THE ROLE OF PEYER'S PATCHES IN THE MODULATION OF IMMUNE RESPONSES

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STATEMENT

The material in this thesis has not been previously submitted for a degree in any University, and, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text.

(Ansaruddin Ahmed).

To my mother and father,

To my wife, Lucy and children,

Sonia, Rana, Victor and Ilka.

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SUMMARY

This thesis is concerned with the characterization and modulation of immune responses to V.cholerae presented by different routes, in mice.

A key question examined is whether the preponderance of IgA found after oral immunization is due to the modulation of the response by cells in the Peyer's patches (PP). An alternative hypothesis examined is whether the antigen is processed in some way by the PP so that it specifically stimulates IgA forming cells.

The Jerne plaque forming cell assay was used for the estimations of antibody responses in spleen and gut lamina propria.

The following are the salient observations:

(1) Although the individual IgM or IgA splenic response in systemically primed and boosted mice is dose dependent, the combination of a very low IgA/IgM ratio and absence of gut response seem to be a function of the route and schedule of the immunization.

(2) Repeated oral immunization followed by systemic boosting leads to a high response in the spleen with a high IgA/IgM ratio. No gut response is detected.

(3) Good responses in the gut are only obtained by repeated high-dose oral priming followed by an oral boosting. The IgA/IgM ratios are highest by this regimen in both spleen and gut lamina propria.

(4) By the nature of IgA/IgM ratios of antibody responses in the spleen it is possible to differentiate between a systemic and a local (intestinal) immune stimulus.

(5) The IgM spleenic antibody responses in mice to V.cholerae following adoptive transfer of PPL from antigen-fed syngeneic donors are profoundly suppressed as compared with the responses obtained in control mice receiving antigen only or antigen plus unprimed PPL from normal conventional donors. The IgA responses in the same experiments are variable, being enhanced, suppressed or remaining unchanged.

(6) When the mixed population of PPL from antigen-fed donors are partitioned into purified T and B cell subsets and are injected separately into the recipients along with the antigen, either T or B cell subset is shown to bring forth concurrent suppression of IgM and enhancement of IgA response in the spleen as compared with results of the control mice described above.

The finding of concurrent IgM suppression and IgA enhancement by gut-presentation of antigen is discussed in the light of earlier evidence. The implication of these phenomena in the immunologic homeostasis of the host is considered.

CHAPTER I

GENERAL INTRODUCTION

General Introduction

This thesis is concerned with the nature of the different types of antibody responses induced by V.cholerae antigen when administered by various routes. In particular, the studies attempt to explain why antigen locally presented in the mouse gut produces IgA antibody almost exclusively whereas, the same antigen, given intravenously leads to the production of IgM and IgG* antibodies predominantly and much less IgA antibody.

Historical perspective : nature of mucosal antibody

In 1919 Besredka published the results of his experiments showing protection of rabbits by oral immunization against the fatal infection caused by Shiga bacillus. Shortly after, Davies (1922) showed that copro-antibody was present in patient's stools in bacillilary dysentery before the appearance of humoral antibodies. The experimental finding of protection was used by Besredka in immunizing thousands of soldiers before and during the First World War. The data in his monograph entitled "Local immunization" (1927) indicated the efficacy of oral immunization in preventing bacillary dysentery. The theory of local immunity proposed by this pioneer investigation was probably not believed for the next 40 years until Heremans (1960) provided an immunochemical basis for it by isolating and characterising a new serum immunoglobulin called β2A or gamma A (different from those already known as gamma G and M). Shortly after Hanson (1961) showed that this immunoglobulin was predominant in milk and had unique

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*IgG antibody responses have not been studied in this thesis.

characteristics. Within the next few years it was categorized as a major immunoglobulin component in human and animal exocrine secretions such as saliva, colostrum, bronchial fluid and intestinal juice (Tomasi and Zigelbaum, 1963; Cebra and Robbins, 1966; Heremans and Vaerman, 1971). Tomasi et al. (1965) demonstrated that its unique characteristics are due to the presence of a polypeptide which was called transport piece, now secretory component (SC). This secretory immunoglobulin (sIgA) is synthesized in plasma cells of the local mucosa (Tomasi & Bienestock, 1968), as a dimer (11S form) containing an additional polypeptide J chain (Koshland 1975) which probably has the function of binding the SC. (Eskeland and Brandtzaeg, 1974). The epithelial cells synthesize SC (Brandtzaeg, 1974, 1978; Crago et al, 1978) and the dimeric IgA after binding to the SC at this site is transported across the epithelial cells to be released at the apical surface into the gut lumen. (Allen et al, 1973; Nagura et al, 1979). These observations on the production pathways of specialized sIgA molecules are in keeping with the fact that specific immunological resistance to mucosal infections may be found without demonstrable serum antibody. For example, IgA antibody to various food antigens and pathogens like bacteria and viruses were found in local secretions and correlation of protection was best found with this local antibody rather than the circulating one, (Tomasi & Bienestock, 1968).

In an extensive series of investigations the groups of Vaerman (LeMaitre-Coelho et al., 1977; Jackson et al., 1978; LeMaitre-Coelho et al., 1978 a, b), Hall (Orlans et al., 1978; Hall et al., 1979; Birbeck et al., 1979) and

Underdown (Fisher et al., 1979; Socken et al., 1979) have shown that dimeric IgA after being synthesized at a particular local mucosal site is transported selectively and rapidly across the hepatic parenchymal cells into the bile. Consequently ligation of the common bile duct gives rise to a marked increase of the IgA concentration in the systemic circulation (LeMaitre-Coelho et al., 1978a). This mechanism serves to increase the concentration of IgA in the gut further and emphasizes the distinction between the circulating immunoglobulin concentrations and those locally in the intestine.

Kinds of antibody responses by various routes of antigen presentation

As studies into the kinds of antibody responses by various routes of antigen presentation constitute the initial part of the project the subject may briefly be reviewed with a special emphasis on what happens when antigen is presented through the gut.

Pierce and Gowans (1975) attempted to find out the best regimen of immunization with cholera toxoid in rats which would establish a high density of antitoxin-containing cells in the lamina propria (LP). They found that the highest number of antitoxin containing cells (ACC) per mm^3 of LP was obtained by challenging the rats intraduodenally after priming intraperitoneally with purified toxoid and complete Freund's adjuvant. The next best regimen was oral priming followed by intraduodenal challenge. Intraperitoneal priming followed by intraperitoneal challenge led only to a very weak primary type of response. Oral priming followed by intraperitoneal challenge produced a moderately low, albeit secondary type of response as judged by qualitative

criteria of immunohistochemical staining.

In a study of the cellular kinetics of the intestinal immune response to cholera toxoid Pierce (1976) used a similar experimental design and obtained almost identical results.

Bloom and Rowley (1979) using various immunization schedules with V.cholerae in mice found that in spite of good primary and secondary plaque forming cell (PFC) responses in the spleen by intravenous immunization, the local response in both the IgM or IgA classes were below the level of detection. The best local response was obtained by priming with four large oral doses followed by an intravenous boosting on day 14 after the last day of priming. This immunization schedule, for obtaining best gut response is at variance with that of Pierce and Gowans (1975) and that used in the present work (vide Table 3.3, Chapter III) in that, it used i.v. instead of intraduodenal or oral route for the challenge. However, it also emphasizes the oral route of presentation of antigen for eliciting the best local immune response.

Using ferritin as an antigen by subcutaneous or intra-peritoneal routes in germ-free adult mice Crabbe et al., (1969) found anti-ferritin containing plasma cells mainly in lymph nodes and spleen but few in gut lamina propria. These specific antibody containing plasma cells in the extraintestinal tissues belonged mainly to IgM class after a single and IgG1 class by multiple stimulations. All of the few plasma cells in the gut were of IgA class but the circulating antibodies by repeated immunization were mainly IgG1 and IgG2. Conversely, mice immunized through drinking water possessed abundant plasma cells in the gut mucosa,

less in mesenteric lymphnode and much less in other lymphoid tissue. Whatever their location, these cells appeared to be exclusively of IgA type and the circulating antibody was found to be IgA. The results highlighted the role of gut as a site of antibody production and selective commitment to the IgA synthesis.

Allowing germ free mice to drink SRBC suspension Bazin et al., (1970) demonstrated that substantial numbers of plaque forming cells (PFC) per 10^6 nucleated cells appeared in extraintestinal lymphoid tissues of spleen and mesenteric lymphnodes after both primary and secondary stimulations. The responses were characterized by a very high preponderance of IgA-forming plasma cells over those producing IgM or IgG antibodies. There was no response in axillary, brachial and inguinal lymphnodes.

Using conventional mice and the same antigen administered by oral or intragastric routes Andre et al., (1973) demonstrated a similar finding in spleen.

Mestecky et al., (1978) demonstrated selective stimulation of immune response in the salivary and lachrymal glands by immunochemical methods in four human subjects who ingested capsules containing killed Streptococcus mutans. Specific antibodies were detected in the salivary and lachrymal secretions within one week which continued to rise up to the second week and then gradually declined during the ensuing two months. In other experiments the pronounced antibody rise peaked earlier at day 10 after an oral challenge. The antibody was associated with only IgA and no serum response was detected after either of the immunizations.

Peyer's patches as the main source of IgA precursor cells for lamina propria of the gut and their association with the induction of local antibody responses

The evidence cited above clearly indicates that to get the best specific IgA response in the gut mucosa and even in extraintestinal lymphoid tissues like spleen and mesenteric lymph nodes, antigen must be presented locally to the gut. This route of antigen presentation can also lead to the secretion of specific sIgA antibody in distant mucosal surfaces such as in mammary glands (in rabbits; Montgomery et al., 1974: in humans; Goldblum et al., 1975) in salivary and lachrymal glands (Mestecky et al., 1978), as mentioned previously . How can the importance of the oral route of antigen presentation be explained ?

A number of investigators have been able to stimulate the production of specific IgA antibody in mammalian secretions (Blackman et al., 1974; Montgomery et al., 1974; Yardley et al., 1976 and Goldblum et al, 1975) but in no case did the experimental design identify the precise site in lymphoid tissue where this occurred.

Peyer's patches (PP) have been demonstrated to have a role of antigen-sampling in the gut. Macromolecules and even bacteria may pass intact through the specialized dome epithelium and reach the follicular area of B lymphocytes (Bockman and Cooper, 1973; Carter & Collins, 1974).

Robertson and Cebra (1976) prepared a series of rabbits with isolated 20 cm. Thiry-Vella loop, each bearing or lacking a PP, keeping the mesenteric attachment and thereby the neurovascular supply intact. The rabbits were rested for seven days after this initial surgery. Then followed administration of antigens (2,4-dinitrophenylated keyhole

limpet haemocyanine, DNP-KLH) (400µg) and heat-killed Salmonella typhimurium ($4x10^7$) on day 0 and day 4. A specific IgA antibody-rise was observed in the test loop with the major peak developing during days 4-8 and falling to a modest peak during 12 to 20 days. Total sIgA, total antibody and total IgA antibody concentrations in loop secretions and serum were monitored by radio-immunoassay using insoluble antigens. S.typhimurium was used as an adjuvant. Almost all the anti-DNP present in the secretions was IgA of dimeric or higher polymeric form. The maximum concentration of IgA anti-DNP often rose 5 to 10 fold over the pre-stimulation values; but when the loop lacked PP the increase in anti-DNP in secretions was either slight or nil. During the 20-40 days when the loop-secretions were monitored anti-DNP of any isotype could not be detected in serum.

Using the allotypic determinants on the L chains of rabbit Igs as markers of their cellular origin Craig and Cebra (1971) first determined that PP and appendix were enriched sources of cells that could repopulate the gut lamina propria of lethally irradiated rabbits with IgA plasma cells. Relative to peripheral lymph nodes, blood and spleen, PP were shown to contain many more immediate precursors of IgA plasma cells. This was assessed either in vitro by monitoring the appearance of IgA plasma cells in mitogen-stimulated microculture of the various lymphoid tissues or in vivo by estimating the number of IgA plasma cells in the spleens of recipients receiving transfer of cells from the tissues. (Craig and Cebra 1971, Jones et al., 1974; Craig and Cebra, 1975). To establish the origin of the cells producing IgA antibody to cholera toxoid and to define the role of the antigen in

their distribution Husband and Gowans (1978) performed experiments by a challenge restricted to a defined segment of the small intestine of a rat primed intraperitoneally with purified toxin plus Freund's complete adjuvant. The results showed that the anti-toxin-containing cells (ACC) which appeared in the draining thoracic duct lymph (TDL) after the challenge of a loop were almost all of IgA specificity and their numbers were proportional to the length of intestine exposed to the antigen. When PP were removed from a loop before challenge, cellular response was abolished, whereas lymphadenectomy of the draining mesenteric lymphnodes did not affect the response significantly. This indicated that ACC originated exclusively from PP. Counts of ACC in gut lamina propria of challenged and saline control loop and the time course study of their appearance led the authors to conclude that though intestinal ACC can migrate into lamina propria independent of antigen, antigen has a profound positive effect on the location, magnitude and persistence of the response.

Unique characters of IgA in comparison to those of IgM and IgG: reasons for its existence and advantage in the evolutionary process.

IgA is very old in its existence in the evolutionary process. On the basis of comparison of amino acid sequences of human α γ and μ C terminal regions Chuang et al.(1973) concluded that the α chain might have diverged from the μ chain some 200 million years ago. Demonstration of IgA in chickens by Lebacq - Verheyden et al.(1972) supports this view because appearance of mammals and divergence of

birds from reptiles are thought to have occurred about 175 million years ago. How does the possession of a sIgA system at the mucosal surface improve the survival value of an animal relative to another animal lacking it ? What does IgA do especially, that IgM or IgG does not do or does less ?

Up to now the evidence for the mechanisms by which IgA can protect animals from the gastrointestinal invasion of numerous microbial and food antigens are as follows: (a) inhibition of bacterial adherence to a variety of mucosal surfaces and thereby prevention of colonization which is required for subsequent pathophysiologic changes leading to disease (Vibrio cholerae to gut mucosa, - Freter, 1970, Streptococci to oral mucosa, - Williams and Gibbons (1972) Enteropathogenic E. coli to gut mucosa in pig - Jones and Rutter, 1972 and Wilson and Hohmann, 1974) (b) (i) complement - independent inhibition of the fraction of bacterial population that are adsorbed to the mucosa not affecting the total count of intestinal vibrio population (Fubara and Freter, 1973), (ii) Alteration of growth pattern of streptococci coated with salivary IgA (Brandtzaeg et al., 1968), (c) Antitoxin activity : Presence of anti-cholera toxin activity in 11s IgA-antibody fraction of rabbit intestinal homogenates (Kaur et al., 1972); Inhibition of binding of cholera toxin to mucosal epithelium (Fubara and Freter, 1972) (d) Virus neutralization (Keller and Dwyer, 1968, Ogra et al., 1968), (e) Antigen exclusion : Antibody in secretions may block or reduce the capacity of the gut mucosa to absorb the antigens present in the lumen and thereby dispose of them from the surface of the mucosa. There seems to be an initial formation of antigen-antibody complex and

subsequent degradation or prevention of absorption due to the physically large complex. (Cornell et al, 1971, Walker et al.1974, Andre et al., 1973).

All the above protective functions are not unique properties of IgA. Given their local availability at the mucosal surface both IgG and IgM , including even their $F(ab)_2$ fractions probably could give similar types of protection with comparable efficacy. (Steele et al, 1974; Heddle and Rowley, 1975). But the evolutionary process gives IgA an advantage over IgM and IgG at secretory surfaces in two respects. Firstly, it is quantitatively by far the most abundant Ig class at that site and secondly, it is not degraded by the proteolytic enzymes in the G.I. tract like IgM and IgG. In addition, breast-fed infants enjoy significant degree of protection in the gut against microbial and food antigens through secretory IgA antibody in colostrum and mother's milk. Indeed, it has been shown that infants retain sIgA specific antibody activity in faecal extracts. (Haneberg, 1974).

Two other characteristics of IgA antibody are its inability to induce opsonization due to lack of its binding to FC receptors on phagocytes (e.g. neutrophils, blood monocytes or alveolar macrophages) and to activate complement. In addition, there is evidence that it can even block other Ig classes from reacting with antigen in complement-mediated bactericidal (Russell-Jones et al., 1980, MacLeod Griffiss, 1975) amd hemolytic assays (Kearney and Hallyday 1970). Its relative inertness in these two functions as compared to IgG and IgM has been shown by Rowley's group. Equivalent

amounts of IgA, IgG, IgM had relative bactericidal activities of 1:6:250 and opsonic activities of 1:7:200 (Rowley, 1977).

When complement is activated in a tissue, in this case the gut mucosa, by IgG and IgM specific antibodies, various biologically active mediators of inflammation are liberated. These phlogistic peptides cause vasodilatation and increased capillary permeability. Factors for chemotaxis, macrophage activation, and migration inhibition etc. causes accumulation as well as disruption of neutrophilic polymorphs and macrophages. These inflammatory reactions are necessary for the removal of the non-self noxious antigens e.g. pathogens, but when these "heightened and frightened" reactions occur throughout an organ on a wide scale its function is impaired and different grades of allergic inflammatory diseases are created (Coombs and Gell, 1975). In fact it is surprising that gut mucosa (along with other mucous surfaces) is not in a state of constant inflammation due to the continual onslaught of food and microbial antigens.

It is tempting to imagine that a system is needed to protect secretory surfaces against complement-fixing antigen-antibody reactions and the IgA system was evolved with the endowment of special properties like (a) inability to activate complement (b) quantitative preponderance at the local site and (c) resistance against proteolytic enzymes, as a result of selection pressure for the protection against the continual onslaight of food and microbial antigens. This protective mechanism should be such that to maintain immunologic homeostasis the host tissue is not inflamed,

the antibody itself is not destroyed, necessary nutriments, even if they are antigenic, are allowed to be absorbed after proper handling and the symbiotic microbial flora is kept intact.

The regulation of humoral immune responses.

The subject of humoral immunoregulation is briefly reviewed in the following paragraphs. Special stress is given to helper and suppressor cells because the most important aspect of this thesis is the finding that modulator cells in PP of orally primed mice help in creating a 'local type' of antibody response on adoptive transfer. The role of the small intestine in modulating immune responses on local presentation of antigen is also discussed.

During the days of Landsteiner and Heidelberger (1930's) the concept of antibody and its behaviour were very simple. The reactions of antigen and antibody were demonstrated to occur in a simple, quantitative chemical way. The nature of antigen was considered to be the prime factor that was responsible for antibody production by stimulating the cells into whose receptors the determinants could fit exactly like a key to a lock. But this simple concept was unable to account for the 'degeneracy' of immune responses that was observed consistently in later years. In quest of explaining this lack of reproducibility in immune responses in terms of affinity for the antigen under very similar experimental conditions it was gradually demonstrated that a specific immune response is the result of an extremely complex process and one antigen can stimulate a large

proportion of the lymphocyte pool. For example, a single particulate antigen like SRBC may stimulate Ig synthesis in even upto 10% of mature B lymphocytes.

As a result of an enormous body of recent research a revolutionary change seems to have appeared now in the concept of immune surveillance in the higher vertebrates. It comprises an intricate network of cellular and humoral antagonists in the form of helper and suppressor cells, antibodies and auto anti antibodies (idiotypes). In addition, a group of less specific cell-borne products called lymphokines or factors constitute another poorly understood immunoregulatory mechanism. Many aspects of these concepts are still full of ambiguity. Examples are the mechanism of induction of antibody formation, mode and target of action of suppressor cells and the role of anti idiotypes, to name but three .

Before going into the details of cellular interactions it is necessary to keep in mind the basic framework of an immune response. For most of the antigens (Thymus dependent) the basic interaction is between a B-cell which produces antibody and a T-cell which helps it to do so. (Miller and Mitchell, 1968; Claman and Mosier, 1972). The help of a third adherent macrophage like cell is necessary for the induction of the response by trapping the antigen and presenting it to the lymphocytes in a specially immunogenic processed form. (Cozena et al., 1971; Feldman, 1972). Certain Thymus-independent polymeric antigens, of course, can directly activate B cells and do not need T cell help.

Helper T cells.

The need for thymus-derived helper (T) cells in humoral immune responses has been recognized from the early sixties. (Miller, 1961; Martinez et al., 1962, Claman et al., 1966). The specificity of the activity of T cells in T-B co-operation was proved by the elegant thymus cell "education" experiment of Mitchell and Miller (1968) in which the activity of the T helper cells was amplified in a primary set of irradiated mice by antigen contact prior to mixing them with bone marrow-derived (B-cells)plus antigen and injecting the mixture into a second irradiated recipient. Test recipients having initially educated thymus cells (T helpers) produced large numbers of hemolysin-forming cells in comparison to very small numbers in controls having none. Further light has been shed on the nature of co-operation, particularly at the molecular level, by the classical experiments with hapten-carrier conjugates. Both Mitchison (1971) and Rajewsky et al.(1969) have demonstrated that primed B-cells can mount a secondary antibody response against a hapten bound to a protein carrier only when T cells primed to the same carrier (helper cells) are also available. These findings led to the theoretical postulate of linked associative recognition in the T-B co-operation (Bretscher, P.A. 1972).

It has been found by immunochemical and serological analysis that the recognition of an epitope by T cells on the carrier portion of an antigen is mediated by Ig-like receptor molecules(Marchalonies et al., 1972). T cell receptors have been found to have functional homology with

B-cell-derived immunoglobin idiotypes (Eichmann and Rajewsky, 1975; Binz and Wigzell, 1975). All of the several T helper cell clones in mice described upto now possess the same phenotypic cell surface profile of Thy-1^+ Lyt 1^+ 23^- (Von Boehmer, 1980), some with and some without Ia markers. The MHC region imposing restriction on them is I-A (Miller, 1980). Their amplifying functions appear to be mediated by subdivisions of the above subsets e.g. those that generate helper activity for B cells are Lyt^+23^-,Qa^- and those that amplify suppressor cell function are Lyt^+23^-,Qa^+.

