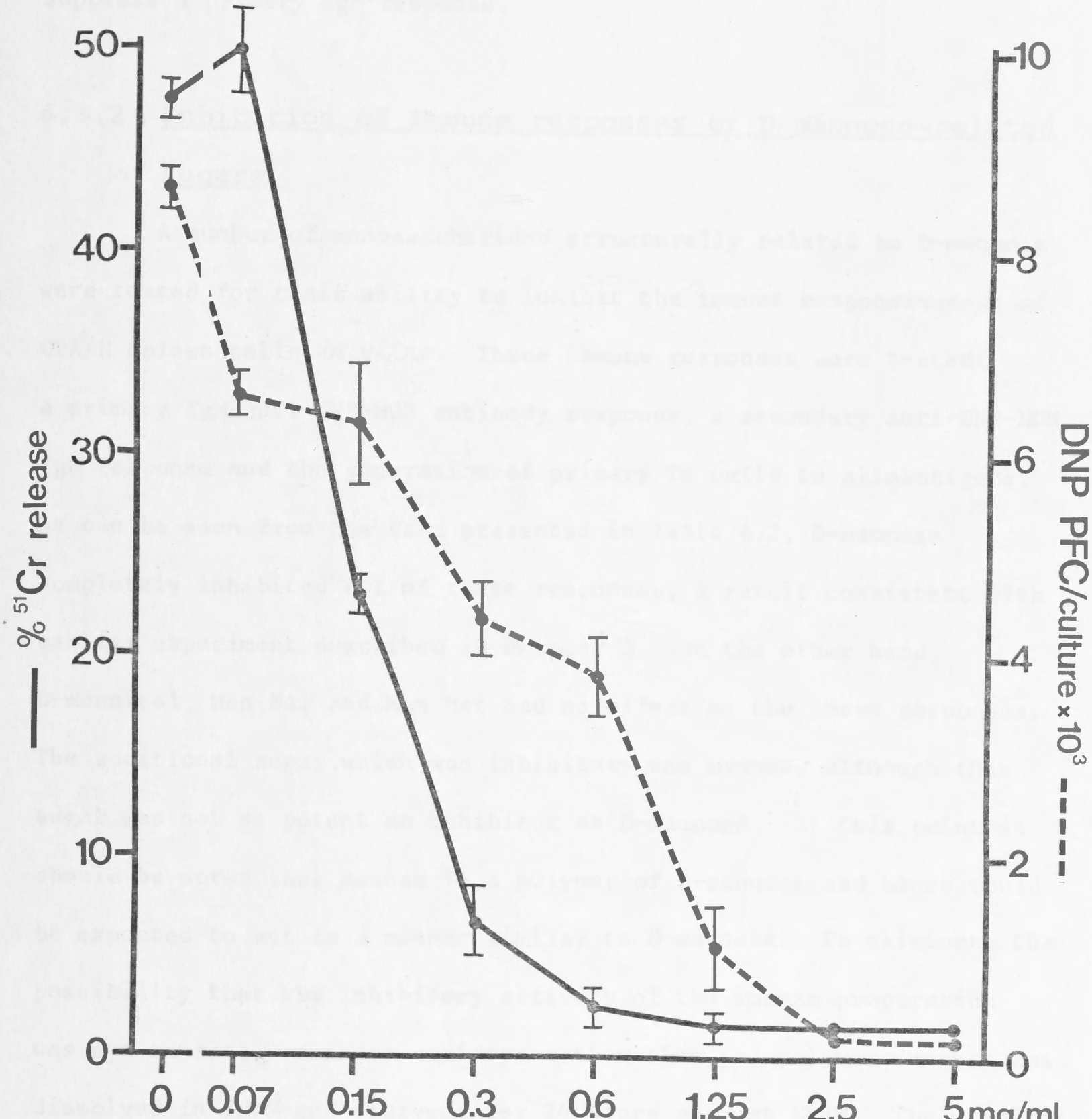
Table 6.1

Inhibition of in vitro T-cell independent antibody responses by D-mannose

Antigen	DNP PFC/Culture		(% Inhibition)
	No sugar	D-mannose	
DNP-Ficoll	1,163 ± 45	220 ± 36	81
TNP-LPS	1,648 ± 177	768 ± 18	53
DNP-beads	948 ± 70	460 ± 61	52

D-mannose (5mg/ml) was present throughout culture. Anti-DNP-PFC were assayed after 3 days of culture. Results are expressed as means and standard errors of four determinations. The response of control cultures to which no antigen was added was 50 ± 5 PFC/culture.

FIGURE 6.1



Ability of different concentrations of D-mannose to inhibit the day 3 anti–DNP–MON antibody response and the day 5 generation of Tc cells in vitro
Vertical bars are standard errors of means (n=4)

0.15 mg/ml (∿ 0.6mM). Figure 6.1 also shows that complete inhibition of the Tc cell response required only 2.5mM D-mannose whereas a higher concentration (10mM) of the monosaccharide was needed to completely suppress a primary IgM response.

6.3.2 Inhibition of immune responses by D-mannose-related sugars

A number of monosaccharides structurally related to D-mannose were tested for their ability to inhibit the immune responsiveness of CBA/H spleen cells *in vitro*. Three immune responses were tested: a primary IgM anti-DNP-MON antibody response, a secondary anti-DNP-MON IgG response and the generation of primary Tc cells to alloantigens. As can be seen from the data presented in Table 6.2, D-mannose completely inhibited all of these responses, a result consistent with earlier experiment described in chapter 3. On the other hand, D-mannitol, Man NAc and Man Met had no effect on the three responses. The additional sugar which was inhibitory was mannan, although this sugar was not as potent an inhibitor as D-mannose. At this point it should be noted that mannan is a polymer of D-mannose and hence would be expected to act in a manner similar to D-mannose. To eliminate the possibility that the inhibitory activity of the mannan preparation was due to free D-mannose residues rather than the polymer, mannan was dissolved in EMEM and dialysed for 24 hours against EMEM. The resulting solution was equally inhibitory as undialysed mannan (Table 6.2), indicating that the polymer was responsible for the inhibition.

