



IMMUNE RESPONSE TO VIBRIO CHOLERAE
IN THE MOUSE

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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.

(L.D. BLOOM).

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ABSTRACT

Immune Response to Vibrio cholerae in the Mouse

This thesis is primarily concerned with the principles underlying immunization against cholera. Evidence is presented in the Introduction that it is the stimulation of immunity in the intestine which protects against this disease. Accordingly, a study was made of the factors required to stimulate a local immune response to Vibrio cholerae.

For this work a systematic survey was performed of the systemic and local immune responses of mice to a selection of V. cholerae vaccines and vaccination schedules. The two main assay methods were the enumeration of antibody forming cells in the spleen and the mucosa of the small intestine by the Jerne technique, and the measurement of antibody levels in the serum and intestinal juice by haemagglutination.

The major findings were as follows:

1. Adhesion of virulent V. cholerae to the mucosa of the small intestine precedes infection in the infant mouse.
2. Antibody prevents the infection in infant mice by reducing the numbers of organisms adherent to the mucosa, and probably mediates this by blocking the adhesion.

3. A strain of V. cholerae which tends to be more adherent to the mucosa than another also tends to persist in the intestine of adult mice for a longer period of time following oral administration. This was related to the greater immunogenicity of an adherent strain when given as an oral vaccine.
4. The most significant immune responses in the intestine were obtained with multiple oral vaccinations followed by an intravenous boosting dose. The superior vaccines were prepared from living V. cholerae of an adherent strain.
5. Evidence was found of an immuno-regulatory mechanism which induced cyclic variations in the immune response to V. cholerae. It was associated with a soluble suppressive factor in the serum.

These findings are discussed in terms of their implications for oral immunization. Other points of discussion include the actions of antibody which are mediated in the intestine and the role of immuno-regulation.

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CHAPTER 1



CHAPTER 1

LITERATURE REVIEW

Part A Local Immunity in the Gut

1.1 Introduction

Oral vaccination would appear to be the rational way to immunize against an enteric infection, and the success of the Sabin poliovaccine supports this view. However, oral vaccines have been used for many years but in general with disappointing results. There is an obvious need for a greater understanding of the response of the gut associated lymphoid tissue to antigens presented to the gut. Such an understanding should lead to improvements in oral immunization.

This part of the literature review will outline what is known about the immune functioning of the gastrointestinal tract. It will also draw attention to the large gaps in this knowledge, in particular the details relating to the stimulation, differentiation and migration of Peyer's patch cells. The immune effector mechanisms of the gut constitute another poorly understood area, for the environment of the gut appears to be hostile to a number of immune functions which are important systemically.

1.2 Peyer's Patches

1.2a Introduction : The Peyer's patches are specialized lymphoid organs which play an important part in gut

immune responses. This was illustrated by Robertson and Cebra (1976), who prepared rabbits with pairs of Thirly-Vella ileal loops, one with a Peyer's patch and one without. There was an IgA response in both loops to an antigen placed in the one with the Peyer's patch; antigen placed in the loop devoid of a Peyer's patch evoked little response in either loop.

The immune functions ascribed to Peyer's patches, to be discussed in this section, have also been ascribed to the lymphoid tissue of the rabbit appendix (reviewed by Parrott, 1976).

1.2b Antigen uptake : The gut takes up small amounts of antigen presented at its lumenal surface. Antigens taken up include soluble proteins like horseradish peroxidase and bovine serum albumin (Walker, Isselbacher and Bloch, 1972); particulate substances such as ferritin and india ink (Bockman and Cooper, 1973); and whole cells of yeast, bacteria and viruses (Raettig, 1968).

Antigen enters through the specialized epithelium overlying the Peyer's patches. This epithelium is cuboidal (Faulk, McCormick, Goodman, Yoffey and Fudenberg, 1971), and has a capacity for the uptake of particulate antigen (Bockman and Cooper, 1973). The antigen is taken up by pinocytosis (Walker, Isselbacher and Bloch, 1972; Bockman and Cooper, 1973). It has been reported that large particles work their way into the Peyer's patch by passing between the

epithelial cells, a process termed 'persorption' (Raettig, 1968). Many workers have failed to find evidence for the transfer of antigen across the epithelial tight-junctions, and query the existence of 'persorption' (Walker, Isselbacher and Bloch, 1972).

Salmonella (Carter and Collins, 1965) and shigella (La Brec and Formal, 1961) both penetrate the intestinal epithelium, and do so through the Peyer's patch epithelium. The significance of this penetration for the local immune response is unknown.

Whereas the Peyer's patches retain particulate antigens such as shigella (La Brec and Formal, 1961), killed particulate toxoplasmas (Strannegard, 1967) and ferritin (Crabbe, Nash, Bazin, Eyssen and Heremans, 1969), the retention of soluble antigens, like bovine serum albumin, has not been shown (Bienenstock and Dolezel, 1971). Either there is particularly poor antigen retention, due to a lack of organised antigen-trapping reticulum in the Peyer's patches (Bockman and Cooper, 1973), or the rapid degradation of soluble antigens makes them difficult to detect.

1.2c Peyer's patch immunocyte development : Extensive B-cell proliferation without in situ maturation to plasma cells is a notable feature of Peyer's patch immunocyte development (Waksman, Ozer and Blythman, 1973). This observation is compatible with the theory

that the function of Peyer's patches is to populate the lamina propria with plasma cells (Craig and Cebra, 1971), discussed in Section 1.3.