Possible mechanisms of T-B co-operation

The precise mechanisms by which B cells differentiate finally into antibody forming cells with the collaboration of specific and non-specific T helper activity are still uncertain. One cannot expect to understand the concept of T cell help without asking a general question : how do the T cells regulate by enhancing or suppressing, or their concurrent effects, the final outcome of immune reactions ? There has been extensive research in this area but clarity has not yet been achieved. Particularly, the role of various 'factors' isolated from one set of lymphocytes that may have effects on the others is still full of ambiguity.

T-B co-operation may take place by direct cell-to-cell contact or by the mediation of soluble factors released by T cells. These is also evidence for two types of factors, specific and non-specific for the antigen.

The direct cell contact mechanism was demonstrated by

Phillips and Waldmann (1977) using microculture system of Lefkovits. Adding low numbers of KLH-specific T cells in microcultures containing excess number of TNP-primed B cells and counting the number of B cell clones producing specific challenge with antibody by TNP-KLH they demonstrated by Poisson formula that a single helper T cell can help only one TNP-specific B cell and thus the co-operation was a cell-to-cell localized phenomenon. If T cell factors that were expected to diffuse freely were responsible, several B cell clones would have been stimulated by a single helper T cell.

Antigen-specific T cell (helper and suppressor) factors, which are capable of replacing specific T cell functions, are divided into two categories according to their biologic activity. They are helper factors and suppressor factors and can replace T_h and T_S cells in various immune responses. They possess properties like antigen specificity and in some cases express idiotypic determinants associated with VH regions of Igs (Mozes and Haminovich, 1979; Gerinain et al., 1979). These properties are in common with those of isolated T cell receptors. But the main difference between them is that factors express determinants coded for the H-2 complex but receptors do not. Helper factors express I-A region determinants whereas suppressor factors express determinants of I-J region. It has been demonstrated that the IgM response of DNP-primed B cells to DNP-KLH is the same when the helper T cells are in the same compartment with or are kept separated from B cells by a semipermeable membrane. The inference is that the T cells release a specific soluble helper factor which

diffuses into the B cell compartment. In addition, the specific soluble factor either alone or forming complex with the antigen can bind to the macrophage-surface and then co-operate with hapten-primed B cells. A comparable specific T cell factor which can replace T cells in vivo has been described by Taussig (1974). But this factor has been found in only a small number of systems and is not widely reproducible.

Non-specific helper T cell factors have many different names pertaining to the assays used for their detection, but they are collectively known as lymphokines. All of them are glycoproteins and exert diverse types of effects on lymphoid and hemopoetic cells predominantly influencing their growth and differentiation. They are secreted by T cells in response to stimultion of antigens or mitogens like Concanavalin A. In the majority of cases T cell responses are macrophage dependent so it is possible that the macrophage may also release similar factors.

The non-specific factors are generally classified into three main categories or groups. One group of factors act in the process of triggering of lymphocytes by antigen, the targets being T and B cells. They do not promote prolonged culture of T cells. The second group members are growth factors for activated T cells of both helper and cytotoxic type. They can maintain long-term growth of activated T cells. The third group of factors act synergistically with the members of the first group and antigen to activate B cells and then enhance their differentiation into antibody secreting plasma cells.

The exact role of these non-specific T cell helper factors in the T-B cell co-operation remains ill understood, but their importance in the growth and differentiation of lymphocytes seems to be fairly well-accepted.

The B cell triggering in response to external antigen may be outlined by the following theoretical models:

(i) Critical matrix.

Antigen forms a critical matrix by attachment with B-cell Ig receptors through its repeating epitopes and thus produces a single activating signal '1'. A multi-valent attachment can produce an exponential increase in the strength of the binding between the antigen and the cell. An appropriate receptor aggregation by itself may be sufficient to trigger B-cell proliferation and differentiation. Obviously a highly polymeric antigen with respect to a single determinant will have an advantage in this way. However, in the case of a T-dependent antigen the multivalent attachment is formed by binding to macrophages via cytophilic T-dependent factors specific for the carrier determinants and the matrix is created.

(ii) One non-specific signal (Coutinho and Möller).

T -independent antigens are mitogenic inherently, for example bacterial LPS is a polyclonal mitogen. Surface Ig of B cells is not directly involved in signalling to the cells. Ig receptors seem to play an indirect role of antigen-binding and concentrating mitogenic signals in the vicinity of specific B cells. In the case of T-dependent antigens the T cells supply the activating signals by way of recognizing carrier determinants.

(iii) 2-signal model, the second signal being non-specific (Bretscher, Bretscher & Cohn). Antigen provides the first specific signal by binding to the Ig-receptors of the B-cell and by this step inactivates it, i.e. produces tolerance. However, the cell will be 'turned on' for antibody synthesis by a simultaneous second signal supplied by T cells on recognizing carrier epitopes or by a polyclonal B-cell activator like LPS. This signal may be transmitted by a close apposition of two lymphocytes against a sandwiched antigen or through a macrophage that serves as the final helper cell, having first bound to its surface, antibody or antibody-like 'factor' molecule secreted by the activated T cells. Alternatively, the second signal may be provided by the 'FC' equivalent part of the antigen binding molecules secreted by the T cells; while any such second signal for a given response is usually considered to be non-specific in character, the initial stimulation of both T and B cells are evoked by specific binding with the particular antigen concerned.

T suppressor cells

The generation of T suppressor (T_S) cells in response to specific or non specific stimulation of the immune system is well documented. They can be distinguished from other subsets of T cells by their phenotypic surface markers $Lyt1^-23^+$, Ia^+ (coded by I-J region of the H-2 complex). They are characterized by extreme radiosensitivity and inactivation by a low dose of cyclophosphamide and antilymphocyte serum. Their activity has been demonstrated in IgM (Gershon and Kondo,

(1970) and IgG and IgE (Tada et al., 1972) specific antibody responses. This holds true in case of T-dependent as well as T-independent antigens (Baker et al, 1970; Baker et al., 1979; Warr et al., 1975). They may be obtained in the process of priming (Tada et al., 1975), tolerance induction (Basten et al., 1975b), mitogen stimulation (Dutton, 1975) and in allotype (Herzenberg et al 1976) and idiotype suppression (Eichmann, 1975). When activated by mitogens Ts cells evoke non-specific suppression in both humoral and CMI responses, but in most other cases they show specific inhibition in the immune responses to the antigen which activated them. They require, like T_H cells, linked associative recognition in their specific activity. Consequently suppression cannot be achived when DNP-KLH primed cells are treated in the presence of non-related carriers such as DNP-HGG (Tada, 1974; Basten, 1974). But in contrast to other T cell subsets, Ts cell activities do not appear to be H-2restricted and they can function in allogeneic hosts(Greene et al., 1978). They have the ability to bind to the native antigen and that may be a reason why antigen which can bypass the macrophage system selectively activates Ts cells. The examples are excess antigen, highly deaggregated forms of antigen and antigen that fails to associate with Ia self determinants on macrophages in a low responder host. (Howard and Mitchison, 1975). Ts cells play a crucial role in various phenomena of immune responses like antigenic competition, induction and maintenance of immunological tolerance and prevention of allergic and autoimmune diseases.

Suppressor factor.

An antigen-specific suppressor factor has been extracted from T_S cells (Tada et al., 1975, 1977.; Taniguchi and Miller, 1978). The factor is a protein of molecular weight 35,000 to 55,000 daltons, has specificity and affinity for antigen and possesses a determinant coded by the I-J region of H-2 complex. Other characteristics are similar to those possessed by specific T helper factors that have been described (Vide page 16).

Immunological Tolerance.

Before discussing regulation of immune responses by suppression the nature of immunological tolerance should be reviewed briefly. An immunological tolerance or unresponsiveness is defined as the specific failure to respond to a secondary challenge with an antigen. This is the other side of the coin of the specific immune response giving rise to antibody formation and generation of effector T cells. It is an acquired characteristic and tolerance to self antigens is not inherited, for the offspring of two histoincompatible parents retains the ability to recognize alloantigens of either of the parents.

Two views are held to explain the mechanism of tolerance : induction of specific clonal deletion by antigen and mediation of tolerance by specific suppression. Neither of the two mechanisms probably can stand on its own right to explain all types of immunological tolerance and the assessment of their relative importance in different

situations comprises an important part of modern immunology having prospects of clinical application.

Induction of tolerance in immature cells.

Tolerance was first classically described by Owen (1945) for transplantation antigens which is a good model for the studies on self tolerance. He showed that dizygotic cattle twins having placental fusions in utero became permanent chimeras with respect to their erythrocytes and hemopoetic cells. Skin grafts were accepted in between themselves but rejected from unrelated cattle. The conclusion drawn was that if the lymphoid tissues of an animal in an early critical period of development is exposed to an antigen, tolerance would be induced. Subsequently Billingham et al.(1953) experimentally created transplantation tolerance, in the same principle, by injecting neonatal mice of one inbred strain (A) with hemopoetic stem cells from another partially histoincompatible strain (AxB) F1. The A strain mice then accepted skin grafts from B strain. The critical factor in this classical transplantation tolerance is the immaturity of the lymphoid cells and not the immaturity of the recipient mice. This can be proved by the tolerance obtained using immature cells from adult mice in the reconstituion of lethally irradiated F1 mice with equal numbers of bone marrow stem cells from A and B strains. After 6 to 8 weeks the T cells of the recipients will be tolerant to each other's transplantation antigens, reject a third party skin graft and will possess normal

immunological functions. This 1:1 ratio of the donor cell types in the recipients will continue for many months. In addition, tolerance to protein antigens can be induced in immature B cells from adult bone marrow and neonatal spleen but not in mature B cells from adult spleen (Nossal and Pike, 1975; Metcalf and Klinman, 1976).

Kinetics of tolerance in immature cells and the importance of persistent antigen.

Weigle (1971) using human gamma globulin (HGG) in normal mice to produce tolerance and adoptive transfer of tolerant T or B cells in irradiated mice showed that both T and B cells were tolerized. T cell tolerance was induced within 2 days and persisted for about 150 days whereas B cells took about a week for the induction of tolerance and lost it by 50 days.

Persistent antigen is required for maintenance of tolerance because primary lymphoid organs keep on generating new clones of lymphocytes. Why there is long-lasting tolerance in a Medawar experimental protocol of classical transplantation tolerance is explained by the fact that 1-2 per cent F1 cells continue to persist indefinitely in the recipient A strain mice that received F1 cells neonatally. The pattern of spontaneous breaking of tolerance in Weigle's experiment may also be explained by the gradual waning of antigen concentration with time below a certain level which was necessary to tolerize newly generated clones of antigen-reactive cells. The late recovery of T cells from tolerance as compared to

B cells was because T cells were selectively tolerized by low dose antigen.

Induction of tolerance in mature cells.

The mature lymphocytes in the peripheral lymphoid organs of an adult animal have passed the critical stage in early ontogeny when contact with antigen could lead to their clonal elimination. Obviously it is difficult to induce tolerance in such cells. For producing tolerance in mature B cells the immune events related to the participation of T cells in B cell triggering must be bypassed. One way to do this is to use high doses of thymus independent (TI) antigen which can directly act on B cells and render them tolerant. For example, a quantity 10 to 100 fold higher than its immunogenic dose of pneumococcal polysaccharide is tolerogenic to B cells in vitro, although certain lower concentrations are immunogenic. The mechanism for this phenomenon is not known but it is thought that B cell inhibition requires some critical degree of epitope density at the cell surface probably created by cross-linking of membrane Ig receptors. B cells initially cultured with non tolerogenic amounts of TI antigen in presence of an appropriate concentration of specific antibody or its $F(ab)_2$ fraction may, during a subsequent challenge with the antigen, be rendered unresponsive. Failure of monovalent Fab fragment to produce a similar tolerance supports the view that this kind of tolerance is evoked by epitope-cross linking on the B cell surface. TI antigen when coupled to a hapten can produce anti-hapten tolerance and in this case, the

more the number of haptens per conjugate molecule the more tolerogenic it is. This has been shown in vitro experiments by Feldmann (1972) using DNP and polymeric flagellin.

Induction of tolerance to thymus dependent (TD) antigens.

Tolerance can be induced in adult animals to TD soluble protein antigens by intravenously injecting very high or low doses of their deaggregated form. The high dose induces unresponsiveness in T and B cells, but low dose preferentially tolerizes T cells (Mitchison, 1964; Weigle, 1971). The mechanism of bypassing antigen presentation by macrophages and co-operation from T cells is believed to occur in this condition also as the aggregated or polymeric portions of the protein antigen are discarded. Whether the B cells are eliminated or are simply blocked by excess antigen in this system of unresponsiveness is not known. But activity of specific suppressor T cells has been demonstrated by cell-transfer experiments in syngeneic mice (Weber and Kolsch, 1973).

Tolerance may also be achieved by the use of immuno-suppressive agents like X-irradiation or drugs e.g. methotrexate or cyclophosphamide in association with TD antigens.

Mechanism of tolerance induction : Clonal deletion versus T cell suppression.

The proponents of T suppressor activity have challenged the conventional theory of clonal deletion to explain themechanism of classical transplantation tolerance

as was achieved by Medawar's group in the form of induction of tolerance in neonatal A strain of mice to B strain alloantigen by the injection of (AxB)F1 spleen cells. To check critically the contribution of T suppressor cells in this system it was demonstrated that serum or cells from the tolerant A mice could not prevent the rejection of B skin grafts on normal A mice or help inducing tolerance to B alloantigens. In addition, the graft versus host (GVH) reaction in F1 mice receiving small dose of normal A cells could not be prevented by the administration of A cells tolerant to B alloantigens. (Brent et al.,1976; Brooks, 1975). The inference was drawn that, if T suppressor cell activity was the main factor in this kind of tolerance all the experiments would have given positive results and the GVH reaction should have reduced. In addition, in the experiment leading to the generations of chimeric mice by injecting lethally irradiated F1 adults with equal doses of both A and B cells Von Boehmer et al. (1975) could not demonstrate the evidence for suppressor mechanism. Also tolerance could be abrogated in Medawar's chimeric mice by the transfer of normal syngeneic cells.

Thus the current consensus is that in the experimental design like that of Medawar's in which large dose of transplantation antigen is injected at birth, clonal deletion certainly plays the main role; in fact a lasting 'chimeric' state is achieved in this situation where donor lymphoid cells persist in a stable mixture with those of the receipient. In this way tolerance to self-MHC components

is primarily 'learnt' when T cells differentiate in the thymus in their early life (not necessarily the early life of the host).

T suppressor mechanism

Tolerance in T cells to other antigens and tolerance in B cells may not be the outcome of clonal deletion but rather depend on an active dynamic suppression imposed by T suppressor cells. In transplantation tolerance in adults produced by a cocktail of donor antigen, pertussis vaccine and antilymphocyte serum, a suppressor mechanism has been recognized as the major factor. Low zone tolerance to protein antigens has also been found to be mediated by Ts cells directed against T_H cells. The general unresponsiveness to soluble as distinct from aggregated or particulate antigens may be ascribed to their weak stimulation of T helpers through poor macrophage processing in comparison to effective activation of suppressor cells that do not require macrophage presentation.

Pure deletion obviously may be conceived as a rigid system and will not be capable to accommodate responses for a proper homeostatic control of the immune system which in the later part of the host's life, will have to encounter new self-antigens, antigens cross-reactive with self antigens etc.

There is a homeostatic feed back mechanism for controlling clonal expansion of lymphocytes by the secreted specific antibody. Antibody in this process competes with lymphocyte receptors for antigen. In fact

antigen is catabolized by body enzymes and then the rest of it is neutralized or blocked by the antibody leading to its gradual decrease to a concentration below the level which is necessary to sustain the immune response. In addition to this feedback mechanism further control is rendered by the antigen specific T suppressor cells. The T cell suppression mechanism is very complex and much less understood in comparison to the facts related to T cell help. For example, in addition to the direct activity of the main Ts subset expressing $Lyt1^{-}23^{+}$ and I-J region determinant, there is another feedback suppression mechanism of the specific helper response. Cantor et al (1978) have shown that Lyt $1^{+}23^{-}$, Qa 1^{+} helper cells activate B cells as well as a distinct T cell subset expressing $Lyt1^{+}23^{+}$,$Qa1^{+}$ which in turn suppresses helper activity by a feed-back mechanism.

Both the helper and suppressor cell sets seems to be activated by a multi-epitope antigen in the course of a normal immune response. An optimum net balance between these two antagonistic forces will lead to effective antibody formation, but an inbalance towards the dominance of suppression may lead to immunodeficiency or hypogammaglobulinemia as documented by Waldmann et al. (1977). Conversely, a loss in suppressor T cell function would be associated with formation of excess antibody, allergic reactions and autoimmune diseases.

The network theory : Idiotypic interactions : Anti-idiotypic antibody and T cell helpor suppression.

The selection process which induces clonal expansion

depends on the recognition of the unique receptors on the pecursor cell membrane. This happens through specific interactions with (external) antigens, but the V-region domains of idiotypic and anti-idiotypic determinants (idiotope and anti-idiotope) on the lymphocyte membrane serving as the 'internal images' of antigen can also perform this function. The possible contribution of idiotype recognition in clonal selection was first visualized by Niels Jerne who proposed the hypothesis of network regulation of immune response (Jerne, 1974, 1975).

Jerne explained that assuming there are of the order of 10^7 idiotopes in an individual, one set of combining sites (idiotopes) will almost certainly recognize another set of idiotopes and this initial set of idiotopes, in turn, will be recognized by another set and so on. This is particularly expected to happen because an antigen-antibody interaction is degenerate; that is, one combining site reacts with more than one epitope.

Since the combining sites which recognize the external antigens are the same that are capable of recognizing idiotypes they can be viewed as internal images of external antigens. Thus all immunoglobulin molecules are anti-idiotypic and any antibody-external antigen reaction will have useful internal cross reactivity. In this way, the whole immune system was hypothesized by Jerne as a closed network of constantly "talking" interacting molecules of cell receptor antibodies, anti-antibodies and anti-anti-antibodies etc. It was

also postulated that the idiotype anti idiotype interactions would perform clonal selection by expansion or deletion of specific clones. As a result, the network at a particular time would be in a state of dynamic equilibrium maintained between its contributing elements.

Anti idiotypic antibodies and their role in T-B co-operation.

Anti-idiotypic antibodies were first reported independantly by Oudin (1966) and Gell and Kelus (1967). That a rabbit can generate auto-anti-idiotypic antibodies against its own idiotypes (e.g. an antihapten) and that the resulting antibody reacts only with its own idiotypes was shown by Rodkey (1974). Synthesis of auto-anti idiotypic antibody spontaneously in the course of a normal immune response was also demonstrated by Cosenza (1976) using rough strain of P.pneumoniae bearing the phosphorylcholine epitope. These observations pointed theoretically to the great possibility of the participation of idiotype-anti idiotype system in the T-B cellular interactions.

This postulate was proved subsequently to be true by Eichmann's group (Eichman, Eichmann et al.and Hetzelberger and Eichmann, 1978) with a number of experiments using a carbohydrate determinant on Group A streptococci and in vivo and in vitro techniques. Without going into the details of experimental protocols and results, the overall conclusions are that (i) during clonal expansion of lymphocytes helper or suppressor signals may be delivered by the anti-idiotypic antibodies,

and (ii) though it apparently seems impossible, specific antibody, in fact, may be produced in the complete absence of the streptococcal antigen.

The existence of the elements to form an idiotypic network has been proved beyond doubt. Since the body cannot help making anti-idiotypic responses this network is bound to contribute to the modulation of immune responses. From the point of view of Jerne's network kept at a state of dynamic cybernetic equilibrium an immune response is viewed as a perturbation of the equilibrium due to the introduction of an external antigen and its localized concentration. This leads to clonal expansion of specific sets of cells which in turn start a selection process for appropriate sets of idiotype-bearing and idiotype-recognizing cells propagating a 'ripple' passing through the net with gradual dampening away from its source till the state of equilibrium is re-established.

But just how great is the contribution of this network in the normal immune responses is difficult to ascertain at present. There may be different possibilities, namely (i) it may be an extensive dynamic network in which all cells are constantly passing messages in between them or (ii) it may consist of interactions limited between immediate idiotype partners that may be expected to occur during clonal expansion of cells bearing antigen-

reactive idiotypes. Another crucial factor which is unknown is what is the relative importance of antigen and anti-idiotype in normal immunoregulation. The concluding remark by Strongberg (1980) in the summary on the theme "Idiotypy, Allotypy and network regulation" during the Fourth International Congress of Immunology may be cited here,".....and finally the physiological significance of the idiotype network remain at the present time open questions : it will require some sophisticated experimentation to outline the in vivo role of auto-anti-idiotype antibodies".

An overview of Immunoregulation.

The elements (e.g. antigen, antibody, macrophage, T_H, Ts cells, T-cell factors) and units of regulatory mechanisms(e.g. antigen processing, immune triggering and its control by H_2I gene control, T-B cell co-operation, Ts cell suppression, idiotype network etc.) participating in the humoral immunoregulation have already been discussed. The overall regulatory control may be briefly presented here.

How the extent and diversity of an immune response is controlled in an individual is not yet clear. Humoral responses may, at times, be extremely diverse and abundant, but at some other times be very restricted. The magnitude of the antigen-driven specific clonal expansion in lymphocytes and the resulting recruitment of effector plasma cells varies from antigen to antigen. What rules govern these variations are not easy to define.