6.3.3 Strain specificity of D-mannose inhibition

Since the inhibition of secondary antibody responses by Fuc and Gal NAc-related sugars was highly strain-dependent (chapter 4), it was

TABLE 6.2

INHIBITION OF IMMUNE RESPONSES BY D-MANNOSE AND D-MANNOSE-RELATED SUGARS

TREATMENT	ANTIBODY RESPONSE IN VITRO (PFC/CULTURE)		CYTOTOXIC RESPONSE IN VITRO
	PRIMARY ANTI-DNP-MON IgM PFC/CULTURE[b]	SECONDARY ANTI-DNP-MON IgG PFC/CULTURE[c]	% ^{51}Cr RELEASE/CULTURE[d]
NO ANTIGEN	18 ± 12	13 ± 9	ND
ANTIGEN ONLY	1,513 ± 99	3,875 ± 75	47.5 ± 2.4
D-MANNOSE	8 ± 8	15 ± 6	1.1 ± 0.2
MANNAN	363 ± 118	337 ± 26	25.7 ± 0.9
DIALYSED MANNAN[a]	320 ± 51	ND	ND
D-MANNITOL	1,493 ± 84	4,025 ± 293	48.0 ± 1.7
MAN NAc	1,413 ± 74	3,900 ± 71	39.8 ± 0.6
MAN MET	1,453 ± 97	3,780 ± 178	45.7 ± 0.8

Legends to Table 6.2

[a]Mannan was dialysed against EMEM for 24 hours to remove contaminating D-mannose residues.

[b]Primary anti-DNP-MON IgM response of CBA/H spleen cells was assayed after 3 days of culture (CBA/H).

[c]Secondary anti-DNP-MON IgG response of CBA/H spleen cells was assayed after 7 days of culture. IgM response was <100 PFC.

[d]Ability of primary Tc cells (generated in CBA/H anti-BALB/c MLR) to lyse ^{51}Cr labelled P815 targets was assayed after 5 days of culture.

Sugars were present throughout the culture period at 5mg/ml. Results are expressed as mean and standard errors of four determinations.

necessary to determine whether D-mannose exhibited any strain specificity in its inhibition. In order to test this possibility an *in vitro* secondary anti-DNP-MON antibody response was examined. Data presented in Table 6.3 demonstrated that D-mannose inhibited the generation of anti-DNP-PFC in four mouse strains differing in both their H-2 haplotypes and background genes. Hence the antibody responses of B10.BR ($H\text{-}2^k$), C57B1/6 ($H\text{-}2^b$), Balb/c ($H\text{-}2^d$), and SJL ($H\text{-}2^s$) spleen cells were inhibited to background levels when the cells were cultured with D-mannose for 7 days. A similar lack of strain specificity in D-mannose inhibition was observed when a primary IgM antibody response to DNP-MON was examined.

6.3.4 Timing of D-mannose inhibition

In the experiments described so far, D-mannose was present throughout the culture period. It was important, therefore, to show the time in the response when D-mannose exerted its inhibitory effect. To determine this, the sugar was added and deleted from culture wells each day either for 7 days in the case of the IgG response to DNP-HCY or for 5 days in the generation of Tc cells to alloantigens. Figure 6.2 reveals that in the generation of Tc cells, the majority of D-mannose inhibition occurred on the second, third and fourth day of culture, no significant inhibition being observed when D-mannose was present during only the first 24 hours of culture. For the secondary IgG response, the adding and deleting data indicates that D-mannose exhibited its inhibitory activity on the third day of culture.

The data presented in Figure 6.2 suggests that D-mannose has no effect on the induction of immune responses if only present during the first 24 hours of the culture. However, in a number of experiments

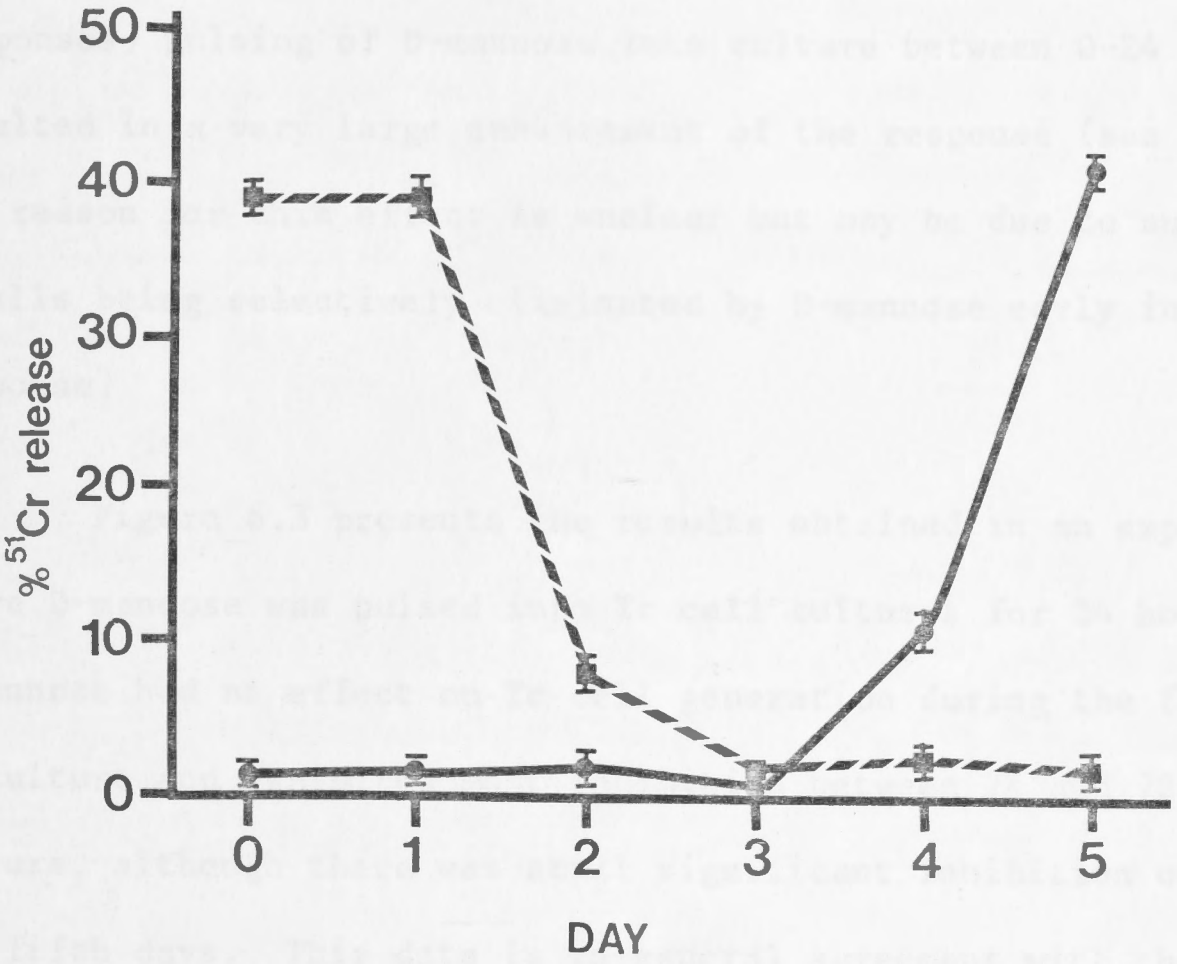
TABLE 6.3

LACK OF STRAIN SPECIFICITY OF D-MANNOSE INHIBITION IN SECONDARY ANTI-DNP-MON ANTIBODY RESPONSES IN VITRO

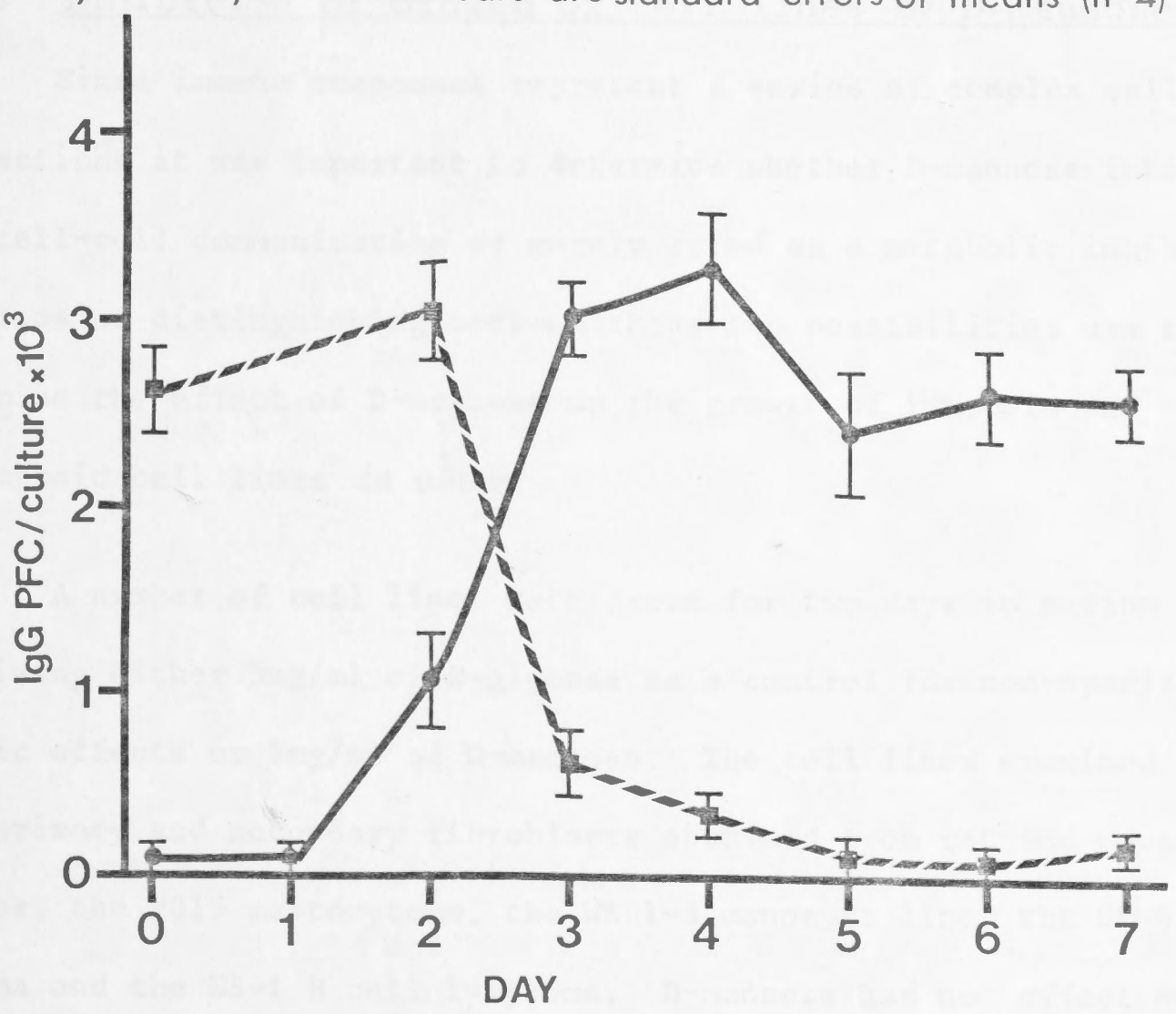
MOUSE STRAIN	HAPLOTYPE	ANTI-DNP-PFC/CULTURE	
		NO SUGAR	D-MANNOSE (5MG/ML)
B10.BR	k	2,517 ± 172	14 ± 8
C57B/6	b	2,928 ± 243	57 ± 20
BALB/C	d	5,400 ± 665	15 ± 6
SJL	s	2,150 ± 225	8 ± 5

PFC RESPONSE WAS ASSAYED AFTER 7 DAYS OF CULTURE. RESULTS ARE EXPRESSED AS MEANS AND STANDARD ERRORS OF FOUR DETERMINATIONS.