It is worth mentioning that modern attempts for understanding immunoregulation have been tried by using oversimplified cybernetic models that have been given mathematical forms. Some of them were capable of predicting cell kinetics, antibody titers and affinities in reasonable agreement with experiements for both primary and secondary immune responses (Bell et al., 1978; Dibrov et al.,1977); but they were still far from serving satisfactory means for understanding complex aspects of immunoregulation primarily for discarding one or more cellular elements in the process for the sake of over-simplification.

Recent evidence also suggests that the various antagonistic functions performed by distinct cell subsets and lymphocyte factors serve the need to switch from one type of immune function to another. Bretscher (1977) proposed a theory on the rational assumption that triggering of immunocompetent cells, including antigen reactive T helper and T suppressor cells, is governed by parameters of antigenic stimulation which in turn depend on the density of foreign epitopes in antigen and its concentration. Different classes of precursor cells require different threshold levels of stimulation by antigen and T_H cells for triggering and the class hierarchy of responses extend from DTH with the lowest triggering threshold, to IgM and finally to IgG with the highest threshold. This hypothesis most elegantly accommodates the role of modulator cells.

The old main theories of immunoregulation such as

(i) self-antigens lead to the deletion of all anti-self reactive clones leaving only those solely able to react to foreign antigens, (ii) precise selection of a very small proportion of specifically reactive clones as a response to foreign antigens, seem to have been overthrown due to the impact of an enormous body of research in the last decade. A new 'paradigm" appears to have started to rule in the '80s. This holds that the diversity of self-epitopes and the degeneracy of binding of antigens to cell surfaces make it inevitable that most or all lymphocytes are initially stimulated in a positive way by self antigens. Also, any response to a foreign antigen will, probably unfailingly provoke a transitory stimulation of anti-self activity. Self-antigens have not been shown to possess systemic chemical differences from foreign antigen, but they do differ in being presented continuously to the host lymphocytes, from the early stage of ontogeny. Thus they may have a much greater impact on the developing repertoire of cells than was thought earlier. Vigorously expanding clones give rise to the formation of counteracting suppressor clones. Cells possessing high-affinity anti-self receptors are strongly suppressed early in ontogeny, but cells with only low affinity for self are much less strongly suppressed and remain capable of reacting to the foreign antigens. Various clones stay extensively interconnected through the "bridges" of antigen and idiotypes. An equilibrium is achieved between the effector cells, modulator cells and constant concentrations of self-antigens. An "immune response" is considered to be a perturbation of the

cybernetic steady state (equilibrium) due to the local concentration of a foreign antigen.

The role of G.I. tract in immunoregulation.

One of the most striking modulating effects exerted by the gut on the humoral immune responses is the tolerance to antigen in the systemic compartment observed when that antigen is presented locally on the mocosal surface. This phenomenon was reported as early as 1911 by Wells who demonstrated that guinea pigs that were fed previously with ovalbumin lost their ability to develop systemic anaphylaxis to it.

It was elaborated further by Chase (1946) in his classic experiments showing inhibiton of DTH to Dinitrochlorobenzene (DNCB) by prior feeding and was subsequently named the Sulzberger-Chase phenomenon.

Though the phenomenon of systemic unresponsiveness to an antigen by its prior feeding has been unquestionably documented, data concerning its mechanism are sparse and not very consistent. Antibody (Kagnoff, 1978; Chalon et al., 1979), immune complexes with IgA as the antibody (Andre et al., 1975) and a humoral suppressor factor (Kagnoff, 1980) have been implicated as the causative agent for this phenomenon; but the present consensus holds that modulator cells like T suppressors (Asherson et al 1977; Mattingly and Walksman, 1978; Miller and Hanson, 1979) and B suppressors (Asherson et al., 1977) play the main role. Elson et al. (1979) performed an important experiment to determine the role of T cells in the control of IgA, IgM and IgG synthesis in various mouse tissues. Ig synthesis in vitro was induced by a polyclonal

activator, lipopolysaccharide; the resulting productions of the three Igs were then measured by double antibody radio immunoassays; in addition, the effect of T cells on Ig-synthesis was determined by adding either ConA or ConA-pulsed T cells to LPS-driven indicator culture. In 7 day cultures, the most IgA was produced in lymphoid cells from P.P., less in cells from spleen and mesenteric lymph nodes (MLN), and little or none in peripheral lymph node (PLN) cells. IgM and IgG were produced approximately in equal amounts in all the tissues, except that IgM synthesis always predominated in the spleen cell culture. There were differences in T-cell regulatory activity for IgA, but not for IgM or IgG, in various mouse tissues. In particular, PP T cells were found to contain a high level of IgA T-cell helper activity compared to that of spleen or PLN; whereas, spleen contained predominantly IgA suppressor T-cells. On the basis of this Ig isotype-specific independent variation of T-cell regulatory activity the authors concluded that a separate subset of T-cells may exist which regulate IgA synthesis and that IgA class-specific T cells are distributed unequally among various mouse lymphoid tissues with a preponderance in the GALT. Gearhart and Cebra (1979) using bacteria-associated and non-environmental antigens and both germ free and conventional mice in their experiments concluded that the preponderance of IgA-specific T helper cells is dependent on an environmental influence. It is postulated that antigens in the gut, derived specially from bacteria, preferentially activate P P T cells by an unknown mechanism

to favour the generation of IgA helper T cells. The latter then selectively amplify the IgA B-cell precursor pool and presumably also expand IgA-memory B-cells.

There has been a sufficient body of data to support separately the two gut-related immune events e.g. (i) systemic unresponsiveness by prior feeding of antigen and (ii) production of secretory antibodies by antigen feeding; but few experiments were done to prove whether these two events occur concurrently in animals exposed to the antigen in the gut. Using soluble (OVA) or particulate (formalin killed Streptococcus mutans) antigens in orally primed and systemically (with CFA) challenged CBA/J mice Challacombe and Tomasi (1980) demonstrated that there was a concurrent production of specific IgA antibody in salivary glands and systemic suppression. All the available data further suggests a logical sequence of events, e.g. an ingested antigen may induce a brisk local IgA response, tending to inhibit further absorption of antigen by the process of so called antigen-exclusion, while concomitantly suppressing IgM and IgG antibody production so as to protect the host by preventing untoward systemic responses to the antigen.

The regulatory role of the liver as an immunological organ specially in the generation of Sulzberger-Chase phenomenon is not clear. Controversy exists as to whether patients with cirrhosis react excessively to parenteral vaccines but there is convincing evidence that patients with liver disease possess higher levels of antibodies to a variety of usual gastrointestinal antigens.

The effects of portacaval anastomosis on the production of splenic tolerance to ingested antigens is another controversial issue; but functional portacaval shunts as exist in cirrhosis has been found to lead hypergammaglobulinaemia.

Tolerance may be transmitted from mother to the offspring via the milk (Auerbach and Clark, 1975). Antigen injected into female mice within 24 hours of delivery could be detected in the colostrum; subsequent absorbtion of the antigen in intact form from the gut could produce tolerization to the specific antigen (Halsey and Benjamin, 1976).

Jarrett and Hall (1979) showed that IgE-specific factors transferred from mother to the young via colostrum could regulate the subsequent development of IgE antibody in the offspring. Evidence for the presence of IgE-specific suppressor cells in P P suggests that orally induced tolerance might lead to therapeutic manipulation of allergic diseases.

Immunoregulation by the gastro-intestinal tract also seems to be a complex process. The nature, timing and quantity of antigen, whether it is processed by epithelium, lymphoid aggregates, liver or some other peripheral lymphoid tissue, existing humoral antibody and the age of the host etc. are just a few amongst the variables that decide whether a particular response will cultimate in immunization or tolerance or the simultaneous existence of both.

In the initial sections of the General Introduction subjects like various kinds of antibody responses following different routes of antigen presentation, the nature of

mucosal antibody, the role of P P in the antigen - specific induction of humoral immunity in the gut and in supplying IgA precursor cells for the lamina propria etc. have been focussed on with relevant evidence and discussion.

General aspects of the regulation of humoral immune responses and the role of G.I. tract in such regulation were dealt with in the subsequent sections.

With this background one may reasonably expect that there should be some mechanism to ensure the preferntial production of larger amounts of IgA over the other major Ig classes when an antigen is presented locally as compared to its systemic application.

To investigate the possible mechanism of immunoregulation in response to antigen impinging on gut the following two hypotheses and principles for testing them were developed:-

1. Antigen is processed locally in such a way that it is mainly active in stimulating antigen-driven IgA committed B cells. The background to this hypothesis has not been discussed in the introduction. This omission reflects the absence of previous work in relation to a novel hypothesis to explain the phenomena following oral immunization.

To test this hypothesis if homogenates of PPs or their killed lymphocytes from antigen fed donor mice are transfused repeatedlyinto syngeneic recipients for priming, and such primed mice are challenged intravenously with the native antigen, the ratio of IgA/IgM antibody forming cells (PFC) in spleen would become higher compared to the ratio in mice receiving systemic priming and boosting

using similar immunizing schedules.

2. Local presentation of antigen stimulates modulators of the immune response, i.e. helper or suppressor cells or both, such that the ratio of IgA/IgM (or IgG)* in the resulting responses are of the 'local' kind.

To test this hypothesis if PP lymphocytes from mice fed repeatedly with antigen are adoptively transferred into syngeneic mice along with native antigen twice at an interval of two weeks, the secondary immune response (by spleen PFC assay), will be modulated ultimately in favour of IgA class dominance as compared to that of IgM class dominance expected by a similar immunization schedule using only antigen or antigen plus PP cells from normal mice.

Aims of the thesis.

The aims of this thesis are to test experimentally the two hypotheses presented above and to discuss the data in relation to the current status of knowledge on the subject of regulation of antibody responses.

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*IgG responses have not been studied in this thesis.

CHAPTER II

MATERIALS & METHODS

ANTIGENS

Bacterial strain, background information.

A strain of classical V.cholerae, serotype Inaba 569B, Vr. Beads VII was taken from the departmental lyophilized culture collection. It was specially selected for the property of adherence to glass Ballotini beads (Selby, Australia; hence the name Beads VII) from which it was not removed by three sequential washings with saline.

The strain was originally obtained from Mr. I. Huq of the International Centre for Diarrhoeal Disease Research, Bangladesh, Dacca, and in 1971, Dr. S. Neoh had serially passaged it ten times through infant mice to increase its virulence. The oral LD_{50} dose for the same animal model decreased from $2x10^8$ to $4.5x10^5$ viable vibrios per mouse by this process. Subsequently the strain was made streptomycin-resistant by selecting on streptomycin-containing (100µg/ml) nutrient agar plates to facilitate animal experiments requiring oral or intestinal applications followed by enumeration on streptomycin-incorporated culture plates with resulting suppression of indigenous gut flora.

Selection for property of adherence.

The one most important property of an intestinal pathogen which can counteract the nonspecific innate defence mechanisms like flushing effects of peristaltic contractions (Dixon, 1960; Freter, 1956; Gibbons, 1977), the continuous stream of secretions assisted by beating of villi (Gibbons, 1977) and continuous desquamation of the epithelial lining (Gibbons and Houte, 1975) is its adherence. Correlation between the property of adherence in V.cholerae and its

virulence in the infant mouse model were shown earlier in this laboratory (Attridge, 1979). For this reason the strain of classical V.cholerae specially selected for this property on Ballotini glass beads, was selected as the antigen.

Maintenance and growth.

This strain of V.cholerae was used for all the experiments and was stocked by freeze drying in skimmed milk in numerous ampoules and stored at -20°. Each month one ampoule was opened and subcultured into 20 slopes of 1% Trypticase-1% saline agar. All cultures were made directly from one of these slopes. Where large numbers of organisms were needed for feeding mice they were grown as shake-cultures for about 10 hours using 1 litre brain heart infusion broth in 5-L conical flasks.

Determination of doses of V. cholerae for various immunization schedules.

Bacterial growth in a culture was estimated from a standard curve relating O.D. at 650 nm to viable count.

Extraction and isolation of lipopolysaccharide from V.cholerae.

The technique was based on hot phenol-water extraction originally described by Westphal et al in 1952. A log-phase culture of V.cholerae was suspended in saline after washing, at a concentration of 20mg/ml. An equal volume of 90% phenol was added and the mixture, at 65°to 68°C, was continuously stirred for 10 minutes before cooling to 10°C in an ice bath. After centrifugation for 15 mins at 3,000 rpm the top aqueous layer was carefully collected avoiding the

phenol layer containing proteins and dialyzed against running tap water overnight to remove dissolved phenol. The LPS was precipitated by stirring into 5 times its volume of cold ethanol. The pellet obtained by spinning in the cold for 30 minutes at 3,000 r.p.m. was dissolved in a small volume of distilled water and LPS was then spun out of this by ultracentrifuging for two hours at 38,000 r.p.m. This crude LPS extract contained about 4 to 5% protein but was adequate for erythrocyte-sensitization.

Dry weight estimation.

A small measured aliquot (o.2 ml) of a new batch of LPS was dried in an aluminium-foil crucible to constant weight. The dose of LPS used for sensitization of SRBC was on the basis of this total dry weight/volume.

Alkali treatment of LPS.

A sample of LPS was initially diluted in distilled water to contain 2mg dry weight/ml and mixed with an equal volume of 0.04 molar NaOH. After incubating overnight at room temperature the pH was adjusted to 7.0. The LPS was then stored at $4^{o}C$ with 0.1% sodium azide.

Standardization of the dose for sensitizing SRBC.

The amount of alkali-treated LPS used for sensitizing SRBC was in the order of 50µg/ml of LPS dry weight in saline in which well-washed SRBC were suspended at a final concentration of 2.5%. For a new batch of LPS the dose was verified for effective sensitization by a passive hemagglution titration of a known anti-V.cholerae serum and PFC assay in

reference to a known batch of LPS.

Antibodies.

1. Rabbit anti-mouse sIgA was kindly provided by Mr. David Haynes who raised it by injecting rabbits with a purified MOPC-315 IgA myeloma protein obtained from Dr. G.J. Russell-Jones. The purification method of the myeloma protein was published elsewhere (Russell-Jones et al, 1980). Briefly, IgA was isolated from a pool of sera and ascitic fluid of MOPC-315 plasmacytoma-bearing BALB/c mice by adsorption to DNP-Sepharose and elution with 0.1M DNP-glycine. Trace amounts of contaminating IgM antibody were removed by an affinity column made of anti-μ chain antibody coupled to CN Br-activated Sepharose. IgG antibodies were removed by a protein A-Sepharose column at pH8. The preparation was finally purified by passing through Sepharose 6B column yielding a major protein peak that eluted between IgM and IgG markers.

The antiserum raised against this purified IgA protein, on double immunodiffusion analysis, showed one precipitin band against normal mouse serum that had reaction of identity with an adjacent band formed by specific anti-mouse IgA but not with those formed by anti-mouse IgM or IgG anti-bodies.

2. Rabbit affinity-purified anti-mouse IgG(L/γchain) antibody was a generous gift from Dr. P.L. Ey. A rabbit was immunized against mouse IgG_{2a}. The resulting antibody was passed through an affinity column made of mouse IgG_1 coupled to sepharose 4B. The anti-γ2a-specific antibody was voided

and the resulting purified anti-mouse IgG was recovered from the column by a low-pH buffer, and concentrated by standard procedures. The final product contained mainly anti-light and some anti-gamma heavy chain antibodies.

3. FITC conjugated goat anti-mouse gamma-globulin.

Fluorescin labelled goat anti-mouse gamma globulin (F-Y Laboratory, U.S.A., Lot 0111C FA/-2306) known to have a working titer of 1/60, was obtained as a gift from Mr. Ian Beckman of the Department of Immunology, Flinders Medical Centre, S.A. It was used at a dilution of 1/50 to stain surface-membrane-immunoglobulin-positive (B cells) cells.

Animals.

As much of the work comprised adoptive transfer of lymphocytes, inbred CBA mice were used for almost the whole work. They were bred and maintained in the animal house of this medical school. Both sexes were used between 6 to 12 weeks of age. Matching of age and sex in groups of mice used in the test and control of an experiment was done as far as practicable depending on supply.

Conventionalized LAC specific pathogen free (spf) outbred white mice, due to their easy availability, were used for setting up or checking reproducibility of methods.

Lymphocyte preparations.

1. Spleen.

A mouse spleen was transferred into a glass tissue homogenized together with 1 ml of Hank's balanced salt

solution (HBSS) with 5% FCS. The spleen was homogenized with three gentle in and out movements of the plunger and strained through a 300-mesh nickel-steel wire guage.

The cells were counted for viability in an improved Neubauer hemocytometer chamber after mixing a suitably HBSS-diluted suspension with a known volume of trypan blue solution.

2. Lamina propria of small intestine

Various techniques of isolation of lymphocytes from the lamina propria of small intestines were tried before a final one was evolved. They were, chronologically, as follows: (i) scraping out of mucosa with a scalpel blade followed by homgenization in HBSS with a glass tissue grinder, (ii) dispersion of the scrapings by brisk agitation in a 10ml vial (Bloom, 1979), (iii) enzymatic isolation of cells by collagenase using a pool of open gut-pieces from at least three mice in a 25 ml conical flask agitating on a magnetic stirrer at 37^{o}C, (iv) collagenase treatment of gut pieces from one mouse in a 10ml vial at 37^{o}C on a magnetic stirrer using a small flea. For brevity, the details of only the finally accepted technique (iv) will be described.

To obtain single cell suspensions of viable, functionally active lymphocytes from murine small bowel lamina propria has not been easy. There has been controversy as to the desirability of adopting mechanical or enzymatic methods of cell disaggregation. But not until Bland et al, 1979, published their comparison between both techniques using antibody-dependent cellular cytotoxicity for assessing functional activity of the isolated lymphocytes was the superiority of

the enzymatic method evident. They demonstrated that mechanical homogenization stimulated the synthesis of prostaglandin E_2 which was responsible for the lack of cytotoxic activity in mechanically liberated lamina propria lymphocytes (LPL).

For this reason an enzymatic method of Cebra et al (1977) was adopted for isolation of LPL after a substantial modification. Clumping of the isolated LPL during washing was a formidable problem. After much experimentation clumping finally could be prevented by selectively depleting HBSS-5% FCS of its Ca^{++} ions by the addition of ethylen glycol-bis-(β-amino ethyl ether) N,N -tetra acetic acid (EGTA), Sigma. It was added just before each centrifugation of the cells for wash. The pellets were also initially resuspended in a small volume of Ca^{++}-depleted medium.

The enzymatic isolation of LPL is described below as a flow-chart:

The small bowel was flushed out with 15 ml of chilled saline using a long blunted cannula fitted with a 25ml syringe, then washed outside also with saline.

↓

All Peyer's patches were cut out with scissors and a toothed forceps whilst keeping the cannula inside the gut.

↓

The gut was slit open longitudinally in its entire length and cut into pieces of about 1 cm. length.

↓

Gut pieces were gently washed with chilled saline in a 10ml vial and kept on ice in a ~ 4 ml of HBSS-5% FCS. When six bottles containing the intestinal pieces from 6 mice were at this stage they were all treated successively as follows:

(a) The bowel pieces were rinsed in Ca^{++}-Mg^{++}-free HBSS containing 0.05% EDTA twice each rinse lasting for about 20 seconds.

(b) 6 ml of 0.5m M dithiothrietol in the same medium as above but pH fixed at 7.6 in presence of 30mM HEPES was added in each bottle and treated for 6 to 8 minutes at room temperature with a gentle but almost continous shaking by hand. The Dithiothrietol solution was then discarded, ~ 6 ml of HBSS-5% FCS was added and kept on ice for 5 minutes with a few gentle swirling movements before being discarded.

(c) 8 ml collagenase solution (10 units per ml) in HBSS-20% FCS (pH 7.6 in presence of 30 mM HEPES) was then added.

The bottle content was then stirred very gently at 37°C for 15 to 30 minutes, increasing opalescence being a guide to the degree of detachment of mucosal cells.

↓

Each of the liberated cell suspensions containing gut material from one mouse was strained in succession through a 100 mesh wire gauze and a pledget of cotton wool placed on a small funnel, and gradually washed down with HBSS-5% FCS up to a volume of 15 ml in a conical graduated centrifuge tube.

↓

After adding and mixing 15 microliter of 0.2M EGTA for selective depletion of Ca^{++} the cells were packed in a table top clincal MSE centrifuge using a swingout head for 6 minutes at 400g.

↓

After discarding the supernatant completely the pellet was resuspended gently in Ca^{++} depleted HBSS-5% FCS manually.

↓

The cells were washed once with 15 ml of HBSS-5% FCS and resuspended in 0.3 to 0.5 ml of EGTA medium as above to prevent clumping. This suspension was either immediately diluted 1/5 or 1/10 in standard HBSS-5% FCS or plated out for PFC assay as soon as possible to avoid keeping the cells in a Ca++ deprived environment.

TABLE 2.1

NUMBERS OF LYMPHOCYTES ISOLATED PER MOUSE

	Mechanical isolation		Enzymatic isolation	
	Homogenization in a glass tissue grinder	Brisk agitation in a 10ml vial	A pool of gut-pieces from 3 mice in a conical flask placed in the centre of a stirrer with bigger magnetic flea	Gut pieces from a single mouse in a 10ml vial placed at the periphery of the stirrer with a small flea
Lamina propria	$1.85\pm 1.3\times10^{6}$	$5.2\pm1.9\times10^{6}$	$5.8\pm 2.4\times10^{6}$	$4.2\pm 3.3\times10^{6}$
Peyer's patches	$\leq 6\times10^{6}$	N.D.	Pool of PP 1.4×10^{7} (normal mice) 2.8×10^{7} (orally primed)	N.D.

Range of cells isolated per mouse gut by different methods.