FIGURE 6.2



Influence of the time of addition and deletion of D-mannose on the generation of Tc cells in vitro The sugar was either added (▌) or deleted (▚) on each day of culture and all cultures assayed for Tc activity on day 5 Vertical bars are standard errors of means (n=4)



Influence of the time of addition and deletion of D-mannose on a secondary anti—DNPP PFC DNP MON antibody response in vitro The sugar was either added (▌) or deleted (▚) on each day of culture and all cultures assayed for anti—DNP PFC on day 7.

(two out of five for IgG responses and one out of three Tc cell responses) pulsing of D-mannose into culture between 0-24 hours resulted in a very large enhancement of the response (see Table 6.4). The reason for this effect is unclear but may be due to suppressor T cells being selectively eliminated by D-mannose early in the response.

Figure 6.3 presents the results obtained in an experiment where D-mannose was pulsed into Tc cell cultures for 24 hour periods. D-mannose had no effect on Tc cell generation during the first 24 hours of culture and exhibited most inhibition between 24 and 72 hours of culture, although there was still significant inhibition on the fourth and fifth days. This data is in general agreement with the addition and deletion experiment depicted in Figure 6.2.

6.3.5 Inhibition of growth of cell lines by D-mannose

Since immune responses represent a series of complex cell-cell interactions it was important to determine whether D-mannose interfered with cell-cell communication or merely acted as a metabolic inhibitor. One means of distinguishing between these two possibilities was to determine the effect of D-mannose on the growth of lymphoid and nonlymphoid cell lines *in vitro*.

A number of cell lines were grown for two days in medium containing either 5mg/ml of D-glucose as a control for non-specific osmotic effects or 5mg/ml of D-mannose. The cell lines examined were primary and secondary fibroblasts obtained from rat and mouse embryos, the P815 mastocytoma, the WEH1-3 monocyte line, the EL-4 thymoma and the NS-1 B cell lymphoma. D-mannose had no effect on the

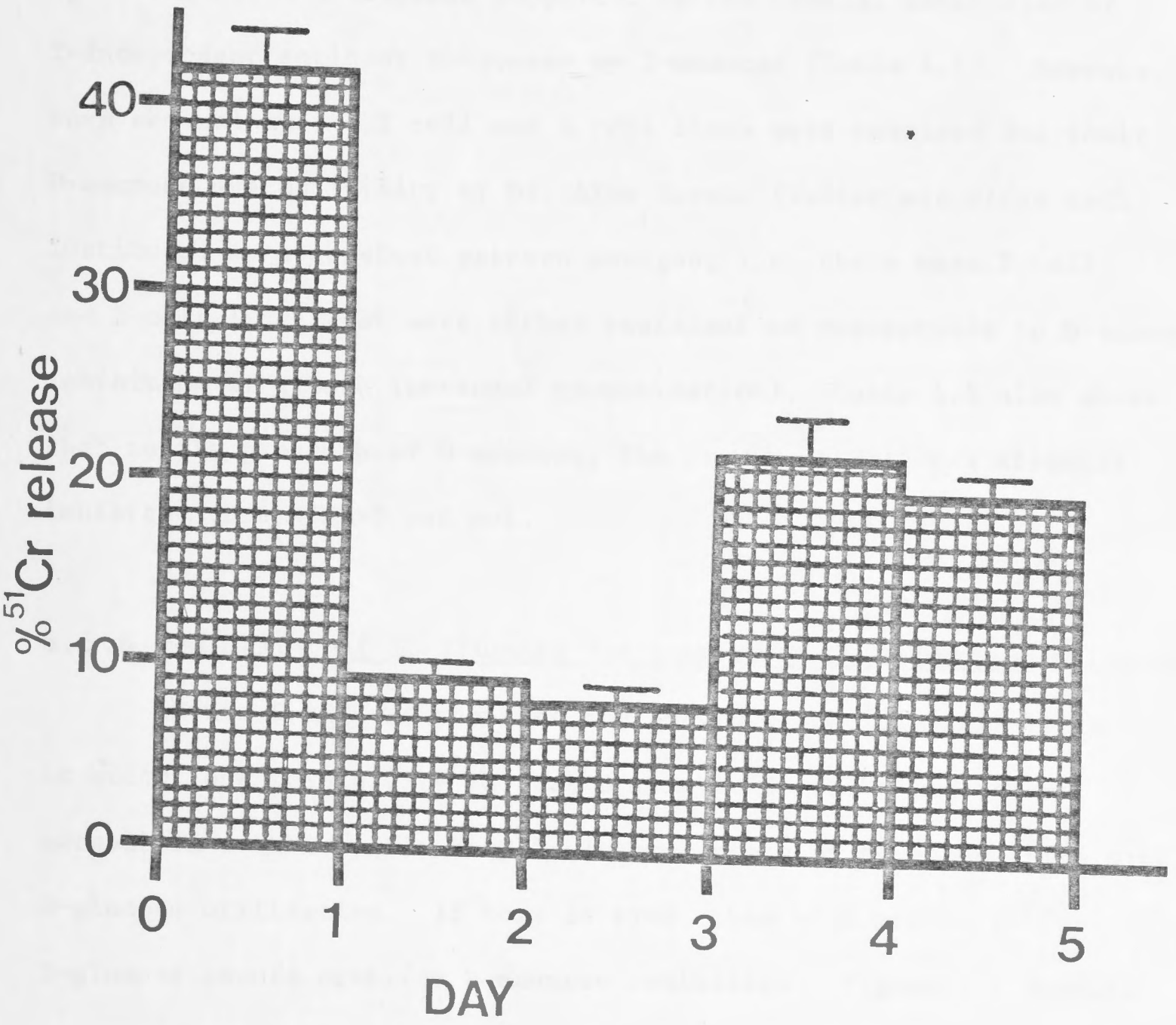
TABLE 6.4

ENHANCEMENT OF IMMUNE RESPONSE IN VITRO BY A 24 HOUR PULSE OF D-MANNOSE

ANTIBODY RESPONSE IgG DNP PFC/CULTURE		CYTOTOXIC T CELL RESPONSE % ^{51}Cr RELEASE	
NO SUGAR	D-MANNOSE	NO SUGAR	D-MANNOSE
2,600 ± 92	14,168 ± 1,478	57.1 ± 1.0	88.8 ± 4.4
24,155 ± 1,106	90,325 ± 2,063	59.2 ± 0.8	56.3 ± 3.2
3,375 + 504	6,250 ± 755	47.6 ± 1.2	49.8 ± 2.1
2,608 ± 270	3,065 ± 250	38.5 ± 1.0	39.2 ± 0.7
15,465 ± 1,100	13,600 ± 943		

D-MANNOSE (5MG/ML) WAS PULSED INTO CULTURES BETWEEN 0-24 HOURS. IgG PFC WAS DETERMINED AFTER 7 DAYS OF CULTURE, % ^{51}Cr RELEASE AFTER 5 DAYS OF CULTURE. RESULTS ARE EXPRESSED AS MEANS AND STANDARD ERRORS OF FOUR DETERMINATIONS.

FIGURE 6.3



Effect of pulsing cultures for 24—h periods with D-mannose on the generation of Tc cells in vitro Vertical bars are standard errors of means (n=4) on day 5.

growth of any fibroblasts. In contrast, variable effects were obtained with lymphoid cell lines, the growth of the thymoma EL-4 being completely inhibited by D-mannose whereas the B cell line NS-1 being barely affected (Table 6.5). At face value, this result suggests a selective inhibition of T-lymphocyte, rather than B lymphocyte growth by D-mannose, a conclusion supported by the partial inhibition of T-independent antibody responses by D-mannose (Table 6.1). However, when several murine T cell and B cell lines were examined for their D-mannose susceptibility by Dr. Alan Harris (Walter and Eliza Hall Institute) no consistent pattern emerged, i.e. there were T cell and B cell lines that were either resistant or susceptible to D-mannose inhibition of growth (personal communication). Table 6.5 also shows that in the presence of D-mannose, the growth of P815 was strongly inhibited, but WEHI-3 was not.

6.3.6 Ability of D-glucose to override D-mannose inhibition

Since D-mannose inhibits the growth of certain cell lines *in vitro*, one possibility is that D-mannose perturbs D-glucose metabolism either by blocking D-glucose uptake or by interfering with D-glucose utilisation. If this is true, then high concentrations of D-glucose should override D-mannose inhibition. Figure 6.4 depicts an experiment where cells were cultured with decreasing concentrations of D-mannose in the presence of D-glucose (5mg/ml) or in the presence of α-menthyl-mannoside (5gm/ml). It can be seen that D-mannose was, in terms of concentration, a 4-8 fold less effective inhibitor when mixed with D-glucose than when added alone. Thus D-glucose overrides D-mannose inhibition, whereas α-methyl-mannoside had no effect.