The mononuclear cells in the single-cell-preparations from lamina propria, spleen and Peyer's patches that looked morphologically like lymphocytes during viable counts by Trypan blue exclusion are called lymphocytes for the plaquing assays of this thesis. In the latter two tissues, which are predominantly of lymphoid nature, the error is likely to be small. With the cell-preparations from lamina propria the probable large errors in true lymphocyte counts would be cancelled out by the fact that similar critera were adopted in the cell-preparations of the control mice in each experiment.

Table 2.1 compares the efficacy of the methods used for the isolation of LPL. Brisk agitation in a 10 ml vial gave a significantly greater yield of cells than homogenization with a glass tissue grinder.

The efficacy of the enzymatic method when a pool of gut pieces from three mice were treated in a flask was not much better than using gut pieces of a single mouse in a 10 ml vial.

Peyer's patches.

Though initially PPL were isolated mechanically using a glass tissue-grinder the enzymatic method was finally adopted using pooled PP harvested from 10-15 mice at a time.

Enzymatic method of isolation of PPL

Peyer's patches from the small bowels of all the mice, after adequate intraluminal and external washings with chilled normal saline, were cut out from outside with small surgical scissors and collected in a 10ml conical flask in HBSS-5% FCS

under ice. Normal saline was used in case of cells that were destined to be killed by formalin.

Two quick rinses in Ca^{++}-Mg^{++}-free HBSS containing 0.05% EDTA and treatment in 0.5mM dithiothreitol in the same medium for ~ 6 minutes were the same as described in the technique with LPL.

PP were transferred in a small wide-mouthed bottle (about 4 cm long and 2 cm wide at the mouth) and were minced thoroughly in a few drops of collagenase solution into ~ 1 mm^3 pieces with the help of a curved scissors. The minced suspension was treated with collagenase solution twice for 20 and 15 minutes and cells harvested using similar methods adopted for the isolation of PPL.

Viability of cells in lymphocyte preparations.

Viable cells in the lymphocyte preparations were counted by the Trypan blue exclusion method on a hemocytometer. In all the cell preparations the viability was always more than 95% except for the Xylocaine-recovered B cells from the antibody coated plastic surface of the tissue culture flask during T and B cell separation. The viability in this case was about 90%.

Separation of T and B cells by the use of a plastic surface coated with anti-Ig antibodies ('Panning')

The most efficient method to separate cell-subpopulation with defined characters from a mixed lymphoid cell population utilizes differentially expressed cell surface markers. For example, a T cell-enriched subpopulation may be obtained by deleting the B cells from a mixed population by means of

their specific surface membrane immunoglobulins. This type of negative selection may be done by complement-mediated lysis of the particular subpopulation using an antibody against a specific cell-surface marker, rosetting them out with the antibody coated SRBC (direct rosetting, Parish et al, 1974, Walker et al., 1979; or based on a hapten-sandwich modifcation, Wofsy et al., 1978) or allowing them to adhere to an antibody-coated flat plastic surface (Mage et al., 1977; Wysocki and Sato, 1978). The method of Mage has the advantage that it is simple and rates of cell recovery are high. It can also be used in the way of positive selection (e.g. recovery of B cells that adheres to the antibody-coated surface). In fact, it appears to be capable of partitioning between T and B cells from a mixed population and those separated cells can be used simultaneously for either functional assays or transfer experiments. So this method was adopted as described by Lewis and Kamin (1980), without any major modification for partitioning Peyer's patch cells from orally primed mice into T and B enriched subpopulations for use in adoptive transfer experiments.

Principle of the method.

A polystyrene tissue culture flask, serving as an insoluble flat matrix, was first coated with rabbit affinity-purified anti-mouse IgG (L/γ) antibody. A mixed cell suspension from PP of orally primed mice was added to the flask after removal of the antibody and washing the flask surface. B cells bound to the antibody coated surface, but T cells did not. The highly enriched non-adherent T cells were then gently pipetted out. After proper washing of the flask again,

the adherent B cells were finally detached with the help of xylocaine and recovered for use.

Special materials and reagents.

(i) Plastic tissue culture flask. Corning glass No. 25100

(ii) Xylocaine hydrochloride (Lidocaine brand), 5 ml ampoule containing 100mg, diluted 1 in 5 in Ca^{++} Mg^{++}-free PBS for working strength of 5 mg/ml

(iii) FITC-cogugated goat antimouse gamma-globulin

(iv) Rabbit affinity-purified anti-mouse IgG (L/γ chain) antibody.

Details of the antisera No. (iii) and (iv) have been given earlier (page 44-45).

The cell-separation technique is described in flow chart:
Precoating of plastic flask with anti-Ig.

Two ml. rabbit affinity-purified anti-mouse IgG (L/γ chain) antibody at a concentration of 1 mg antibody/ml in PBS was added to a flat plastic culture flask and kept on a level rack at 4^{o}c for at least 18 hours.

↓

Immediately before use the antibody was carefully pipetted off and transferred to another flask to be used serially six times.

↓

The flask was washed three times with 5 mls of cold PBS, kept cold and used within two hours.

T cell enrichment

A single-cell lymphocyte suspension was prepared from a pool of PP of orally-primed donor mice as described before (page 49). The cell-suspension was adjusted to a

concentration of 1.5×10^7 viable PPL/ml. 3 ml or less of this PPL suspension was added to the ready-to-use antibody precoated flask and was incubated on a level table for 30 minutes at room temperature (20°-25°C).

↓

The flask was given a few gentle swirling movements and incubated similarly for another 30 minutes.

↓

The non-adherent T cells were gently resuspended by rocking movements of the flask and were carefully harvested by a pasteur pipette taking care not to dislodge the adherent B cells from the bottom.

↓

The enriched T cells were used for adoptive transfer after one wash in HBSS-5% FCS.

B cell enrichment (by removal of adherent cells).

After harvesting the non-adherent T cells the adherent cell layer was washed gently four times with 5 ml of Ca^{++}-Mg^{++}-free PBS taking care as before not to dislodge the cells.

↓

2.5 ml of xylocaine solution (5µg/ml) in the same PBS was added to the flask and allowed to stand 10-12 minutes at room temperature on a level table.

↓

Using a pasteur pipette the adherent cells were dislodged by vigorously pipetting up and down. (This step was repeated once more when satisfactory removal of cells was not obtained the first time).

↓

Finally the flask was rinsed with HBSS-5% FCS and the cells were washed once before using them for adoptive transfer. The cells were washed two more times before immunofluorescent staining.

Determination of the purity of T enriched PPL subpopulation: Percentage of SmIg positive cells (B cells) by immunofluorescent staining.

Aliquots comprising ~ $5x10^6$ viable cells of T and B enriched subpopulations, as well as the pre-separation mixed cells from Peyer's patches or spleen were taken in conical centrifuge tubes and washed twice in PBS.

0.05 ml of the working solution in PBS (1/50) of the FITC-conjugated goat anti-mouse gamma globulin was then added to each tube, mixed thoroughly and incubated on ice for ½ hour. The cells were then washed three times by centrifuging in swing out head to remove all unbound conjugates.

The pellet was suspended in one drop of glycerol-glycine mounting medium and the cells were examined on a microscope slide under a cover-slip sealed with nail varnish.

Fluorescence positive cells (due to stained SmIg) in each field were counted promptly after excitation with ultraviolet epi-illumination using suitable filters.

The total lymphocytes were counted next in the same microscopic field using tungsten light and the percentage of fluorescent cells with reference to the total lymphocytes was determined for each of the subpopulations and the mixed suspension.

Data supporting the efficacy of the cell separation method.

Lewis and Kamin (1980) in describing the method commented that the procedure should give a 15 to 30% yield

of T cells from mouse spleen containing 5% or less SmIg-positive cells.

In the pilot experiment using a spleen cell suspension the yield of nonadherent T cells was 34.5%. This T cell population contained only 4% SmIg-positive B cells. These results were very similar to the above data and the percentage of T cells was almost exactly the same as has been well documented by others (35%; Hudson and Hay, 1980). The average by other non-adherent T cells in Peyer's patch lymphocytes by 6 experiments was 30.74 ± 4%. In this T cell population the average content of SmIg-positive cells in 3 of the 6 experiments by immunofluorescent examinations was 6.97 ± 1.25%. Similar kind of data on mouse PPL are not available. The recovered adherent B cells enumerated in two experiments using spleen cells in one and PPL in the other were 41.4% and 60% respectively. Amongst these adherent cells SmIg positives were 66.8% and 80% respectively. In these two experiments the combined nonadherent and adherent cells accounted for 76% and 93% respectively of the total mixed cells applied to the antibody-coated flasks.

ASSAYS.

Direct bacterial hemagglutination : Quantitation of the property of adherence in V.cholerae.

Five-hour young cultures of different strains of V.cholerae in nutrient broth were centrifuged and washed once in saline. Their thick saline suspensions of ($5x10^{10}$ to $1.5x10^{11}$ viable vibrios/ml) equal optical densities at 650 nm were diluted in isotonic PBS on a microtiter plate (Cook Engineering, Philadelphia) by serial two-fold dilutions

in 25 microliter volums. A well-washed suspension of 0.5% SRBC in saline was added in 25 microliter volumes to all the wells containing the serial dilutions of the bacterial suspensions, incubated for 30 minutes at 37°c followed by 30 minutes at room temperature (15 to 20°c).

A nonenteropathogenic and nontoxigenic strain of fecal E.coli was titered by the identical procedures as control. While the selected adherent strain of V.cholerae 569B, Beads VII showed in triplicate determinations a reciprocal titer of 512-1024 the control E.coli strain had a titer of <2

Indirect hemagglutination

Sensitization of SRBC with LPS.

Well washed 2.5% (v/v) SRBC were suspended in saline containing 50 µg/ml (w/v) alkali-treated purified LPS. The reactants were incubated at 37°c for two hours on a roller drum. The sensitized erythrocytes (LPS-SRBC) were washed three times and adjusted to 0.75% (v/v)

Hemagglutination (HA) titration.

Anti-V.cholerae antibody was diluted serially on microtiter perspex plates (Cook Engineering), mixed with equal amounts of the LPS-SRBC suspension followed by incubation for 1 hour at 37°c. After keeping the plates for 1 hour at room temperature end points of the titration were read.

Indirect hemagglutination was mostly used either as an indicator for sensitization of SRBC with LPS prior to Jerne PFC assay or to compare the efficacy of a new batch of extracted LPS with reference to a an earlier batch.

PFC assay of Jerne and Nordin.

Jerne PFC assay was used for analysis of the antibody responses in all the experiments included in this thesis.

In this assay lymphocytes from an immunized animal are mixed with a dense suspension of indicator SRBC and spread in a thin layer of gel (Jerne and Nordin, 1963) or liquid matrix (Cunningham, 1965). The indicator SRBCs are prepared by attaching the specific antigen (in this case V.cholerae-LPS) to them. Following incubation the plasma cells secrete antibodies which sensitize the indicator SRBC surrounding the antibody forming cells. When complement is added plaques or holes appear against the background of erythrocyte suspensions due to the lysis of the indicator SRBC indentifying single specific antibody-forming cells. This permits counting all of the crop of specific plasma cells in a measured volume of a lymphocyte preparation even if the crop is a small minority of the total population.

The above method (direct method) allows enumeration of cells secreting IgM, which is a high-efficiency antibody class in terms of its complement-mediated lytic property.

Lymphocytes secreting other Ig classes like IgG or IgA can only be identified by plaquing with the help of the corresponding amplifying anti-Ig sera. Other Ig classes, theoretically speaking, will require more than one adjacent molecule to trigger the lytic complement pathway and so the

small amounts secreted by the plasma cells are not capable of producing plaques. The anti-Ig antibodies of the amplifying serum attach to the specific antibody molecules that were fixed to the indicator SRBC during the first incubation. These bigger sized Ig-anti-Ig complexes attached to the erythrocytes fix complement on subsequent application and bring out plaques by hemolysis. The number of cells forming Ig-class specific indirect plaques may be computed approximately by subtracting the number of direct plaques from the total number measured by indirect plaquing. But for precise enumeration an indirect assay should be performed using reagents that inhibit the formation of IgM plaques.

It should be mentioned here that the commonly held assumption that the direct method enumerates only the IgM producing cells may not be wholly true specially in the case of the secondary immune responses. Pasanen and Makela (1969) demonstrated that some of the anti-NNP direct plaques with densely coupled indicator cells were due to the production of IgG; on the other hand, some IgM-producing cells may not form plaques directly (Plotz et al 1968, Sell et al,1970).

A modification of the original Jerne technique using agar gel and a petri dish was adopted. One of the main disadvantages of the gel-plate method, namely the consumption of large amounts of reagents (specially the expensive facilitating anti-sIgA) was obviated by the use of small petri dishes (diameters: 50 and 45 mm) instead of standard ones (diameter: 85mm). The greatest advantage of the gel-plate method namely the high number of plaques countable with accuracy (~700 in standard plates) was

partially restricted by the use of smaller plates (~200 for 45 mm and 250 for 50 mm plates). The limit of countable number was still far superior to that of other types of microassays (e.g. Cunningham's method) leading to more reliable estimates.

For the enumeration of indirect plaques the single set of plates were used and while counting the direct plaques they were masked by a waterproof fine felt-pen. During a second count for the indirect plaques following augmentation and second application of complement the masked direct plaques were not counted.

Technical details of the assay.

Indicator SRBCs coated with V.cholerae-LPS (vide page 56) and lymphocyte single-cell suspension from spleen (vide page 45) or lamina propria of small intestine of mouse (vide page 46) were prepared as described before. A requisite amount of 0.7% agarose in RPMI medium containing 10% decomplemented fetal calf serum (FCS) was kept in a water bath at 48°C. This was made by mixing equal amounts of hot (48°C) 2X RPMI-20% FCS and 1.4% agarose in deionized double-distilled water, boiled for 30 to 40 minutes and cooled to the same temperature. The required number of 5 ml disposable serologic plastic tubes were kept warm in the water bath labelled and arranged according to the protocol of the assay.

A volume of 50 microlitres of 30% indicator SRBC were distributed in 6 to 10 tubes. Immediately before plating, 0.1 ml of suitably diluted lymphocyte preparation was added to a tube containing the indicator erythrocytes that was just lifted from the water batch. One ml. of agarose in RPMI was

quickly transferred to the tube with the help of a shortened and marked pasteur pipette which was kept heated in the water batch After mixing the reagents by 2 or 3 rapid but gentle rolling movements between the palms the mixture was poured in a petri dish (diameter: 50mm) avoiding bubbles, quickly spread into a thin layer by rocking movements in different directions and placed to solidify on a level surface. Reciprocal dilutions of 10,100 and 500 for spleen and neat, 5 and 10 for lamina propria were usually used for the assay. After gelling of the agar in all the plates of the experiment they were incubated for 1 to 1¼ hours at 37°c followed by addition of 0.7 ml of 1:25 guinea pig complement per plate and a further incubation of 30 to 45 minutes. Following removal of the complement by a pasteur pipette connected to a faucet the direct plaques were counted and masked carefully by fine-pointed felt pen so that they could be avoided during counting IgA plaques for the second time.

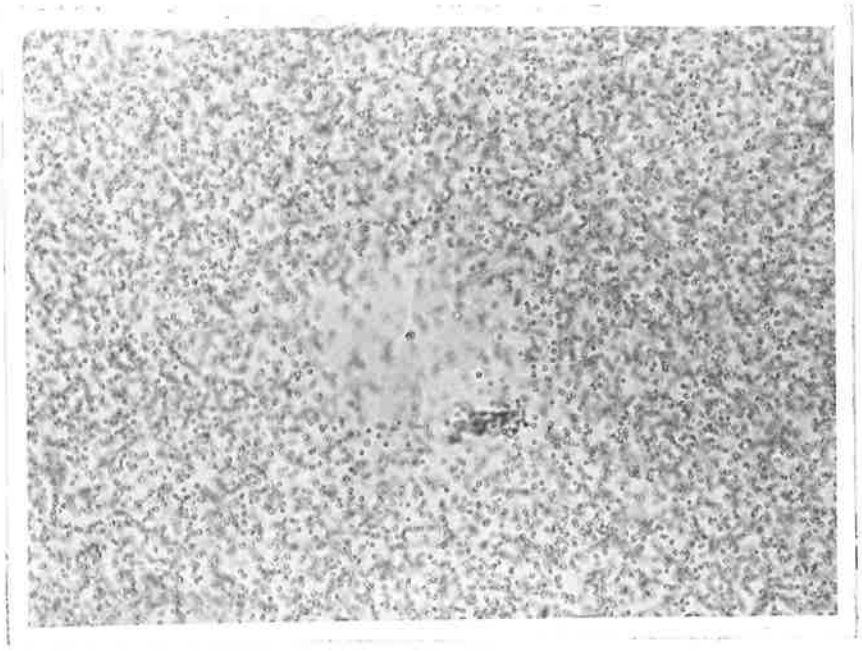
Now 0.7 ml of the optimum dilution of the facilitating anti-IgA serum was added to each plate, incubated for 45 to 60 minutes, serum poured off and again incubated for 30 minutes with complement. After discarding the complement the indirect plaques that were recognised with confidence with the help of a 'magni-viewer' or 'Optivisor' were counted and marked off with a different coloured felt pen and the doubtful plaques were encircled singularly or in batches of not more than three. These doubtful plaques were all examined with the low-power dry lens of a microscope from the back of the plate. A true plaque always possessed a lymphocyte in the centre of the zone of hemolysis but a false plaque had things like an air bubble, undissolved agar particle, clumped cells, suspected protozoal body producing hemolysins

etc. During counting of direct plaques the number of false plaques were negligibly small; but during counting of indirect plaques if the doubtful plaques were not examined carefully there could be an error of up to 30% depending upon the total number of plaques obtained. With experience, of course, confidence in identifying true plaques with the visual aid of only an Optivisor increased greatly (see photographs for microscopic pictures of true and false plaques).

Immunization schedule.

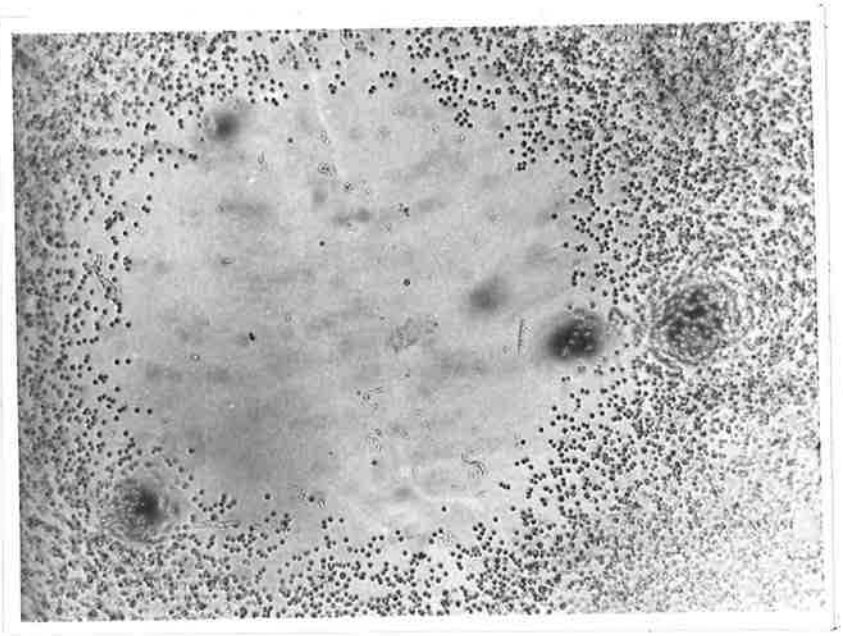
The general experimental design has been described in the preamble of each chapter concerning a particular group of experiments. The details of immunization schedules have also been given in protocols in tabular forms facing the tablesof experimental results.

Those details, though expected to be present in this chapter, are not included here to avoid repetition.

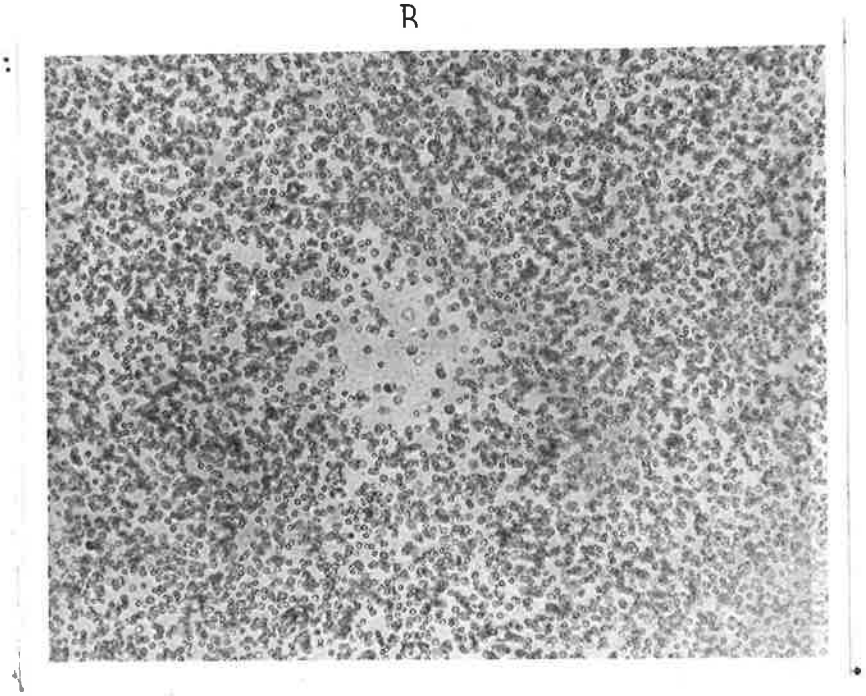


A true hemolytic plaque showing a centrally situated antibody-forming plasma cell possessing a typical eccentric nucleus. It is an indirect plaque facilitated by anti-IgA serum and of rather less than medium size. Not infrequently some erythrocytes, more commonly in indirect plaques, may remain intact in the hemolytic zone as seen in this photomicrograph. (They are not sharp because of their presence at other levels than that of critical focusing). (Total magnification is about 2000 times and all the photographs shown are of the same scale).