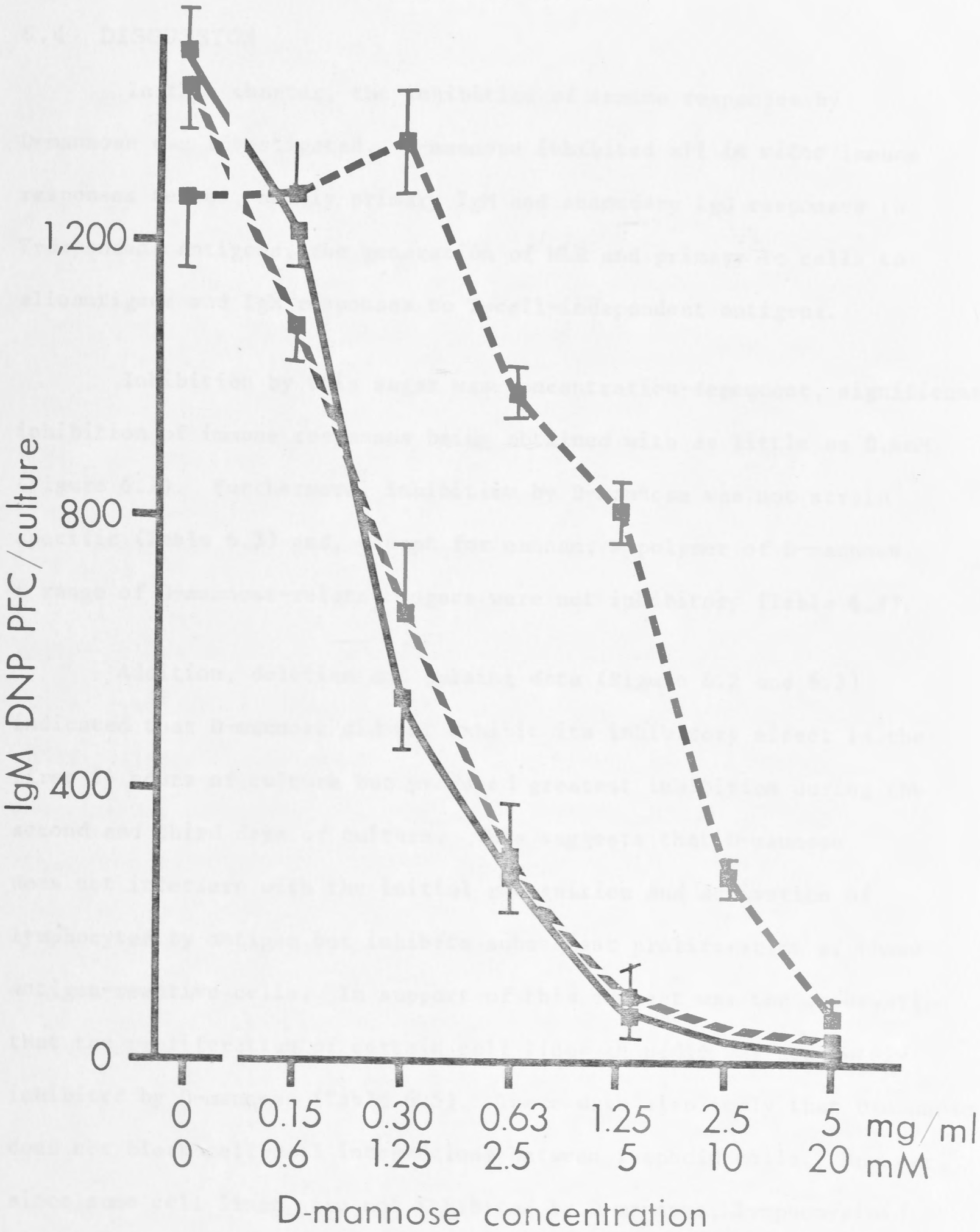
Table 6.5

Ability of D-mannose to inhibit the growth of cell lines

Cell Line	Cell Number/Culture		
	D-Glucose	D-mannose	Starting inoculum
Primary rat fibroblasts	$5x10^8$	$5.2x10^8$	$5x10^5$
Secondary mouse fibroblasts	$1.9x10^8$	$1.8x10^8$	$5x10^5$
P815 (mastocytoma)	$1.2x10^7$	$3.3x10^5$	$5x10^5$
WEHI-3 (monocyte)	$6x10^6$	$3x10^6$	$1x10^5$
EL-4 (thymoma)	$3x10^6$	$1x10^5$	$1x10^5$
NS-1 (B cell lymphoma)	$6x10^5$	$3.6x10^5$	$1x10^5$

Cell lines were seeded into small flasks in the presence of glucose or mannose (5mg/ml) and allowed to grow for 48 hours. Results are expressed as the mean cell number per flasks based on four determinations.

FIGURE 6.4



Inhibition of primary anti-DNP-MON antibody response by D-mannose (—). D-mannose in the presence of D-glucose (5mg/ml) (- - -) and D-mannose in presence of the methyl mannoside (5mg/ml) (— —). PFC response was assayed after 3 days of culture. Vertical bars are standard errors of means (n=4)

6.4 DISCUSSION

In this chapter, the inhibition of immune responses by D-mannose was investigated. D-mannose inhibited all *in vitro* immune responses tested, namely primary IgM and secondary IgG responses to T-dependent antigens, the generation of MLR and primary Tc cells to alloantigens and IgM responses to T-cell-independent antigens.

Inhibition by this sugar was concentration-dependent, significant inhibition of immune responses being obtained with as little as 0.6mM (Figure 6.1). Furthermore, inhibition by D-mannose was not strain specific (Table 6.3) and, except for mannan, a polymer of D-mannose, a range of D-mannose-related sugars were not inhibitory (Table 6.2).

Addition, deletion and pulsing data (Figure 6.2 and 6.3) indicated that D-mannose did not exhibit its inhibitory effect in the first 24 hours of culture but produced greatest inhibition during the second and third days of culture. This suggests that D-mannose does not interfere with the initial recognition and activation of lymphocytes by antigen but inhibits subsequent proliferation of these antigen-reactive cells. In support of this concept was the observation that the proliferation of certain cell lines *in vitro* was completely inhibited by D-mannose (Table 6.5). These data also imply that D-mannose does not block cell-cell interactions between lymphoid cells. However, since some cell lines are not inhibited by D-mannose, lymphomyeloid cells must be heterogeneous in their response to D-mannose.

The findings presented here indicate that D-mannose in some way perturbs the metabolism of lymphoid cells. This notion is further supported by the observation that the inhibitory activity of D-mannose

was overridden by D-glucose (Figure 6.4). An interesting feature of this experiment is that D-glucose needed to be in a substantial molar excess to overcome D-mannose inhibition, i.e. D-mannose still completely inhibited immune responses when an equimolar concentration of D-glucose was present (Figure 6.4).

Thus D-mannose probably interferes with the metabolism of D-glucose by cells. There are two possible ways in which this could occur. First, D-mannose could bind and block the D-glucose carrier on the surface of cells. This seems unlikely, since D-mannose is readily transported into rat thymocytes (Hume *et al.*, 1981) hamster cells (Dahl and Morse, 1979) and human cells (Ganda *et al.*, 1979) by the D-glucose carrier. It is possible, however, that mannan inhibits immune responses by blocking D-glucose uptake, although there is the additional possibility that mannan is hydrolysed to D-mannose by enzymes released by cells analogous to the cleavage of maltose by disaccharidases released by rat thymocytes (Hume *et al.*, 1981).

Secondly, D-mannose may interfere with cell metabolism. Mannose is phosphorylated by mannokinase and then converted to glucose-6-phosphate by an isomerase (Slein, 1950). This possibility could be tested by comparing the metabolism of D-mannose and D-glucose in cell lines that are either susceptible or resistant to D-mannose inhibition. This was, in fact, done by Dahl and Morse (1979) who compared two chinese hamster cell lines: CHO-K1 derived from ovary and resistant to D-mannose inhibition and V79 from foetal lung whose growth was inhibited by D-mannose. The authors found no difference in the uptake of D-mannose by the two cell lines, no defect in the pathway of energy

metabolism either using D-mannose as substrate or in the presence of D-glucose and no defect in DNA synthesis. However, V79 was not able to hydrolyse complex D-mannose-containing polysaccharides due to a lack of the appropriate mannosidases and cell death probably resulted from accumulation of these polysaccharides inside the cell. Whether this explains the inhibitory effect of D-mannose on lymphocyte proliferation remains to be determined.

Nevertheless, it is somewhat surprising that D-mannose inhibits the proliferation of lymphomyeloid cells since human cells of brain, adipose tissue, liver and pancreas utilise D-mannose as an energy source as readily as D-glucose. However, free D-mannose would not be normally available to these cells since it is poorly transported across the intestine (Ganda *et al.*, 1979). Nonetheless, D-mannose appears to exert a profound effect on lymphomyeloid proliferation in the mouse giving rise to two possible uses: firstly, it may act as a selective immunosuppressive agent and secondly, it may have a use in chemotherapy of tumors since it affects a number of cell lines and has been reported to have an antitumor effect in mice when administered orally or by intraperitoneal inoculation (Gonzales and Amos, 1977).