A



B



Two examples of false hemolytic plaques. A : A false plaque probably caused by an air bubble. Note the absence of a central cell.
B : a small hemolytic zone with quite irregular margins. There are more than one leucocyte present, but none at the centre.

CHAPTER III

CHARACTERIZATION OF ANTIBODY RESPONSES TO V.CHOLERAE IN MICE BY DIFFERENT ROUTES OF ANTIGEN PRESENTATION USING JERNE PFC ASSAY.

Preamble

As the main theme of the thesis was modulation of immune responses to V. cholerae in mice a prerequisite was to perform a series of preliminary studies to establish different types of antibody responses to V.cholerae in mice using various immunization schedules.

The initial protocols were chosen on the basis of results obtained by Dr. L. Bloom working previously in this laborary (Bloom-Thesis, 1979).

These experiments were performed using four broad immunization patterns : (1) both priming and boosting by intravenous route, (2) oral* priming followed by parenteral boosting, (3) priming and boosting by oral route, and (4) intraperitoneal (ip) priming with LPS from V.cholerae followed by oral boosting. Living V.cholerae was used as antigen in all these experiments except in i.p. priming followed by oral boosting in which phenol-water extracted purified LPS from V.cholerae was also used.

Jerne's hemolytic PFC assay was exclusively used in these studies because this assay is one of the most sensitive and direct methods (when expressed in PFC per defined number of viable lymphoid cells) to quantitate Ig-class-specific antibody responses on a comparative basis simultaneously in the spleen as well as gut lamina propria. The efficiency achieved in performing this critical assay during these initial

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*All 'oral' primings and boostings mentioned in this thesis have actually been done by intragastric feeding with a blunt-end needle instead of using the true oral route, as, for example, is done by mixing Ag with food or water. For convenience the 'intragastric' route has been referred to as 'oral' throughout this thesis.

experiments ensured its use in further studies on immunoregulation by gut presentation of antigen.

In these experiments the estimations of antibody responses were restricted to the peak or near peak periods of antibody responses. The times were selected on the basis of preliminary results. On days when spleen and gut assays were to be done simultaneously, spleen assays had to be carried out by pooling spleens of all the mice of the day's experiment, the results being, theoretically, equal to arithmetic means of unperformed individual estimations. As inter-mouse variations have not been studied here, this particular data has to be interpreted with caution in the search for generalisations on the various immune responses observed.

Experimental results

(1) Priming and boosting both by i.v. route.

Systemic (splenic) and local antibody responses in gut lamina propria to V. cholerae in mice after intravenous priming with 10^6 (three injections), 10^7 and 10^8 V. cholerae followed by a booster of 10^7 (10^8 in 10^8-primed group) intravenous V. cholerae are summarised in table 3.1. In each of the groups of experiments 5 to 6 mice were used and the results are tabulated using geometric means of IgM and IgA PFC per 10^7 spleen cells or lamina propria lymphoid cells (LPL) obtained in individual mice. The lowest and highest observations in each experiment are given in parentheses to show the range.

The table shows that the magnitudes of both IgM and IgA responses in the 10^6-priming group of experiments at peak or near peak-response periods of day 5-6 after the

TABLE 3.1

ANTIBODY RESPONSE TO *V. cholerae*

AFTER I.V. PRIMING AND BOOSTING

(Geometric means, range in parentheses)

Immunization schedules			PFC response/10^7 spleen cells		
Priming	Booster~14 days after last priming	Day of assay after booster	IgM	IgA	IgA/IgM
10^6x3 times at weekly intervals	10^7	5 (n=6)	903 (302-3793)	278 (67-827)	1/3.2
		6 (n=5)	619 (140-4372)	224 (45-1970)	1/2.4
		8 (n=6)	327 (180-2216)	59 (37-309)	1/6.7
10^7, once	10^7 **	3 (n=5)	N D	N D	
		5 (n=5)	9975*	2539*	1/3.9
		7 (n=5)	8733*	1931*	1/4.5
		9 (n=5)	7265*	1600*	1/4.5
10^8, once	10^8	4 (n=6)	8646 (5538-14,239)	840 (541-1818)	1/10.3
		6 (n=6)	5685 (1904-17,521)	344 (128-1580)	1/16.5

n= number of mice in the experiment
* A pool of spleens of all the mice in the experiment was assayed
** Mice were individually assayed for gut response in these experiments. Except some not significant IgA plaques on day 3 after booster, no gut plaques were detected on other days.

ND -Not done

booster are much lower than those with the 10^7 and 10^8 priming groups. The average of the geometric means of IgM PFCs on day 5 and 6 in the "10^6-group" is 9.4 and 13 fold less than those, on day 5, of the "10^7-group" and the average on day 4 and 6 of the "10^8-group" experiments respectively. Similarly, the average of IgA PFC numbers on day 5 and 6 of the "10^6-group" was 10.1 and 3.3 fold less than those on day 5 of the "10^7-group" and day 4 of the "10^8-groups respectively. The difference between IgA results of 10^6-group on day 6 and that of 10^8-group on the same day, however, was not high.

There seems to be no difference between the day 4-and 6 - IgM responses of the 10^8, and day 5 - IgM response of the 10^7 priming groups, whereas the IgA responses at this time in the 10^8 group are much smaller than those of the 10^7-group. In the 10^6 priming group there was a sharp decline in the IgM PFC number from day 5 through to day 8, with a decrement of more than 30% at each step on day 6 and day 8, whereas, decrease of IgM PFC number in the 10^7-group from day 5 through to day 9 was gradual with an average decrement of 15% at each step. Similarly the drop of IgA PFC count from day 5 to day 8 in the 10^6-group was 79%, but the equivalent drop from day 5 to day 9 in the 10^7-group was only 36%. The interesting finding is that the IgA/IgM ratio on both days 4 and 6 in the 10^8-group are much smaller than those on day 5 in the 10^7 and on days 5 and 6 in the 10^6 priming groups. Perhaps the most significant observation in this series of experiments, using systemically primed and boosted animals at varying doses of antigen, is that the IgA/IgM ratio has always been well below unity, varying from 1/2.4 through 1/16.5

Examination of the results during near-peak responses suggested that increasing the dose of the antigen produced a decrease in the IgA/IgM ratio.

After two injections at 2 weeks' interval the antigen dose of 10^7 V. cholerae appeared to be more efficient than the dose of 10^8 when antibody responses of both IgM and IgA were taken into consideration. If only the IgM response was considered these doses appeared to have a roughly similar effect.

Although the best systemic response was obtained in the "10^7-group" (the best amongst the three schedules included in table 3.1) there was no antibody response in the lamina propria of the guts of 5 mice on days 5, 6 and 9. Only on day 3, when a spleen assay was not done, was an IgA response just detectable and even this, by the nature of the significant lowest limit of the assay, may be considered negligible. (Vide footnote of table 3.2).

(2) Oral priming followed by parenteral boosting

Table 3.2 shows the systemic and local antibody responses after oral priming and parenteral boosting. Mice fed $5x10^{10}$ V.cholerae three times at weekly intervals and boosted intravenously with 10^7 V.cholerae ~ 14 days after the last feed produced quite high IgM and IgA responses in spleen. The IgM responses were comparable to those obtained in groups of mice that were systemically primed once and boosted with combinations of live V.cholerae doses of either $10^7/10^7$or $10^8/10^8$ for priming/boosting. The IgA responses, however, were much higher than those obtained in any one of the systemically primed and boosted

TABLE 3.2

ANTIBODY RESPONSE TO *V.cholerae*
AFTER ORAL PRIMING AND I.V. BOOSTING
(Geometric means, range in parenthesis)

Immunization schedule	Day of assay after booster	PFC/10^7 viable spleen cells			PFC/10^7 Viable lamina propria lymphoid cells		
		IgM	IgA	IgA/IgM	IgM	IgA	IgA/IgM
$5x10^{10}$, oral 3 times at weekly intervals	3 (n=4)	6283 (1818-16,070)	6796 (1919-22,400)	1.08/1	(14) (13-55)	(99) (81-254)	7.1/1
↓	5 (n=6)	2803 (1382-7722)	3013 (1712-9754)	1.07/1	Nil	(46) (25-293)	20/1 (approx)
Boosted with 10^7 i.v.~ 14 days after last priming. * *	6 (n=5)	875*	905*	1.03/1	Nil	Nil	
	9 (n=5)	739*	406*	1/1.82	Nil	Nil	

n= number of mice used in the experiment
* A pool of spleens of all the mice was assayed.
Nil. Not a single plaque detected in any of the mice.

() For the sake of calculating geometric means as well as IgA/IgM ratio, a value of 50% of the lowest possible positive result has been assigned to a 'nil' determination when the criterion of positivity is taken as at least one plaque in any of the two plates having the least diluted or neat cell suspension.

But for assessing significance of a particular result this criterion has been taken as at least one plaque in each or two plaques in any one of the 2 plates having the neat cell suspension. Depending upon the cell-yield from a particular gut this significant lowest limit of the assay varied roughly from 14 to 110 PFC/10^7 viable cells. So, in any experiment a geometric mean value of 100/10^7 viable cells may be considered as 'not significant' and is put within a bracket.

** Vide text for more similar experiments showing consistently negative gut response. Chapter III, page 66

groups of mice. (vide table 3.1). The rates of fall in both the IgM and IgA responses were very sharp and almost identical. The falls from day 3 to 5 and from day 5 to 6, in both Ig classes, were approximately 55% and 70% respectively of their day 3 and day 5 values. The fall in IgA responses continued up to day 9 declining by 55% of its day 6 value but the fall in IgM up to day 9 became slower falling by 15%. Due to high numbers of IgA PFCs the IgA/IgM ratios of the spleen responses on days 3, 5 and 6 were higher than unity. Though this ratio was relatively close to unity only on day 9 after booster (1/1.82) it was higher than the highest value (1/2.4) obtained in the systemically primed and boosted mice (vide table 3.1).

In spite of quite high splenic responses in both the Ig classes the orally primed and intravenously boosted mice showed only negligible IgM and IgA gut responses on day 3 and IgA response on day 6 after the booster. The geometric mean values of IgM (14) and IgA (99) PFC/10^7 viable lymphoid cells of lamina propria were actually just within the range of lowest significant positive results obtained by the PFC assay using enzymatically isolated cell-preparations from lamina propria of the gut. (vide foot note of table 3.2).

In the hope of demonstrating significant antibody response in the gut lamina propria 12 more experiments were performed using similar immunization regimens. The boosting procedure, by intravenous injection of 10^7 V.cholerae 14 days after the last oral priming, was kept identical but the methods of oral priming were varied. Among these variations were inclusion of an oral dose of 10^{10}

TABLE 3.3

ANTIBODY RESPONSE TO *V. cholerae* AFTER ORAL PRIMING AND BOOSTING

(Median values of gut results, range in parentheses)

Immunization schedule		Day of assay after booster	PFC/10^7 spleen cells			PFC/10^7 lamina propria lymphoid cells		
Priming	Booster		IgM	IgA	IgA/IgM	IgM	IgA	IgA/IgM
$5x10^{10}$ oral, 3 times at weekly intervals.	$5x10^{10}$ oral, 10^{7+} i.v. ~5 weeks after last oral priming	5 (n=3)	Not done (ND)	ND	-	(53*)	2000*	37.8/1
		6 (n=3)	"	"	-	Nil	887*	20.6/1 (approx)
$5x10^{10}$ oral, 5 times within 12 days, **	$5x10^{10}$ oral ~3 weeks after the last feed. **	4 (n=4)	206 *	773 *	3.7/1	(62) (55-166)	979 (62-1916)	15.8/1
		5 (n=3)	10 *	813 *	81.3/1	(10)	201 (60-427)	33.5/1
		5 (n=5)	139 *	2061 *	14.8/1	(23) (16-80)	305 (119-916)	13.4/1
					Average ratio 33.1/1			Average ratio 20.9 ~~61.1~~/1

* A pool of either spleens or guts of all the mice in the experiment was assayed

** Prior feeding of ½ saturated solution of $NaHCO_3$

() Values within bracket are not significant (Vide Key to table 3.2)

n = Number of mice used in the experiment.

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V.cholerae in some experiments, using 1 to 3 feeds for priming, neutralization of stomach acidity by prior feeding with a 50% saturated solution of $NaHCO_3$, shortening the time period between repeated feedings for priming etc. In spite of all these variations in the priming, no significant IgA or IgM gut antibody responses were obtained in these experiments.

However, responses in the spleen of orally primed and intravenously boosted mice were interesting. In 5 experiments where a dose of 10^{10} V.cholerae was given the IgM PFC were higher than those of IgA with an average IgA/IgM ratio of 1/1.82; whereas, the feeding of $5x10^{10}$ V.cholerae produced in most of the individual and all of the median values a ratio above unity, except in the declining response of day 9 after boosting.

(3) Priming and boosting both by oral route.

Table 3.3 summarizes the systemic and local responses to V.cholerae in mice using the oral route for both priming and boosting except in the first two experiments in which an additional intravenous booster of 10^7 organisms was used. Mice were fed three times, at weekly intervals, with $5x10^{10}$ V.cholerae and boosted ~ 5 weeks after the last priming with $5x10^{10}$ V.cholerae orally plus an i.v. injection of 10^7. These showed a high and moderately high (2000 and 887 PFC/10^7 cells) IgA response in the gut lamina propria on day 5 and 6 after the booster. There was a negligible IgM response on day 5 and none on day 6.

Adding two more doses to the oral priming as well

as distributing the 5 feedings within a span of 12 days and boosting only by the oral route 3 weeks after the last feed gave a comparable IgA response (median 979 PFC) in the gut lamina propria on day 4 after the booster. Similar results were achieved on two other occasions with moderate IgA gut responses on day 5 after the oral booster given 3 and 5 weeks following the last priming. IgM responses in the gut in these three experiments though detectable were so low that they were considered negligible by the criterion mentioned earlier. Consequently the IgA/IgM ratios in the gut in these experiments were 15.8/1, 33.5/1 and 13.4/1 respectively. The systemic responses in these 3 experiments (not done in the first two), though not high in magnitude, were remarkable in showing very high IgA/IgM ratios e.g. 3.7/1, 81.3/1 and 14.8/1, similar to the IgA/IgM ratios seen in the gut.

(4) I.p. priming with LPS in CFA followed by oral boosting with LPS or V.cholerae

Lastly table 3.4 summarizes the systemic and local Ab responses to V.C. in mice when primed intra-peritoneally by one injection of ~ 100 to 150 microgram of purified phenol-water extracted lipopolysaccharide (LPS) from V.cholerae in CFA and boosted after 3 weeks by oral feeding with either 1 mg of LPS or $5x10^{10}$ live V.cholerae. In mice boosted with oral LPS very weak IgM and IgA responses were observed in the spleen but there was no plaque of either isotype in the plates containing lamina propria

TABLE 3.4

ANTIBODY RESPONSE TO *V. cholerae*
AFTER I.P. PRIMING WITH LPS+CFA
AND ORAL BOOSTING WITH LPS OR VIBRIO

Priming	Booster (3 weeks after priming)	Day of assay after booster	PFC/10^7 viable spleen cells			PFC/10^7 viable gut cells	
			IgM	IgA	IgA/IgM	IgM	IgA
~100 to 150 microgram of LPS plus CFA i.v.	1 mg LPS orally *	5 (n=4)	99	16	1/6.2	nil	nil
Do	$5x10^{10}$ live *V. cholerae** orally	5 (n=5)	480	121	1/4	(18)	(12)

() Not significant. Vide Key to table 3.2

* Prior feeding of ½ saturated solution of $NaHCO_3$

cells. In contrast, in mice boosted with live organisms, about 5 times greater IgM and 7 times greater IgA splenic responses were obtained in comparison to what was found in LPS-boosted mice. The magnitudes of the splenic IgM and IgA responses in these experiments were much smaller than those in any of the previous immunization schedules. It is interesting to note that the splenic IgA/IgM ratios in these two experiments, contrary to expectations, were quite low e.g. 1/6.2 and 1/4 respectively.

Discussion:

The observation that the IgM and IgA responses in mice primed systemically three times with 10^6 V.cholerae followed by a booster of 10^7 were lower than in those from mice primed once only and then boosted with either of the combinations of $10^7/10^7$ and $10^8/10^8$ V.cholerae was unexpected. Indeed, repeated systemic priming with low dose bacterial antigen followed by a booster is a standard recipe for raising high-titre anti-bacterial serum.

In between groups of mice primed and boosted with $10^7/10^7$and $10^8/10^8$ organisms respectively there was no significant difference in the IgM responses but the IgA response in the former group was much higher than that of the latter group.

The variations in the magnitude of isotype specific responses found in these three systemic immunization

schedules seems to depend upon the level of immunising doses, especially those used for priming.

But the most important qualitative character, on the positive side of the response, that was observed in common amongst all the groups of systemically primed and boosted mice was the very low IgA/IgM ratios of the responses varying from 1/2.4 through 1/16.5. The other qualitative character, on the negative side of the response, was that observed in the group ($10^7/10^7$); although showing the best splenic responses there was an absence of significant PFC responses of both the isotypes in the gut lamina propria. This unique combination of qualitative characteristics of the immune responses seems to be the function of this particular immunization regimen, namely intravenous priming and boosting.

In mice repeatedly primed orally ($5x10^{10}$, 3 times) and systemically boosted (10^7 i.v.) the magnitude of IgM response in the spleen was remarkable, being comparable to those obtained by systemically primed and boosted mice (vide table 3.1). The magnitude of IgA response was still higher resulting in a conversion of the systemic (splenic) response into a 'local kind' characterised by a high IgA/IgM ratio reaching above unity. But contrary to earlier observations (Bloom., Thesis, 1979) no better than a very weak and negligible antibody response in the gut lamina propria was obtained. This point was checked and rechecked further by 12 more experiments each containing 3 to 5 mice with variations in the methods of oral priming

as well as using LAC spf and CBA mice from another stock to obviate the possibility of any species-specific innate lack of response in the animals. In spite of all these manoeuvres, none of these latter experiments could show a gut response above a GM value of 50 PFC/10^7 lymphoid cells.

A significant gut response, of almost exclusively IgA isotype, could only be elicited by orally primed and orally boosted groups of mice as summarized in table 3.3. Amongst the 5 experiments giving such results the first two groups of mice also had a simultaneous i.v. challenge; however, since i.v. boosting following oral priming did not produce an IgA response in the gut (table 3.2) one can eliminate here, any possible contribution by the 'simultaneous i.v. challange'.

Though IgM gut responses in 4 out of 5 experiments using orally primed and boosted mice were detectable, their magnitudes were below the level of significance. Obviously the gut response in this schedule (Table 3.3) shows the highest IgA/IgM ratios with an average of 61.6/1. Concurrently, the greatest shift of the systemic splenic response qualitatively into the 'local' character was obtained with an average IgA/IgM PFC ratio of 33.1/1 (table 3.3).

By analyzing the data of IgA/IgM ratios of the numbers of splenic PFC obtained in all the experiments done under various immunization schedules a cut off point of 1/2 in the ratio of a splenic response can be fixed above which any ratio of a splenic response may arbitrarily be defined as 'local' kind. Conversely, a splenic response showing a smaller ratio than 1/2 may be designated 'systemic' type.

Since splenic assays for IgA/IgM PFC ratios clearly allowed us to distinguish between immunization regimens leading to a 'systemic' or 'local' type of response and since early attempts at plaquing cells of the gut lamina propria were unsuccessful, splenic assays were used for much of the future work. When later, success in gut plaquing was achieved (after using the oral route of boosting) the validity of accepting the spleen assay to distinguish between a systemic and local response was confirmed.

Contrary to expectations no gut antibody response was observed in the mice primed i.p. with LPS in CFA followed by oral LPS booster. A challenge of similar primed mice with oral live V.cholerae showed a detectable but insignificant gut response. The reason for this I believe, is that the LPS behaves as a linear T-independent antigen and this kind of antigen is known to produce predominantly IgM class of antibody. Thus the 'systemic' nature of the splenic responses obtained in these experiments was probably a reflection of this phenonemon.

CHAPTER 1V

EFFECT OF INTRAVENOUS ADMINISTRATION OF ANTIGEN PROCESSED IN PEYER'S PATCHES ON THE IMMUNE RESPONSE OF RECIPIENT MICE.

Preamble

The primary role of Peyer's patches (PP) in supplying antigen-sensitive IgA precursor cells to the lamina propria of the gut, the association of PP with the antigen specific induction of humoral immunity in the gut and antigen handling on the mucosal surface were discussed in Chapter I. Indeed these tiny non-encapsulated gut associated organised lymphoid tissues serve as prime sites of antigen-sampling and subsequent interaction between antigen and immunocompetent T and B cells. This latter interaction controls the final systemic and local immunologic responses to oral antigen and maintains the balance between tolerance and immunity.

With these considerations as a background, we have been looking for a possible mechanism in the gut which produces the 'local' kind of antibody response by mucosal presentation of antigen. This led to the concept of "processed antigen" which presumed that antigen could be processed locally in such a way that it is mainly active in stimulating antigen-driven IgA-committed B cells.

To test this hypothesis, the following experiments were performed. Donor mice were fed thrice with $5x10^{10}$ V.cholerae per feed at weekly intervals. Fourteen to sixteen hours after each feed PP were dissected, washed three times with saline, disrupted by alternate freezing and thawing, and injected intravenously into syngeneic recipients on three occasions, each mouse receiving, on the average, PP material derived from one

donor mouse. These recipients were boosted two weeks after the last priming with intravenous 10^7 V.cholerae. Spleen and gut lamina propria were assayed for IgM and IgA PFC on days 5 and 6 after boosting.