The literature contains several other reports of the inhibitory effects of D-mannose and D-mannose-related sugars on cellular processes. Thus D-mannose blocked the binding of glycoproteins by alveolar macrophages (Stahl *et al.*, 1978), mannose-6-P inhibited the uptake of lysosomal enzymes into human fibroblasts (Kaplan *et al.*, 1977), D-mannose blocked the cytotoxicity of natural killer (NK) cells against solid tumors in mice (Stutman *et al.*, 1980) and mannose-6-P inhibited human NK cell activity *in vitro* (Forbes *et al.*, 1981). These phenomena

are unlikely to be related to the inhibition of immune responses reported here since they appear to deal with the blocking of recognition structures on the surfaces of cells rather than inhibiting cell metabolism. However, Muchmore *et al*, (1980) have also reported that D-mannose inhibits *in vitro* immune responses although the significance of these observations was not discussed.

6.5 SUMMARY

Data presented in this chapter and chapter 3 indicates that D-mannose inhibits a variety of immune responses *in vitro*, such as primary and secondary antibody responses to T cell dependent antigens, primary antibody responses to T cell-independent antigens, MLR and generation of Tc cells to alloantigens. D-mannose also inhibited the growth of some lymphomyeloid cell lines and lacked strain specificity in its inhibition. These results combined with the timing of D-mannose inhibition and the ability of D-glucose to overcome the effects of D-mannose indicated that unlike Gal NAc, Fuc and Gal, D-mannose inhibits responses by interfering with cell metabolism.

CHAPTER 7

Summary and Conclusions

The work described in this thesis was undertaken to test the hypothesis that cell-cell collaboration can be mediated by MHC-controlled carbohydrate-protein interactions. As a first step (chapter 3), a variety of simple sugars were tested for their ability to inhibit a number of immune responses *in vitro*. Gal NAc, Fuc and Glc Nac inhibited IgG responses to hapten-carrier conjugates in CBA/H cultures, but had no effect on any other *in vitro* response tested.

In contrast to sugars which specifically inhibited IgG responses *in vitro*, D-mannose completely inhibited all *in vitro* immune responses tested in all mouse strains examined (chapter 6). Hence, D-mannose was a nonspecific inhibitor and in fact did not block cell-cell interaction, but interfered with cellular metabolism. In this context, it is important to rule out nonspecific effects when sugar inhibition of immune responses is analysed, since, in our hands, a number of other sugars (e.g. amino-sugars, D-ribose) proved to be nonspecific inhibitors.

A range of monosaccharides were subsequently tested for their inhibitory effects on the *in vitro* IgG responses of a variety of mouse strains. (Chapter 4) a number of strain-specific inhibitors emerged and the inhibition of IgG levels by these sugars was found to be controlled by the I-J and/or the I-C sugregions of the H-2 complex. The simplest explanation for these observations is that induction of IgG responses involves carbohydrate structures, recognition of which is controlled by the I-J and/or I-C subregions.

A detailed analysis of the mechanism by which Gal NAc inhibited IgG responses supported the concept that the sugars were blocking interactions that were required for IgG secretion, Adding, deleting

and pulsing Gal NAc and Fuc in the cultures revealed that these sugars needed to be present in a short time period just before IgG was secreted in order to exert their inhibitory effect. Moreover, Gal NAc exerted its effect during this short time period by abrogating the ability of antigen-generated TRF to restore the antibody response of T cell-depleted cultures (chapter 3).

Other strain-specific inhibitors (i.e. Fuc, Man NAc and Gal) also exerted their effect in this manner. The activity of the TRF, which was active across H-2 barriers, was inhibited by Fuc in $H\text{-}2^k$ strains, and by Man NAc and Gal in $H\text{-}2^b$ and $H\text{-}2^d$ strains (chapter 5), a pattern identical to that seen in the unfractionated spleen cell cultures (chapter 4). This observation again supported the view that an interaction between TRF and its target B cell, resulting in an IgG response, occurs via H-2-controlled carbohydrate-lectin binding and that the addition of appropriate sugars to the cultures competitively inhibited lectin binding.

The next step was to determine whether the lectin was expressed on the surface of the target B cell, or was the TRF molecule itself. (N.B. A lectin is defined here simply by its ability to bind sugar molecules; whether it is also capable of agglutinating cells is unknown.) Using TRF from $H\text{-}2^b$ cultures and T cell-depleted spleen cells from antigen-primed $H\text{-}2^k$ mice, it was found that Gal and Man NAc but not Fuc inhibited the reconstitution of the IgG response. Since Gal and Man NAc were $H\text{-}2^b$ specific inhibitors (i.e. they blocked $H\text{-}2^b$ TRF) then it follows that the $H\text{-}2^b$ TRF was a lectin and the TRF molecule mediated its effect by interacting with carbohydrate structures on target B cells. Moreover, since the inhibition by Gal and Man NAc was controlled by the I-C subregion, the lectin (TRF) is either coded for

or controlled by gene(s) in the I-C subregion. By corollary, the TRF from H-2^k cells is a lectin controlled by the I-J and/or I-C subregion and is specific for Fuc and Gal NAc. Since Gal NAc inhibited TRF from all mouse strains and was the strongest inhibitor, TRF must be primarily specific for Gal NAc. It should be noted that inhibition of TRF in H-2^k strains appears to be controlled by two subregions - I-J and I-C. Whether these regions together control/or code for a single lectin, or two separate lectins, both of which are involved in IgG responses, could not be resolved.

From the work presented in this thesis, very little can be deduced about the nature of the carbohydrate-defined receptors on the target B cells. It is likely that Gal NAc or a structurally similar monosaccharide is the dominant component of the TRF receptor on all target cells. Furthermore, Fuc may be an additional component of this receptor on H-2^k cells. At least two possibilities exist in terms of H-2^b and H-2^d target cell receptors: either they contain Gal NAc, Gal and Man NAc as components, (an unlikely possibility since Man NAc is believed to be absent from mammalian carbohydrates), or Gal NAc is linked to its subterminal sugar in such a way as to sterically resemble Man NAc and Gal. One way to analyse carbohydrate-defined TRF receptors would be to treat B cells with various glycosidases and then determine their ability to absorb out TRF molecules, since TRF can be absorbed on whole spleen cells at 4^oC (chapter 5).