However > 75% of the mice that received intravenous frozen and thawed PP homogenate died and eventually the experimental procedure had to be abandoned. Subsequently, a single cell-preparation (by collagenase-disaggregation) was obtained from the PP of mice fed as described above, which were taken about 50, 26 and 14 hours respectively after each of the three feedings. The cells were killed in 1% formalin-saline and injected intravenously to the recipients on the three occasions. The other steps remained the same. The killing of the cells by formalin-contact prevented the formation of progeny clones in the recipients so that all the resulting effects may be ascribed only to the transferred processed antigen.

There were significant differences in the two experimental designs. In the former case the antigen, processed or native, that was (hypothetically) present intercellularly in either the interfollicular or the intrafollicular regions of the PP of the fed mice would have been transferred into the recipients. But in the later design, due to the collagenase digestion and subsequent washing of the cells, only that portion of the antigen which was very intimately attached to the PP cells had the chance to be transferred into the recipients.

Two groups of control mice were included. The first group received three intravenous doses each of 10^6 V.cholerae followed by a booster of 10^7 V.cholerae intravenously in the same schedules as the test. The

second group comprised unprimed mice which received only the same booster on the day of boosting the other groups. The post-feeding amount of antigen that might have been taken up by the PP of the donor mice in a process of antigen-sampling would not have been large and, in any case, no attempt was made to measure it. The priming dose for the control mice ($1x10^6$ V.cholerae) was kept quite small to be comparable with that (unknown) of the test group.

The main aim of the experiment was to find out whether priming with the antigen from the PP of fed mice could bring out a high IgA/IgM ratio of antibody responses in the test mice in comparison to the low ratio that was usually obtained in control animals.

Experimental results.

As mentioned in the preamble the original experimental design could not be carried through due to the death of most of the experimental animals that received intravenous frozen and thawed PP homogenate. Only six mice survived. They were divided into two equal groups and were assayed for spleen and gut plaques on days 5 and 6 after the booster of intravenous 10^7 V.cholerae. Obviously the number of mice were not adequate for statistical analysis. It was more so for pooling of spleens in the assay. However, the data, summarized in table 4.1, obtained from those six mice were highly interesting and pointed towards the validity of the hypothesis of processed antigen modulating the antibody response to antigen presented by the gut.

TABLE 4.1

MODULATION OF ANTIBODY RESPONSE† BY PROCESSED ANTIGEN

Groups of mice	Immunization (same booster for all groups, 10^7 iv) Priming	Day of assay after booster	PFC/10^7 viable spleen cells IgM	IgA	IgA/IgM
Test T I	PP homogenate (from one orally fed mouse) 3 times at weekly intervals.	5 (n=3)	2562*	3000*	1.2/1
		6 (n=3)	2660*	2320*	1/1.1
Test T II	Formalin-killed PPL (from one orally fed mouse) 3 times at weekly intervals	5 (n=4)	36*	29*	1/1.2
		6 (n=5)	83*	35*	1/2.4
		8 (n=4)	138*	44*	1/3.1
Control C I	10^6 *V.cholerae* i.v. 3 times at weekly intervals	5 (n=6)	903 (302-3793)	278 (67-827)	1/3.2
		6 (n=5)	619 (140-4372)	224 (45-1970)	1/2.8
	* *	8 (n=6)	327 (180-2216)	59 (37-309)	1/5.5
C II	Unprimed normal conventional mice	6 (n=3)	107	(5)	1/21.4

†All the mice of the test groups TI and TII were individually assayed for gut response with complete negative results.

n= number of mice used in the experiment.

* A pool of spleens of all the mice in the experiment was assayed.

** Geometric means with range in parenthesis in these groups of mice.

() The significant lowest limit of assay in spleen was 10 to 30 PFC/10^7 viable spleen cells when the criterion of lowest limit was at least 1 plaque in each of 2 or 2 plaqyes in either of the 2 plates at a 1/10 dilution of a spleen homogenized in 1 ml of medium (vide key to table 3.2)

The table shows that the transfer of PP-homogenate from orally primed mice could create significant IgM and IgA responses in the recipient spleen on day 5 and 6 after the booster. The average number of IgM PFC/10^7 viable spleen cells in the test mice was 3.4 times higher than those in the first group of control mice that had three primings with 10^6 native antigen. Similarly the average IgA response in the test mice was 10.2 times greater than that in the same group of control mice. The second group of control mice was included to determine the nature of primary response on day 6 due only to the booster dose of V.cholerae. This group showed very weak IgM and IgA response in spleen, too small to exert any significant influence to the trend of responses observed in the main experiments.

It is unquestionable that substantial amounts of antigenic material were transferred by way of PP-homogenates into the test recipients as evidenced by the systemic responses obtained which were higher than those obtained in the control groups.

By far the most important finding of this experiment was the high IgA/IgM ratios obtained in the test mice on both the days 5 and 6 (1.2/1 and 1.1/1) which are very close to those (1.08/1, 1.07/1 and 1.03/1 on days 4, 5 and 6) obtained in repeated orally primed mice boosted intravenously with identical doses of V.cholerae, (vide table 3.2, Chapter III). These ratios look significantly different from those obtained in the first control group of mice on the same days after booster; but the level of significance of this difference cannot be ascertained

statistically for reasons given earlier.

There was no evidence of PFC response in the gut lamina propria in any of the groups of mice.

The effect of transfer of processed antigen contained in formalin-killed PP lymphocytes (PPL) from orally primed mice, on the systemic immune responses of recipients, with reference to response in the first group of control mice, has been shown in table 4.1. On days 5, 6 and 8 after boosting both the IgM and IgA PFC responses in the test mice receiving dead PPL from orally primed donors were very much smaller than the corresponding results in the first group of control mice receiving native antigen in a similar schedule. This is quite different from that obtained with PP-homogenates from antigen-fed donors. Though the IgA/IgM ratio of PFC on day 5 after booster showed 'local' and on days 6 and 8 showed 'systemic' character, no confidence could be placed on these findings because the overall magnitude of the responses were so very low that they could hardly be of any significance.

Discussion

The data indicates that a substantial amount of antigenic material was transferred to the recipient test mice via the PP-homogenate from orally primed donors. It was sufficiently large in amount to create significantly higher responses in the test mice than that in the first group of control mice which were

hyperimmunized with three doses of 10^6 V.cholerae followed by a booster of 10^7. By extrapolating the data shown in table 3.1 in Chapter III it can be estimated that the equivalent of 10^6 to 10^7 V.cholerae needed to be transferred through the PP homogenate to give the observed response. The data also tended to prove that the transferred antigenic material was qualitatively different or altered to produce a 'local' kind of response having an average IgA/IgM ratio above unity.

In contrast, the antigen transferred into the recipients by formalin-killed PPL from orally primed mice, was only large enough to produce a response sufficient to be recognized above the effect of 10^7 V.cholerae in the previously unprimed 'booster control' group.

The magnitudes of splenic PFC responses were so low and near to the limit of lowest significant positive results of the splenic PFC assay that no confidence could be placed on the characters of antibody responses found in the second group of test mice by the criterion of IgA/IgM ratios.

CHAPTER V

MODULATION OF SYSTEMIC ANTIBODY RESPONSES TO *V.CHOLERAE* IN MICE BY CONCOMITANT ADOPTIVE TRANSFER OF PEYER'S PATCH LYMPHOCYTES FROM ANTIGEN-FED SYNGENEIC DONORS.

Preamble

The experiments described in this chapter were designed to test the alternative hypothesis outlined in Chapter I for explaining the predominance of IgA antibody response after immunization via the gut. This hypothesis is that locally presented antigen leads to the emergence of modulator cells in the gut lymphoid tissue which modulate the antibody response ultimately to an IgA preponderance i.e. possessing a 'local' character.

The experiment designed to test this hypothesis was as follows. Live PPL were isolated from donor mice fed three times with $5x10^{10}$ V.cholerae at intervals of a week, on day 4 or 5 after their last feed of antigen. They were mixed with $1x10^{8}$ V.cholerae and injected intravenously twice into syngeneic recipients with an interval of two weeks between injections. The numbers of IgM and IgA PFC/10^{7} viable lymphocytes in the recipient spleens were determined by Jerne's hemolytic PFC assay during the peak antibody response period after the second booster injection. The numbers of IgM and IgA PFC were also determined in control mice injected either with a mixture of PPL from normal conventional mice and V.cholerae or only with V.cholerae. The former was designated as 'true control'; the latter as 'antigen control'. Another control experiment was included using spleen cells instead of PPL from normal mice. No significant difference was

found between the results of this group and the results of the other control groups. For this reason the results of test groups of mice were not statistically compared at all with those of the spleen cell control groups. The antigen only and spleen cell control groups were included for some supporting data that could check the validity of the true controls which they did satisfactorily.

Experimental results

Details of the modulation of IgM and IgA antibody responses to V.cholerae in recipient mice (measured by PFC assay in the spleen) by the concomitant administration of PPL from V.cholerae-fed mice are summarized in table 5.1

This table contains the results of all the individual controls, as well as the median values of PFC per 10^7 viable cells with lowest and highest observations of each experiment. This seems to be too detailed to be comprehended at a glance and is furnished rather for an archival purpose when the reader wants to have a critical examination of data, specially the range of variations in each experiment. A simpler table 5.2 is generated out of the details in which only the true controls I and II are lumped together and only the median values of test observations are given. The symbolic arrows indicating suppression/enhancement in reference to true controls I and II and antigen control are, of course, retained.

This table shows that compared to the results obtained in the antigen control and two true control experiments (I & II) the transferred PPL from fed mice caused a profound suppression of IgM PFC response in all four test experiments; the one on day 4 and the three on day 6 after the booster. A similar effect was observed on day 8 after booster as compared to the results in the experiment with antigen control. (True control experiments were not done on day 8). By a comparison of geometric means of the median values of true controls and test experiments it was found the IgM PFC response in test mice was inhibited approximately 12 times on day 4, 5.64 times on day 6 and 2.25 times on day 8 after the booster.

The IgA response in the four test groups however gave variable results. In the first test group T1 there were

Protocol for the experiments shown on table 5.1

Type of experiments	No. of mice in each experiment	Priming (i.v.)	Interval between priming & boosting, in days	Boosting (i.v.)
Antigen only control	6 (day 4) 7 (day 6 & 8)	Ag^{**} only	15	Ag^{**} only
True Control I	8	Ag + $3x10^6$ PPL from normal conventional mice	13	Ag + $4.5x10^6$ PPL from normal conventional mice
True Control II	8	Ag + $3x10^6$ PPL from normal conventional mice.	15	Ag + $2.5x10^6$ PPL from normal conventional mice
Spleen cell control	7 (day 4) 8 (day 6)	Ag + $2.5x10^6$ spleen cells from normal conventional mice	14	Ag + $5x10^6$ spleen cells from normal conventional mice
Test experiments TI	6	Ag + primed* PPL from one mouse (not counted)	14	Same as priming. cells not counted.
T2	7 (day 4 only)	Ag + $3x10^6$ primed PPL.	16	Ag + $5x10^6$ primed PPL.
T3	8	Ag + $5x10^6$ primed PPL.	17	Ag + $5x10^6$ primed PPL.
T4	6	Ag + $2.7x10^7$ primed PPL.	25	Ag + $1.7x10^7$ primed PPL.

*Syngeneic donor mice were fed 3 times at weekly intervals, each feed consisting of $5x10^{10}$ live V.cholerae. Live PPL were obtained by collagenase treatment on days 4 or 5 after the last feed for transfer into the recipients simultaneously with the Ag.

Ag^{**} = $1x10^8$ live V.cholerae; the same dose was used in all these experiments.

TABLE 5.1

MODULATION OF ANTIBODY RESPONSES*
BY TRANSFER OF
LOCALLY PRIMED PEYER'S PATCH LYMPHOCYTES

Types of experiments	Day 4 after booster		Day 6 after booster		Day 8 after booster	
	IgM	IgA	IgM	IgA	IgM	IgA
Ag Control	8,445 (5,537-14,239)	817 (541-1,818)	7,422 (1,904-17,521)	243 (128-1,580)	1,208 (425-9,493)	96 (21-269)
True Control I	12,971 (2,149-19,293)	953 (182-1,592)	7,826 (1,851-13,951)	533 (127-752)		
True Control II	12,183 (6,588-31,325)	1,170 (472-1,764)	6,175 (1,539-15,744)	388 (62-2,471)		
Spleen Cell Control	12,314 (2,173-23,816)	1,088 (560-2,415)	4,312 (929-39,378)	283 (83-3,861)		
Test Expts T1	1, 983 (400-5,545) ↓↓↓	4,565 (3,093-10,841) ↑↑↑	918 (516-1,708) ↓↓↓	2,142 (537-4,333) ↑↑↑	535 (268-1,470) ↓	1,110 (309-1,752) ↑
T2	1,803 (730-4,532) ↓↓↓	600 (222-12,000) ↓				
T3	958 (737-2,279) ↓↓↓	568 (232-866) ↓	1,459 (237-4,532) ↓↓↓	374 (74-872) ↓↓↓		
T4	356 (100-463) ↓↓↓	528 (288-729) ↓↓	1,306 (560-2,835) ↓↓↓	368 (149-650) ↓↓↓		

*Median values of numbers of PFC/10^7 viable spleen cells : range in parentheses. Analysis of variance by Mann-Whitney U test with the level of significance at $p = \leq 0.05$
Arrows directed downwards and upwards indicate suppression and enhancement respectively in reference to the results of control experiments: true control I(----), true control II (=====) and antigen only control (——) . Arrows indicate significance at $p < 0.05$
Ahlborg pp A. Ahmed

TABLE 5.2

MODULATION OF ANTIBODY RESPONSES
BY TRANSFER OF
LOCALLY PRIMED PEYER'S PATCH LYMPHOCYTES

(Median values of number of PFC/10^7 viable spleen cells)

Types of experiments	Day 4 after booster		Day 6 after booster		Day 8 after booster	
	IgM	IgA	IgM	IgA	IgM	IgA
Average of true controls I & II Normal PPL+Ag.	12,577	1,061	7,000	460	1,208	96
Test experiments. Locally primed PPL + Ag. T1	1,983 ⇣⇣↓	4,565 ⇡⇡↑	918 ⇣⇣↓	2,142 ⇡⇡↑	535 ↓	1,110 ↑
T2	1,803 ⇣⇣↓	600 ⇣				
T3	958 ⇣⇣↓	568 ⇣	1,459 ⇣⇣↓	374 ⇣		
T4	356 ⇣⇣↓	528 ⇣↓	1,306 ⇣⇣↓	368 ⇣		

Analysis of variance by Mann-Whitney U test with the level of significance at $p \leq 0.05$.
Enhancement : arrow upwards; suppression : arrow downwards in reference to true control I ⇢
true control II ⇢ and only antigen control ⟶ . Arrows indicate significance at $p<0.05$

Vide page facing table 5.1 for experimental protocol.

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significant enhancements over all groups of controls at all the times of testing. (True control experiments were not done on day 8). On the other hand the IgA responses in the other test groups T2, T3 and T4 remained unchanged or were reduced compared to the controls though, as seen in the table 5.2 these reductions were significant only in comparison to one or the other of the control groups.

One apparently paradoxical finding in the results of different controls was that the numbers of IgM PFC/10^7 viable splenocytes in the first and second true controls on day 4 were much higher than that of the antigen control. The levels of significance of analysis of variance by Mann-Whitney U test were 0.06 and 0.76 respectively.

The suppression of the IgM response, and variable changes in the IgA response are depicted in the bar-graphs (Figures 5.1 and 5.2).

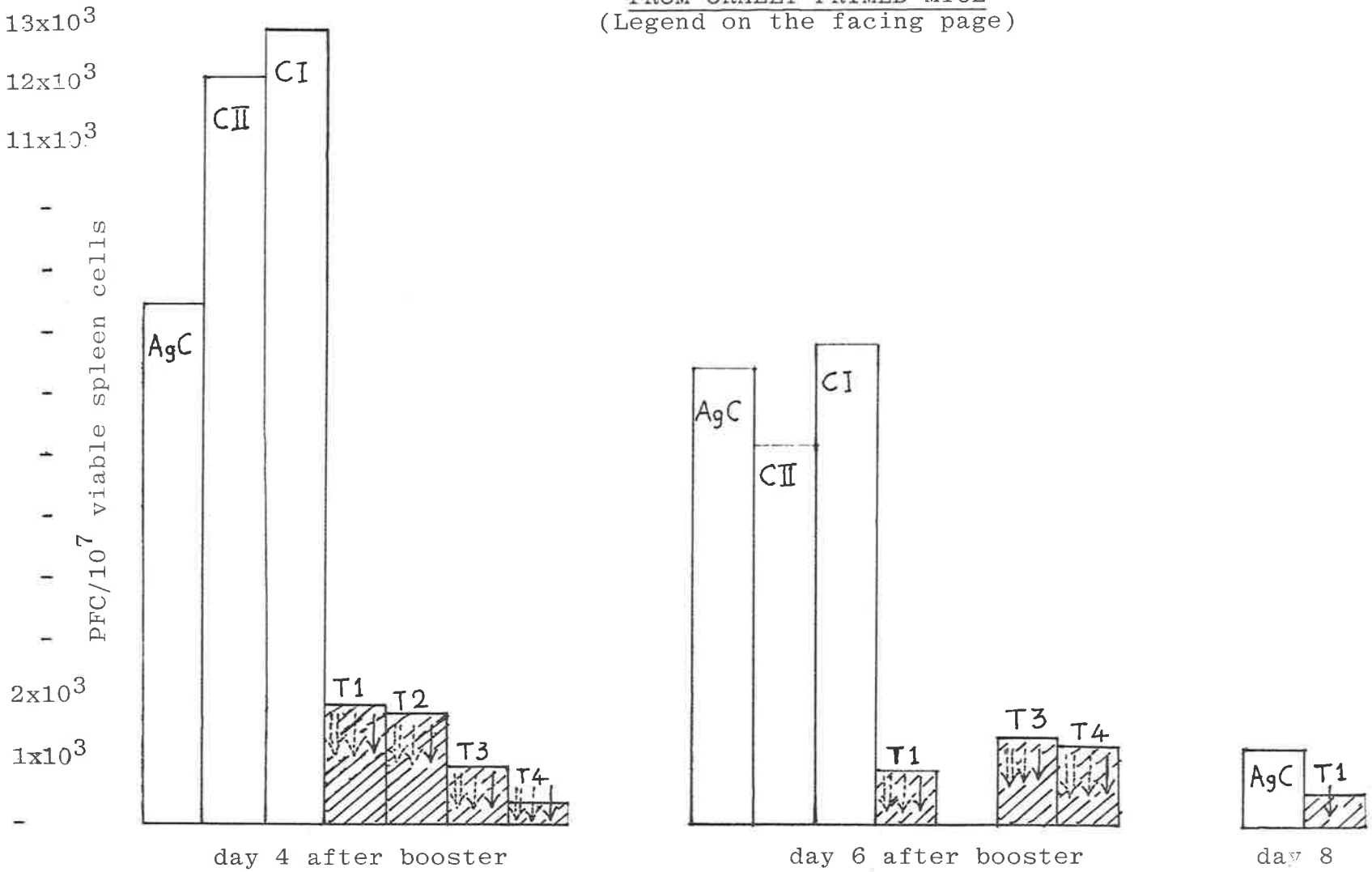
Discussion

A clear inference which may be drawn from these experimental findings is that the PPL of the orally primed mice must have contained adequate numbers of IgM class specific suppressor cells able to cause a profound and unidirectional suppression of the IgM response in spleens of the recipient mice. This was shown by all the test experiments on all the days after boosting when compared with the results of almost all the control experiments. Even if it was expected that both suppressor and helper cells with capacity to modulate IgM response were transferred simultaneously through the mixed population of PPL from fed mice, the initial number or subsequent clonal expansion of the suppressor cell set established a clear preponderance over the helper cell set to explain this kind of inhibitory effect on IgM synthesis

FIGURE 5.1

MODULATION OF IgM ANTIBODY RESPONSES BY TRANSFER OF PPL FROM ORALLY PRIMED MICE

(Legend on the facing page)



LEGEND TO FIGURE 5.1

Median values of number of IgM PFC/10^7 viable spleen cells are plotted.

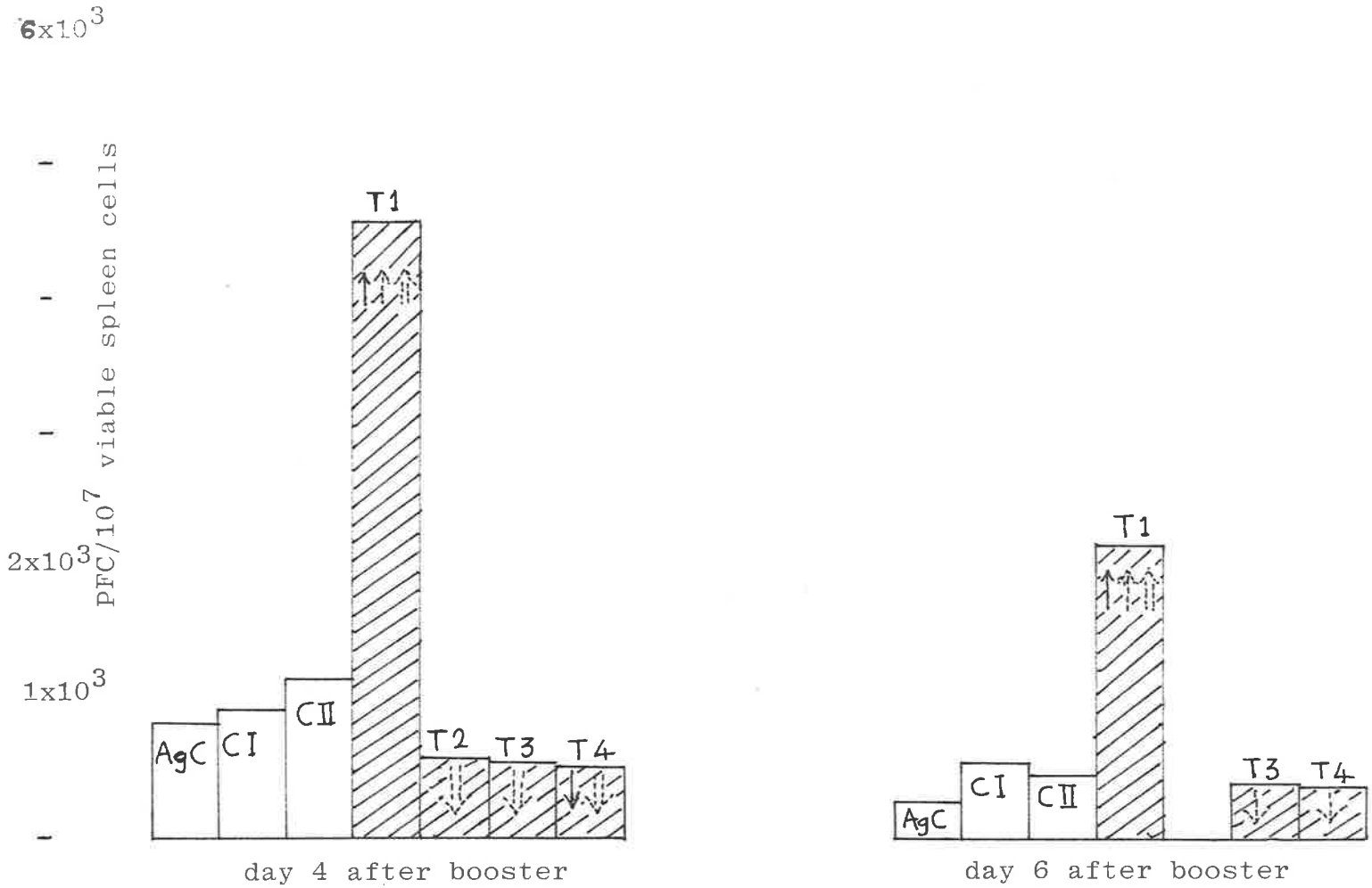
Test groups (shaded bars) are compared with true controls I, II and Ag Control (Open bars)

⇓ / ↓ / ↓ indicates significant suppression in reference to true controls, II, I and Ag Control respectively.

FIGURE 5.2

MODULATION OF IgA ANTIBODY RESPONSES BY TRANSFER OF PPL FROM ORALLY PRIMED MICE

(Legend on the facing page)



LEGEND TO FIGURE 5.2

Median values of number of IgA PFC/10^7 viable lymphocytes are plotted.

Tests (shaded bar) are compared with true controls I, II and Ag Control (open bar)

 indicates significant suppression/ enhancement in reference to true controls, II, I and Ag Control respectively.

in the recipient mice.

Another important characteristic of this inhibitory effect on IgM synthesis was that the degree of suppression was most pronounced (12 fold decrease of IgM PFC as compared to those of true control experiments) during the peak period of IgM antibody response (i.e. day 4 after booster) and gradually decreased as the antibody responses decreased in the control mice.

As IgM PFC in the true controls were markedly higher than those in the antigen control groups on day 4 after the booster, suppression of a lower magnitude on that day would have appeared significant in comparison to true controls but non-significant in comparison to antigen control groups. However, the actual suppression was so great that they were significant with regard to all of the control groups.

A comparison between the numbers of PPL from fed mice that were administered in test mice group T2 through T4 and the number of IgM PFC obtained in these groups suggests that probably there was a direct correlation between the numbers of PPL injected and the magnitude of suppression obtained on day 4 after the booster i.e. the peak response day in control animals.

The IgA responses obtained in the four different test groups of animals were not unidirectional. The first test experiment T1 was unique in that on all the days 4, 6 and 8 after booster significant enhancement was observed in comparison to responses in all control groups (only antigen control done on day 8). But

there were variable responses in the other three test groups which showed little change or suppression. The possible causes of these variable responses and the implications of their final nature will be discussed in the last chapter.

The two most important immune events that are thought to occur as a result of antigen presented to the gut, namely (i) suppression in systemic antibody responses and (ii) enhancement of IgA antibody (with a conversion of the splenic response into a 'local' kind) seemed to have occurred concurrently in the recipient mice in these experiments. The direct proof was obtained by the results of the test experiment T1 in which both suppression of IgM and enhancement of IgA splenic response occurred concurrently on all the days 4, 6 and 8 after the booster. The indirect proof was the conversion of the 'systemic' kind of antibody responses in control mice into a 'local' kind in the test mice when the sum total of data from all test experiments was taken into account.

CHAPTER VI

MODULATION OF SYSTEMIC ANTIBODY RESPONSES TO V.CHOLERAE IN MICE BY CONCOMITANT ADOPTIVE TRANSFER OF PURIFIED T AND B PEYER'S PATCH LYMPHOCYTES FROM ANTIGEN FED SYNGENEIC DONORS

Preamble

In Chapter V it was demonstrated that an adoptive transfer of PPL from repeatedly orally primed mice along with V.cholerae twice at an interval of ~ two weeks created a profound unidirectional suppression in the splenic IgM PFC response of the recipient as well as variable modulation of IgA responses. Further studies aimed at the elucidation of the mechanism by which this suppression of IgM and the modification of IgA response occurred concurrently in the recipient mice was felt to be a logical extension of that experiment.

The experimental design was similar to that used in the test experiments described in Chapter V (vide protocol of table 5.1). The essential difference was that the mixed cell populations from the Peyer's patches were separated into purified T or B cells before injection into the recipient mice together with the vibrio antigen. The T and B cells were separated by 'panning' using plastic surfaces coated with anti-mouse IgG.

In assessing significant suppression or enhancement the results of the same 'true controls' I and II and the antigen control described in Chapter V were adopted.

Experimental results

The effects of transfer of both T and B purified subpopulations from PPL of fed donors into the recipients in the IgM PFC response in spleens are shown in the Table 6.1.

PROTOCOLS FOR THE EXPERIMENTS

SHOWN ON TABLE 6.1

Type of experiments	Priming	Interval between two i.v. inj.s. days	Boosting
Peyer's patch T or B cell transfer	*Ag+2 to $3.3x10^6$ primed ** purified T or B lymphocytes	12 to 14	Same as priming
Only antigen control	Only Ag	15	only Ag
True control I	Ag+ $3x10^6$ PPL from normal conventional mice.	13	Ag+$4.5x10^6$ PPL from normal conventional mice
True control II	do	15	Ag+$2.5x10^6$ PPL from normal conventional mice.

* Ag = $1x10^8$ live V.cholerae; the same dose was used in all the experiments.

** Syngeneic donor mice were fed with $5x10^{10}$ live V.cholerae per feed 3 times at weekly intervals. T and B cells were 'partitioned' from Peyer's patch cells and injected intravenously with antigen into recipients.

TABLE 6.1

MODULATION OF IgM ANTIBODY RESPONSES* BY TRANSFER+ OF LOCALLY PRIMED PEYER'S PATCH T & B CELLS.

Day of Expt. after booster	Antigen control group	True control group I	True control group II	T cell group	B cell group
4	8,445	12,971	12,183	3,816	3,359
	(5,537-14,239)	(2,149-19,293)	(6,598-31,325)	(1,031-17,273)	(1,205-7,225)
				---- p=0.06	⇣ p=0.002
				⇣ p=0.027	⇣ p=0.001
				— p=0183	↓ p=0.004
	n=6	n=8	n=8	n=7	n=6
6	7,422	7,826	6,175	3,332	3,166
	(1,904-17,521)	(1,851-13,951)	(1,539-15,744)	(1,700-43,539)	(1,642-5,786)
				⇶ p=<0.18	
				Discarding the result of the high responder mouse	⇣ p=0.023
				↓ p=0.014	⇣ p=0.047
				⇣ p=0.036	↓ p=0.047
				↓ p=0.005	
	n=7	n=8	n=8	n=5	n=5

*Median values of numbers of PFC/10^7 viable spleen cells: range in parentheses.
Arrows directed downwards indicate suppression and lines directed horizontally indicate no change in reference to the results of control experiments : true control I (----), true control II (====) and only antigen control (——)
n = Number of mice used in the experiment.
+ Table on the facing page shows the experimental protocol.

IgM response after T cell transfer.

T cells created suppression in the recipient mice on day 4 after booster by comparison with true controls but not the antigen control. This suppression was significant at the $p = \leq 0.05$ level in comparison with second group of true controls. With reference to first true control and antigen control groups this suppression did not quite reach statistical significance ($p=0.06$ and 0.183 respectively) though the median of the test group was less than half the median value of either of these controls.

On day 6 the IgM response in the test mice obtained by the transfer of T cells showed suppression with regard to all the control results though it fell short of statistical significance because of one super-responder mouse. Among 5 mice in the experiment the super responder had 43,539 IgM PFC/10^7 viable splenocytes whereas these numbers in the other four mice ranged between 1700 to 4204. This particular result was obviously an unusually high one and in spite of the ranking test for the analysis of variance an otherwise significant suppression effect appeared to be non significant. Table 6.2 clearly shows that this unusual result is way above the natural distribution that was observed in the IgM responses by different ranges of PFC numbers when the results of all the recipient mice receiving total, T and B PPL from fed donor mice were taken into consideration. On the basis of these observations there is a strong case for considering the suppression obtained as being highly significant as would be the case if the results of this exceptional mouse were excluded

TABLE 6.2

Frequency distributions of IgM responses at different ranges of PFC numbers in all the test mice that received total, T and B PPL-transfer from fed donor mice.

PFC/10^7 viable cells	Day 4	Day 6	Combined on day 4 & 6
< 100	nil	nil	nil
100 - 500	8	3	11
500 - 1,000	11	6	17
1,000 - 2,000	13	8	21
2,000 - 5,000	15	5	20
5,000 - 10,000	5	4	9
10,000 - 15,000	1	1	2
15,000 - 20,000	1	1	2
20,000 - 25,000	nil	nil	nil
25,000 - 30,000	nil	nil	nil
30,000 - 40,000	nil	nil	nil
40,000 - 50,000	nil	1*	1*

* The place of the super responder mouse.

from the statistical analysis (as has been shown by the p values discarding the result of the high responder mouse.

IgM response after B cell transfer.

The results of transfer of purified B cells in the test mice was also a clear suppression on both the days 4 and 6 in reference to the results of all the controls except in reference to the results of the antigen only control on day 6 when the p-value reached 0.074.

IgM response after both T and B cell transfers.

As with the results of IgM responses in the test mice obtained in earlier experiments by the transfer of total PPL from fed mice uniform suppression was again found on both days after the booster by transfers of both the purified T and B cells. It is of interest to note that the highest magnitude of suppression was observed by B-cell transfer on day 4 after the booster ($p=0.001$ to 0.004).

IgA response after both T and B cell transfers.

In the IgA response, significant enhancement was seen by the T-cell transfer on both the days in comparison to all the controls except to the second true control on day 4 after the booster. The B-cell transfer, however, created significant enhancement in comparison to all the controls on day 6 but not on the day 4 after booster and also in this particular experiment the magnitude of enhancement was highest. The IgA responses obtained in the test mice on day 4 after the booster

did not show any statistical difference from the corresponding results obtained in any of the controls.

The enhancement in splenic IgA responses in the recipients by the transfer of either T or B cells from PPL of orally primed mice is in keeping with the results of test expt. T1 in Chapter V in which total mixed PPL from fed mice were adoptively transferred using a very similar experimental design. In 3 out of 4 experiments, (i.e. day 4 after booster in T cell-injected and day 6 after booster in both T and B cell-injected recipients,) the enhancements in IgA response were unidirectional with reference to the results of almost all the control experiments. (Vide table 6.3).

Discussion:

By separate adoptive transfers of purified T and B cells from the PPL of orally primed donors in conjunction with antigen, unidirectional suppression in IgM and enhancement in IgA PFC responses were obtained in syngeneic recipients. These results are in full agreement with the results obtained on all the days 4, 6 and 8 after booster in recipients receiving mixed total PPL from fed mice in test experiment T1 (table 5.2,Chapter V). They do not tally with the unchanged level or suppression of IgA observed in similar test experiments T2, T3 and T4. Due to the unique method of partitioning T and B cells from PPL of the same set of orally primed mice by 'panning' with anti-mouse Ig-coated plastic surface and transfer of those T and B cells in syngeneic recipients at nearly identical times the implications of these results showing simultaneous

TABLE 6.3

MODULATION OF IgA ANTIBODY RESPONSES*
BY TRANSFER+ OF
LOCALLY PRIMED PAYER'S PATCH T & B CELLS

Day of Expt. after booster	Antigen control group	True control group I	True control group II	T cell group	B cell group
4	817	953	1,170	1,493	1,373
	(541-1,818)	(182-1,592)	(472-1,764)	(993-5,257)	
				↑ p=0.016	----- p= 0.331
				⇡ p=0.076	⇡ p= 0.286
				↑ p= .007	—— p= 0.120
				n = 7	n = 6
6	243	533	388	723	1,308
	(128-1,580)	(127-752)	(62-2,471)	(533-11,166)	(928-3,666)
				↑ p=0.047	↑ p=0.001
				⇡ p=0.033	⇡ p=0.015
				↑ p=0.026	↑ p=0.015
				n = 5	n = 5

*Median values of numbers PFC/10^7 viable spleen cells: range in parentheses.
Arrows directed upwards indicate enhancement and lines directed horizontally indicate no change in reference to the results of control experiments : true control I (----), true control II (⇡⇡⇡) and only antigen control (——).
n= number of mice used in the experiment
+ The experimental protocol is shown on the page opposite to the table 6.1

suppression of IgM and enhancement of IgA are far greater. It is as if two isotype specific immune events of opposite directions have been caused to occur in the same set of test recipients but so designed that the effects can be observed separately.

CHAPTER VII

GENERAL DISCUSSION

GENERAL DISCUSSION

As mentioned in the general introduction these studies were aimed at the characterisation of antibody responses to V. cholerae given to mice by different routes and elucidating how local presentation of antigen in the gut modulates the antibody response towards a preponderance of IgA as compared to the IgM predominance over IgA following immunisation by the systemic route.

Two hypotheses to explain this change were tested. They were (i) that the antigen entering the gut is processed locally in such a way as to render it likely to generate an IgA response and IgM suppression; (ii) that modulator cells generated in Peyer's patches after administration of oral antigen are responsible for this pattern of response.

Characterization of antibody responses by various routes of immunization.

The two phenomena that characterize antibody responses in mice both primed and boosted by the intravenous route using various doses of V.cholerae are (i) a markedly low IgA/IgM PFC ratio of systemic PFC responses in the spleen and (ii) the absence of any significant local response in the gut lamina propria.

In all the nine experiments with intra-venous immunization schedules using various doses of V.cholerae the IgA/IgM ratios were found always to be very low, none of them rising above the value of 1/2.4 and ranging down to 1/16.5. These observations deal with ratios of Ig-specific responses of two classes and not any individual or groups

of Ig-specific results of a single class, and as there had been not a single case where the ratio reached unity, a definite generalization applied to later experiments was that a low IgA/IgM ratio in the spleen was a characteristic feature of antibody responses to V.cholerae in systemically primed and boosted mice.

The other phenomenon was best observed in the group of mice primed and boosted with i.v. $10^7/10^7$ V.cholerae. Though this group had the best IgM and IgA response in spleen, perhaps as a result of receiving the optimum dose-pairs for immunization, they were unable to show any significant response in the gut lamina propria. This remarkable absence of a mucosal immune response in systemically primed and boosted animals was consistent with similar findings by other investigators (Newcomb et al, 1969; Ogra, 1971; Pierce and Gowans, 1975).

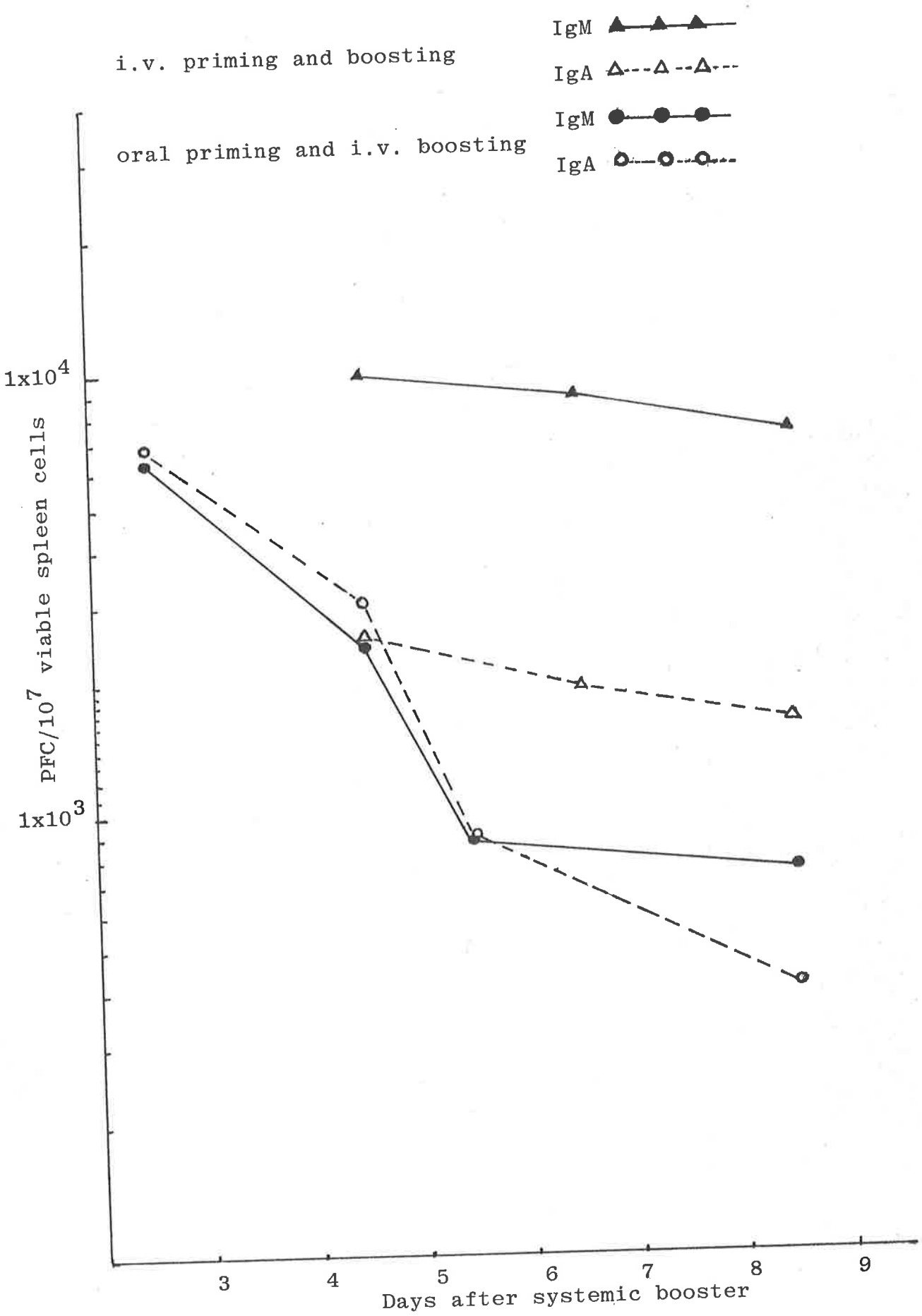
Thus, a combination of these two phenomena namely a low IgA/IgM ratio in systemic antibody response and absence of any significant response in the gut lamina propria seems to be a hallmark of this particular route of antigen presentation i.e. intravenous priming and boosting. The other findings of this immunization schedule like unexpectedly low and short-lived antibody responses in the group of mice repeatedly primed with 10^6 V.cholerae, highest and sustained response in $10^7/10^7$ and comparatively lower IgA response in $10^8/10^8$ priming/boosting groups seem to be functions of dose of the antigen in the process of immunization, particularly the priming dose.

Both the optimum-dose systemically primed/boosted and the high-dose orally primed/systemically boosted groups of mice showed absence of significant antibody response in the gut lamina propria, but their systemic reactions, as observed by PFC/10^7 viable splenocytes, had categorical differences in characters that have been shown in figure 6.1. The IgM response in the systemically primed mice started high on day 5 and reached day 9, with a very gradual slope up to a total drop of only 27% of its starting value. The corresponding IgA response started at about 4-fold lower level than the starting level of IgM but its course ran parallel with the slope of IgM up to day 9 maintaining quite low IgA/IgM ratios all through. In the orally primed group both the IgM and IgA responses started high on day 3 and at about the same level but dropped almost exponentially with roughly coinciding steep slopes up to a drop of 94% in IgA and 88% in IgM of its day 3 values by day 9, maintaining a very high IgA/IgM ratio above unity on the days 3, 5 and 6 after the booster. So, it is clear that an immunization solely by the systemic route elicited a 'systemic' kind of sustained response (with low IgA/IgM ratios); conversely, an oral followed by systemic immunization produced a 'local' kind of short-lived splenic response (with high IgA/IgM ratio). But none of them were effective to create local antibody responses.

An immunization schedule of priming and boosting solely by oral route only was capable of creating definite IgA antibody responses in the gut lamina propria on days 4-6 after booster as shown in table 3.3. Though in the first two experiments i.v. boosting was used in addition to oral boosting,

FIGURE 6.1

COMPARISON OF SPLENIC PFC RESPONSES
IN SYSTEMICALLY AND ORALLY PRIMED MICE
BOOSTED INTRAVENOUSLY



the contribution of the former would appear to have been minimal as no , significant gut response was observed in numerous previous experiments using orally primed and i.v. boosted mice.