An interesting finding in this thesis is the fact that although the TRF activity is inhibited by strain-specific sugars, it is nonetheless capable of helping B cells to respond to antigen irrespective of their strain of origin. The simplest explanation is that

despite the lectins possessing different sugar specificities, the carbohydrate receptors on the target B cells of different strains are sufficiently alike for the lectin to bind crossreactively to all receptors.

Data presented in this thesis indicates that the lectin (TRF) is either coded for or controlled by I-C and/or I-J subregions. Whether the carbohydrate receptor on the target cell is also an I-region-controlled structure is a matter of speculation made possible by the demonstration that some I-C subregion molecules on B cells are carbohydrate-defined antigens (Sandrin *et al.*,1982).

At this point it should be noted that Roseman suggested in 1970 that cellular interactions important for the regulation of biological phenomena may be mediated by highly specific protein-carbohydrate recognition. He proposed that such interactions might occur via recognition of an appropriate carbohydrate determinant or substrate on one cell by a glycosyltransferase on another cell, and that after the attachment of a new monosaccharide residue, the enzyme substrate complex would dissociate and the cells separate. The subsequent demonstration of glycosyltransferases on the surface of intact cells of many kinds has added support to the concept that these enzymes mediate interactions between cells, particularly where carbohydrate recognition has been implicated (reviewed by Pierce *et al.*, 1980).

With evidence for the existence of both carbohydrate-defined and protein-defined H-2 antigens (O'Neill *et al.*, 1981a, Higgins *et al.*, 1980) as well as the need for the involvement of glycosyl-transferases in the synthesis of the carbohydrate antigens, it is

possible that responses controlled by H-2 genes are mediated by H-2 controlled protein-carbohydrate-interactions. In fact, several recent articles have speculated on how MHC controlled glycosyltransferases could mediate antigen-specific and antigen-nonspecific interactions by lymphoid cells (Parish *et al.*, 1981, 1982).

As informational molecules, carbohydrates have considerable scope, particularly in their ability to exist in a variety of spatial configurations, to be linked in a number of different ways (e.g. α and β linkages) and their ability to readily form branched structures. There are, for example, 10^{24} possible structures for a twelve-residue oligosaccharide of three mannose, three Glc NAc, three galactose and three sialic acid residues (weir, 1980). On the basis of these calculations it is possible to envisage subtle changes in the structure of carbohydrate receptor for TRF on target B cells of different mouse strains.

The activity of a number of other lymphokines have also been shown to be blocked by monosaccharides (reviewed by Rocklin *et al.*, 1980). The best defined of these is the macrophage migration inhibition factor (MIF). MIF activity in guinea pigs is specifically blocked by α-L-Fucose (Liu *et al.*, 1981). Furthermore, guinea pig and human macrophages treated with fucosidase no longer respond to MIF. These findings suggest that MIF is a lectin which recognises L-fucose and that L-fucose is a part of a MIF receptor, which may be a gangloiside.

Murine macrophage activation factor (MAF), which is indistinguishable from MIF, is also blocked by a monosaccharide, in this case by D-mannose. The macrophage receptor for MAF could be destroyed by mannosidase treatment indicating that MAF is a lectin

recognising D-mannose and that this sugar is an essential component of the receptor (Yamamoto and Tokunaga, 1981). Although MIF/MAF are active across species barriers, it would be interesting to determine whether species or strain specific sugars could be found to inhibit the activity of these lymphokines and whether these effects could be mapped to the MHC.

The activity of leucocyte-migration inhibitory factor (LIF) which is distinct from MIF (reviewed by Rocklin *et al.*, 1980) could also be inhibited by sugars and L-fucose and Glc NAc have both been postulated to be important parts of the LIF receptor.

Additionally, the activity of a number of other lymphokines could be inhibited by monosaccharides although the mode of inhibition is less well defined. Amongst these are lymphokines inducing skin reactions to antigens (Baba *et al.*, 1979, Amsden *et al.*, 1978), mitogenic factor (Geczy, 1977) and interferon, (Vengris *et al.*,1976).

Hence, in addition to an H-2-controlled carbohydrate-lectin interaction resulting in IgG synthesis described in this thesis, a number of other lymphokines appear to be lectin-like molecules which interact with their target cells via carbohydrate-defined receptors. Whether these interactions are H-2 controlled remains to be seen.

A number of other features warrant brief discussion. Firstly, the TRF described in this work resembles that of other workers in that it is non-antigen-specific, non-H-2-restricted in its action, and is late-acting, presumably supplying the final signal which results in IgG secretion by antigen-activated B cells (Schimpl and Wecker, 1972). However, the TRF described here is coded for or

controlled by the I-J and/or I-C subregions; yet the TRF previously described in the literature does not appear to carry Ia antigens.

To reconcile this difference it could be argued that the I-J and/or I-C subregion-controlled structures on TRF are either serologically silent or very weakly immunogenic. The difficulty in raising antibodies against, and identifying I-C subregion-controlled determinants (Sandrin *et al.*, 1981) supports this notion.

Secondly, the demonstration that IgM synthesis in both primary and secondary antibody responses was not inhibited by any of the sugars which were good inhibitors of IgG responses implies that the IgM and IgG responses are activated by different mechanisms.

Finally, the inhibition of TRF activity by monosaccharides offers a means of functionally distinguishing between TRF and IL-1/IL-2 molecules in culture supernatants. Hence, if the helper activity in a culture supernatant is due to expansion of precursor T cells by IL-1/IL-2 molecules, then the sugar inhibition of IgG synthesis will be specific for the H-2 haplotype of the cultured cells, not of the origin of the supernatant. In contrast, if the "help" is due to TRF molecules, then the sugar inhibition pattern will follow the origin of the supernatant rather than that of the cultured cells.

In conclusion, data presented in this thesis support the concept that MHC-controlled carbohydrate recognition is important in IgG responses to hapten-carrier conjugates. Whether these observations can be extended to other immune responses awaits further investigation.

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The material present in this thesis is or will also be presented in the following publications:

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