Gut plaques were finally obtained using a schedule of repeated oral priming followed by oral boosting.

The ability of oral boosting of orally primed mice to generate an antibody response in the gut lamina propria was at variance with the observations of Bloom (Thesis, 1979) who obtained the highest gut PFC response by i.v. boosting of similarly primed mice, but in accordance with the results of Pierce and Gowans (1975) and Pierce (1976) who could produce recall-response of ACC in gut only by intra-duodenal boosting of orally primed rats. The importance of the oral route for boosting was bolstered by their observations that a still higher recall-response in i.p. primed rats with a toxoid-FCA also could be achieved only by oral but not by i.p. (parenteral) boosting.

The ratios of IgA/IgM in the gut responses of mice primed and boosted both orally were extremely high averaging 61.2/1. In fact, none of the five experiments (vide table 3.3) showing high or moderate IgA PFC numbers showed any IgM PFC response above the range of the significant lowest limit of the assay. This finding is in keeping with a similar observation of Pierce (1976) who demonstrated that virtually 100% of all the ACC, with the morphology of plasma cells, in the gut lamina propria of mice immunized in a similar way with cholera toxoid contained IgA.

Furthermore the ratio of IgA/IgM in the spleen in these animals (33.3/1, highest average splenic IgA/IgM ratio amongst all the groups) immunised purely by the oral route

supports the generalisation referred to earlier, that a high IgA/IgM ratio is suggestive of immunisation via the gut.

The role of processed antigen in PP.

From the experiments involving the transfer of PP homogenate, used to test the hypothesis that processed antigen was a possible mechanism for producing a 'local' kind of antibody responses, two conclusions could be drawn; (i) substantial amounts of antigenic material were transferred into the recipients via the frozen and thawed PP homogenate to adequately prime them for a systemic splenic response in both Ig classes. (This response was higher than that obtained in control mice which received three i.v. doses each of 10^6 V.cholerae for the priming). (ii) the change in splenic response was towards that seen after oral priming revealing a very high IgA/IgM ratio. As no live cells were transferred this is compatible with an effect of antigen processing within the PP. The first observation may be criticised with a query whether the antigen could have been transferred mechanically (e.g. adherent to the mucosal surface of the PP that had resisted saline-washings). However it seems highly improbable that even after three saline-washes more than 10^6 V.cholerae could pass on a dozen tiny PP by simple adherence to the overlying mucous membrane. In addition, a logical explanation may be put forward against this criticism in that if intact V.cholerae in their native

form were transferred, a 'systemic' kind of antibody response having a low IgA/IgM ratio would have been the outcome instead of a ratio above unity.

This observation pointed to the possibility that the hypothesis of processed antigen may be true, but this remains unproven because more experiments could not be done assaying recipient spleens individually to make the data adequate for statistical analysis.

It is not known why the recipient mice died of i.v. injections with PP homogenate. It was certainly not due to any suspended material big enough to cause embolism, because during the later experiments the final injectable material was prepared by homogenization with an ultratarrax, filtered through a 300 mesh wire-netting and mixed with a liberal dose of heparin to obviate any chance of embolism. In most of the mice death was almost instantaneous. This may have been due to a sudden load of cell-disintegration products of nucleic acids and electrolytes, especially potassium.

The splenic antibody responses obtained in the recipients by the transfer of collagenase-deaggregated, formalin-killed PPL from orally primed donors were very low in comparison to those obtained by the transfer of frozen and thawed material. In fact the IgM response on day 6 after the booster was of similar magnitude to that obtained after a single injection of 10^7 *V.cholerae* (which was the control for the booster only). The most important finding of this experiment is the absence of any 'local' character in the splenic response as was obtained by using its analogue material, namely the homogenate of whole PP from donor mice.

As has been mentioned in the preamble to Chapter IV the discrepancies between these apparently similar experiments presumably lies in the difference between their designs. Two possible results of the different schedules spring to mind. In the former experiments the whole of the substantial amount of antigen material that was trapped in the inter- and intrafollicular and even some areas of PP (possibly after processing,) has the chance to be transferred into the recipient to elicit respectable splenic responses and to convert the response into a 'local' kind. Conversely, most of the inter-cellular antigen in the latter experiments with collagenase-deaggregation and cell-washings may have been washed away and only the small amount of possibly native antigen that was intimately sticking to the cells had the chance to be transferred. Consequently the responses in recipient spleens were very low in magnitude and qualitatively mimicked the responses obtained by immunization with native antigen, i.e. with very low IgA/IgM ratios.

Another possible explanation for such a low grade antibody response in the recipients after the transfer of formalin-killed single-cell preparation is that as a result of formalin-treatment an otherwise significant amount of antigen material in the cells was fixed along with its carrier and was consequently unavailable for the stimulation of the lymphoid tissues in the animals into which it was transferred.

Although no method was found to prevent the deaths of mice receiving i.v. injections of the whole PP homogenates during these studies, it is to be hoped that this will be achieved in the future allowing possible substantiation

of this interesting concept of processed antigen.

The role of modulator cells in PP/transfer experiments with mixed PPL.

The studies on the modulation of systemic antibody responses (PFC response in spleen) to V.cholerae in mice by concomitant adoptive transfer of PPL from antigen-fed syngeneic donors (Chapter V) revealed that there was unidirectional profound suppression of IgM responses in all the experiments done on all the days 4, 6 and 8 after the booster in comparison to the results of all the antigen and True control experiments. However, the IgA responses were not unidirectional. They showed enhancement on all the days in test experiments T1 but exhibited suppression or no change in comparison to antigen and two true controls as shown in table 5.2

Before starting a detailed discussion on the experiments described in this chapter, incorporation of the antigen control and spleen cell control in addition to the true control groups I and II needs comment. These were incorporated for supporting data that could show the validity of the true controls. The antigen only control was used to check that PPL from normal conventional mice, per se, would not create results grossly aberrant from those evoked by antigen alone. The spleen cell control was to check whether any particular change in response was purely a consequence of the V.cholerae being in contact with macrophages at the time of injection. These two additional controls served their purpose. Except in the IgM response on day 4 after booster all their other results showed no

significant variation from those of true controls. The paradoxical variation in between different controls on day 4 was that the numbers of IgM PFC/10^7 viable splenocytes in the first and second true controls seemed to be much higher than that of the antigen only control. The levels of significance on analysis of variance by Mann-Whitney U test were 0.06 and 0.076 respectively. No satisfactory explanation can be put forward for this unexpected phenomenon and as noted above it did not reach statistical significance.

The observation of most pronounced degree of suppression in IgM responses during the period of its peak response (12 fold decrease in number of PFC in test mice in comparison to the control results on day 4 after booster) was remarkable. It suggests that the suppressive effect may have been selectively more pronounced on very actively replicating IgM-forming B cell clones.

The modulation of IgA responses in test mice with variable final results of enhancement, suppression or no effect is intriguing. The observation of unfailing enhancements on all the days 4, 6 and 8 after booster in experiments T1 was unique and pointed strongly to a definite biological phenomenon supporting the hypothesis under examination. But the variable results in the other three experiments T2 through T4 were perplexing.

Whether a particular dose pattern of PPL in the two successive injections given to the T1 mice-group was responsible for the impressive enhancement of the IgA response cannot be ascertained because PPL of one mouse on average from a large PPL pool of many were injected both times without counting the cells.

As mentioned earlier in Chapter I (Page 35) Elson et al (1979) in an attempt to determine relative capacities of different types of lymphoid tissues for the Ig class specific antibody synthesis in vitro found that PP cells were by far the most prolific producers of IgA. Also in trying to assess Ig class specific T cell regulatory activity of different lymphoid tissues they demonstrated differences in T cell regulatory activity for IgA, but not for IgM or IgG in various mouse tissues; for instance, the spleen contained predominantly IgA suppressor T cells. In the context to these findings one can attempt an explanation of the variable IgA responses in the test experiments. There might have been a dichotomy in the mechanism of IgA antibody response in the test mice as a result of this particular experimental design. The suppression of IgA response may have been guided by actively generated IgA specific T suppressor cells in the spleen in response to the systemic injection of the live bacterial antigen, whereas, the enhancement was probably the outcome of expanded T helper cell activity passively transferred into the test mice by way of PPL from antigen fed donor mice in addition to fruition of antigen driven IgA B cells maturing into antibody forming plasma cells. An unknown regulatory factor(s) might have tipped the balance either to enhancement or suppression or no change.

Another factor that could have been expected to influence the splenic IgA response in the recipients of test experiments T2 through T4 was the number of donor PPL transferred in them. But an examination of the numbers

of donor PPL actually transferred (Protocol, table 5.1) did not show any such relationship. However, suppressions in IgM responses seemed to have direct correlations with the increasing numbers of donor PPL transferred in the two injections. (Table 5.1 and its protocol).

In spite of the variable changes obtained in IgA PFC numbers in control and test mice, one important final change was observed in the qualitative character of the overall antibody response in the test mice as a result of receiving PPL from fed donors. That is, the conversion of the response from its so called 'systemic' into the 'local' character by the change of the IgA/IgM ratio from a much smaller fraction to up to very near unity or higher. These changes of the isotype ratio in the antibody response have been shown in table 5.3. The ratio of 1/8.7 to 1/12.9 with an average of 1/10.6 in the control mice on day 4 after the booster rose up to 1/3.1 to 2.5/1 with an average of 1.26/1. Similarly on the day 6 after booster the average ratio of 1/15 had a rise up to an average of 1/1.29. One experiment on day 8 after the booster showed a rise from about a fourteenth to almost twice unity. It is pertinent to recapitulate here from Chapter III (Table 3.2 and 3.3) that when mice were primed with repeated heavy doses of antigen by the oral route and boosted by the i.v. or the oral route this isotype ratio of spleen PFC on the average were 1.06/1 (average of ratios on day 3, 5 and 6) and 33.3/1 respectively, though the magnitude of responses were not very high in the latter group. This ratio was still extreme on the side of IgA in the PFC response in the gut lamina propria of the latter group of mice where

TABLE 5.3

IgA/IgM SPLENIC PFC RATIOS* IN MICE RECEIVING PRIMED PPL COMPARED TO RATIOS IN CONTROL GROUPS

Control groups	Day after booster 4	6	8	Test groups	Day after booster 4	6	8
True Control I	1/10.6	1/14.9	ND	T 1	2.5/1	2.3/1	1.7/1
True Control II	1/12.9	1/14	"	T 2	1/3.1		
Antigen Control	1/10.3	1/16.5	1/13.7	T 3	1/2.2	1/4.2	
Spleen cell Control	1/8.7	1/14.6	ND	T 4	1.7/1	1/3.8	
Average ratios of all controls	1/10.6	1/15		Average ratios of all tests	1.23/1	1/1.29	

* GM of IgM and IgA PFC/10^7 viable lymphocytes from all mice in each experiment on a day were used for calculating the ratios

ND - Not done

there were none or negligible number of IgM as opposed to average ~ 900 IgA plaques on day 4 to 6 after the oral booster. Clearly, antigen presented to the gut had modulated even the systemic responses, as monitored by spleen plaquing, to have completely altered the isotype ratio as opposed to that seen in parenterally primed and boosted mice. This qualitative character of isotype ratio of antibodies irrespective of its quantitative magnitude seems to be the hallmark of an antibody response to antigen entering via the gut.

It was mentioned earlier that though substantial data have been generated in support of the two main immune events occurring as a result of local presentation of antigen, namely (i) suppression in systemic antibody responses and (ii) production of IgA antibody in secretory surfaces, few experiments have been done to show that these events occurred concurrently. The present group of experiments, using live V.cholerae as the antigen, is one serving this purpose. This is the first experiment, to my knowledge, in which a live enteric bacterium has been used successfully in proving the fact that these two immune events can and probably do occur simultaneously as has been shown in the recipient mice by transferring PPL from orally primed donors in conjunction with the native antigen. At least, the data of the test experiment T1 on all the three days (day 4, 6 and 8) after the booster, tends to prove the fact directly. The sum total of the data of all four test experiments (T1 through T4) on the average showing conversion of the IgA/IgM isotype ratio of the systemic antibody response from a 'systemic' to a

'local' kind produces strong indirect proof (vide Table 5.3, Chapter V) of this concept.

The use of a live enteric bacterium specially selected for its property of adherence in this experiment as compared to a soluble protein (ova) and dead particulate bacterial antigen (Streptococcus mutans) used earlier (Challacombe and Tomashi Jr 1, 1980), is important for the obvious reasons of its similarity to natural infections of the gastrointestinal tract. The use of V.cholerae, in particular, probably has greater theoretical implications because it causes a disease in which bacterial multiplication with production of exotoxin occurs on the surface of the gut after simple attachment of the organisms by the property of adherence. The clinical manifestations are due to altered physiology caused by the liberated toxins without obvious changes in the anatomical structures of the mucosa. Also, this is why any protective immunity that comes into play in this disease is thought and expected to be primarily active locally.

Transfer experiments with T and B subpopulations of PP.

The experiments described under Chapter VI using adoptive transfers of purified T and B cells separately from PPL of orally primed donors in conjunction with V.cholerae, resulted in unidirectional suppressions in IgM splenic responses by both T and B subpopulations on both the days 4 and 6 after the booster. With the same experimental design unidirectional enhancements of IgA responses on day 4 by only T cell transfer and on day 6 by T and B cell transfers were also demonstrated.

These were the same two observations made previously in experiments using mixed cells from whole PP, the IgM suppression in all the test experiments and IgM suppression plus IgA enhancement in test experiments T1 on days 4, 6 and 8, after booster. (Table 5.2, Chapter V.)

Earlier evidences for gut-induced suppression of systemic response : T suppressor cells.

The systemic suppression, commonly designated as Sulzberger-Chase phenomenon, has been demonstrated in a number of experiments using various types of antigens like oxazolone, ovalbumin, BSA, formalin-killed streptococcus etc. but not a live enteric bacterium as is used in the present project.

As possible mechanisms for this phenomenon various factors like antibody (Kagnoff, 1978), immune complex with IgA as the antibody (Andre et al, 1975), and humoral suppressor factors (Kagnoff 1980) etc. have been implicated; but the present consensus is that modulator cells such as T and B suppressors of GALT probably play the primary role in this phenomenon.

Hanson et al (1979) demonstrated that sera recovered from almost completely tolerant OVA-fed mice could not inhibit antibody responses of normal recipients by the criteria of antibody titers in Farr assays and passive cutaneous amaphylaxis (PCA) assays for IgE antibodies, whereas responsiveness of normal or OVA-primed syngeneic spleen cells was substantially reduced upon transfer into recipients that were previously fed OVA, when compared with transfer into saline-fed recipients. The differences in responsiveness of lymphocytes transferred into OVA- and

saline-fed mice were completely eliminated by irradiation (850R). The conclusion was that the post-feeding state of tolerance was actively maintained by radio-sensitive T suppressor cells and not by antibody or any humoral inhibitory factor of the fed mice. Miller and Hanson (1979) observed that antigen-specific T suppressor cells of either spleen or lymphnodes of OVA-fed donors, but not the serum, could transfer suppression into normal recipients. Ngan and Kind (1978) demonstrated T suppressor cells for IgE and IgG in PP of mice that were made tolerant by oral immunization of OVA. Richman et al (1978) showed that anti-hapten (DNP) responses also were diminished when mice were fed autologous carrier (OVA or keyhole limpet hemocyanin). A tolerant state was observed to be maintained in irradiated recipients by adoptive transfer of spleen cells from intragastrically primed donors and specific anergy could be transferred to normal recipients. That adoptive suppression was mediated by T lymphocytes was proved by nylon wool fractionation and susceptibility of the cells to Anti-Thy 1.2 serum and complement. Rats orally fed with SRBC were found by Mattingly and Waksman (1978) to contain specific suppressor cells to SRBC in PP and MLN after two days of feeding. These suppressor cells blocked IgM and IgG PFC response to SRBC in Mishell-Dutton culture and DTH responses on transfer into syngeneic recipients. They were of the long-lived recirculating T cell type which were eliminated in rats injected in vivo with antilymphocyte serum and were not affected by adult thymectomy or cyclophosphamide.

Evidence for B suppressor cells

There was one convincing experiment by Asherson et al (1977) in support of the existence and activity of B suppressor cells (in association with T suppressors) in mice following oral immunization with the contact sensitizing agents oxazolone or picryl chloride. A prior feeding of oxazolone 14 days before painting with the same agent almost abolished the contact sensitivity and antibody production which normally occurs on painting normal mice. Single feeding with either of the agents evoked suppressor cells in PP and MLN, and three feedings produced suppressor cells in spleen as well. These cells could suppress the passive transfer of contact sensitivity and were found to be presumptive B-cells because they had the ability to form rosettes with erythrocytes coated with antibody and complement and resisted lysis by anti-Θ serum plus complement. In addition to the B suppressor cells, spleen and peripheral lymphnodes of mice fed and painted later contained T cells (suppressors) which, when transferred into normal recipients followed by skin-painting, limited the DNA synthesis in the recipient regional lymph nodes.

Evidence of IgA-specific T suppressor cells.

As has been cited in Chapter I (page 35) in detail , Elson et al (1979) with the aim of determining Ig isotype-specific variation of T cell regulatory activity on B-cell functions found differences in T cell regulatory activity for IgA, but not for IgM or IgG synthesis, in various mouse lymphoid tissues. PP T-cells, particularly, were found to contain a high level of IgA T-cell helper activity compared

to that of spleen or PLN; spleen tissue contained predominantly IgA specific T suppressor cell activity. It is noteworthy that the experiment produced convincing evidence in favour of the existence of IgA specific T suppressor and T helper cells; otherwise all the suppressor activity detected up to date in lymphoid tissues of orally immunized animals appeared to be specific for IgG and IgM as well as CMI responses.

Present results in the light of earlier observations

The salient findings of the purified PP T and B cell transfer experiments given in Chapter VI may now be examined in the light of evidence put forward in favour of various types of modulator cells generated in animals as a result of immunization by gut presentation of antigen. The significant suppression of splenic IgM PFC response on day 4 and 6 after booster by T cell transfer in reference to the results of first and second true controls (Table 6.1) were in agreement with the observations of T suppressor cell activity in many experiments manifesting Sulzberger-Chase phenomenon using various kinds of antigens except live enteric bacteria. Similar significant suppression in IgM responses on both the days after booster by B cell-transfer had corroboration from Asherson's demonstration of B suppressor cells obtained in mice fed with oxazolone or picryl chloride.

The significant enhancements in IgA response in recipient mice by PP T-cell transfer on both the days 4 and 6 after the booster in comparison to the control results

had strong support from Elson's (1979) observation of extremely high IgA-specific T helper cell activity in PP tissue.

The highly significantly enhanced IgA splenic response in the recipient test mice by PP B cell transfer on day 6, but not significantly changed IgA response on day 4 after the booster in comparison to IgA results in the mice of all the control experiments looked apparently paradoxical. The enhanced IgA response in the test recipients on top of a low grade response (as was observed in control mice receiving antigen and normal PPL) expected to have been created by concurrent immunization with antigen was most probably due to the input of an additional crop of plasma cells generated in the second host by the fruition of transferred antigen-driven immediate precursors of IgA B cells. As it took some time in proliferation and differentiation for reaching the stage of fully matured IgA plasma cells - the enhancement in IgA response was manifested in the later assays on day 6 but not on day 4 after the booster.

Concurrent occurence of IgM suppression andIgA enhancement in the test recipients by the transfer of T or B PPL form orally primed donors.

So, it was clearly shown, that as an outcome of adoptive transfer of purified PP T cells from gut presented locally with antigen, a suppression of IgM and enhancement of IgA responses in reference to the corresponding results in control mice occurred concurrently in the same set of test recipients. Similar concurrent suppression of IgM on day 4 and 6 and enhancement of IgA responses on day 6 in another set of

test recipients occurred as an outcome of transfer of purified PP B cells from the gut of antigen fed donors. Both these observations were similar in nature with the demonstration of concurrent occurrence of systemic suppression (by inhibition of the proliferative response in the draining lymph nodes of mice primed intragastrically and boosted subcutaneously) and production of salivary IgA antibodies (immunized only intragastrically) using OVA or formalin-killed streptococcus mutans by Challacombe and Tomasi Jr. (1980).

There is another important facet of the interpretation of these results obtained in the transfer experiments with T and B PPL that can be ascribed to a special aspect of the experimental design. As described in Chapter II (page 50), mixed PPL from the same set of orally primed donors were partitioned into purified T and B subpopulations by the 'panning' technique and were injected into two sets of test recipients in the same sitting. For this reason, as has been emphasized at the end of Chapter VI, the implication of the demonstration of the concurrency of IgM suppression and IgA enhancement in the test recipients by the transfer of either T or B PPL from orally primed donors seems to be appreciably greater than that of a similar finding in experiments in which T or B cells would have been taken from different sets of donors. In this situation, it seems reasonable to extrapolate the data obtained in the two sets of recipient test mice and infer that these two immune events could occur concurrently in a primary set of orally immunized mice as a final outcome of the effects of IgM suppressing and IgA enhancing T and B modulator cells and the fruition of

antigen-driven precursor IgA plasma cells from the PP..

Thus Nature's logical mechanism of the concurrent occurrence of IgM-suppression and enhanced IgA-synthesis by gut presentation of antigen have been seen to occur in the interest of the host. As a result noxious antigens are handled at the gut mucosal surface by exclusion with a brisk IgA response and systemic response of the complement-fixing IgM antibody is tamed down to prevent the allergic damage of the deeper mucosal layers,in case the first line of defence is broken. This kind of balanced co-existence of immunity and tolerance is perpetuated for the maintenance of immunologic homeostasis of the host.

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