Cebra, Kamat, Gearhart, Robertson and Tseng (1977) have conjectured that the distinctive morphology of the Peyer's patches may not provide the interactions necessary for B-cell maturation to plasma cells. They point out that cellular interactions may be hampered by the segregation of T-lymphocyte interfollicular areas from the B-lymphocyte follicular areas (Goldschneider and McGregor, 1973; Faulk, McCormick, Goodman, Yoffey and Fudenberg, 1971). Moreover, there have been reports that Peyer's patches are deficient in accessory adherent cells in some animals (Kagnoff, 1975; Kagnoff and Campbell, 1974). There may also be a positive inhibition of B-cell maturation imposed by the presence of considerable numbers of suppressor T-cells (Kamin, Henry and Fudenberg, 1974).

Attempts to stimulate the appearance of antibody forming plasma cells in the Peyer's patches by a wide variety of antigens and immunization schedules consistently fail (Henry et al, 1970). A small, delayed appearance of plasma cells can be provoked by a direct injection into a patch, but this situation has been described as distorted and unphysiological (Veldkamp, Van der Gaag and Willers,

1973; Bienenstock and Dolezel, 1971).

However, antigen stimulation does activate B-cells, with T-lymphocyte help, in the germinal centres of the patch follicles (Mitchell, Pye, Holmes and Nossal, 1972). The B- blasts have little cytoplasmic immunoglobulin, but most have membrane IgA (Guy-Grand, Griselli and Vassalli, 1974). The activated B-cells share with other lymphoid tissue the potential to generate plasma cells, shown by the transfer of these cells, supplemented with thymocytes, to irradiated syngeneic recipients (Cebra et al, 1977).

The T-cells in the Peyer's patch can also be activated by antigen feeding (Levin, Rosenstreich, Wahl and Reynolds, 1974; Kagnoff, 1975; Muller-Schoop and Good, 1975). Like the B-cells, there is evidence that the T-lymphocytes do not have an effector function in situ (Kagnoff, 1975), but may well have such a function after migrating from the patches (see Section 1.3). The guinea-pig may be an exception, as its Peyer's patch T-cells have the potential to induce a Graft-versus-host reaction, to produce lymphokines upon mitogenic stimulation and to proliferate in response to allogeneic lymphocytes (Levin et al, 1974).

1.2d Differentiation of Peyer's patch immunocytes :

Cebra and co-workers have reported extensive work on the differentiation of immunocytes in Peyer's patches

(in Ciba foundation symposium 46 : Cebra et al, 1977; and Husband, Monie and Gowans, 1977). They have found that there is a preferential development of B-cells bearing the IgA isotype in this site. In one experiment, bone marrow cells bearing almost no surface IgA were transferred to irradiated congenic BALB recipients. The cells proliferated in Peyer's patches to generate clones capable of mounting an IgA response. As a B-cell clone may have a single idiotype but multiple isotypes, the potential of B-cells to express any particular isotype can be readily appreciated (Gearhart, Sigal and Klinman, 1975).

Little is known about the factors in the Peyer's patch environment which favour the expression of the IgA isotype. One speculation is that repeated B-cell division without differentiation is responsible (Cebra et al, 1977). The same group has found that antigen can change the potential of Peyer's patch cells. They stimulated the Patch cells with a variety of antigens in vitro and analyzed the immunoglobulin product of the clones that were generated. It was interesting that antigens normally present in the gut stimulated the generation of large clones making IgA; antigens not normally presented to the gut stimulated the generation of clones making IgM.

1.3 Immunocyte Circulation and Migration1.3a Migration of Peyer's patch immunoblasts to the gut :

It is now known that immunoblasts present in the intestinal lymph enter the circulation and then preferentially migrate to the lamina propria of the small gut. This migration has been shown for B-blasts (Gowans and Knight, 1964; Griscelli, Vassalli and McCluskey, 1969; Hall and Smith, 1970) and for T-blasts (Sprent and Miller, 1972, Guy-Grand, Griscelli and Vassalli, 1974; Ford, 1975; Sprent, 1976). In the lamina propria the B-blasts differentiate to plasma cells, and the T-blasts to small interepithelial lymphocytes (Guy-Grand, Griscelli and Vassalli, 1974).

The route of migration is understood reasonably well. Peyer's patch immunoblasts migrate through the lymphatics to the mesenteric lymph nodes (Jacobsen, Marks, Simmons and Gaston, 1961), and then to the thoracic duct (Griscelli, Vassalli and McClusky, 1969). The thoracic duct immunoblasts enter the circulation and then home to the gut (Guy-Grand, Griscelli and Vassalli, 1974), if they originate from the mesenteric lymph nodes (Griscelli, Vassalli and McClusky, 1969) or the Peyer's patches (Craig and Cebra, 1971, 1975; Rudzik, Perey and Bienenstock, 1975). Some immunoblasts go to the Peyer's patches and mesenteric lymph nodes, rather than to the lamina propria (Guy-Grand, Griscelli and

Vassalli, 1974). Immunoblasts not of intestinal origin do not home to the gut lamina propria, but rather to somatic lymphoid tissue (Parrott and Ferguson, 1974; Hopkins and Hall, 1976; Hall, Hopkins and Orlans, 1977).

1.3b Circulation of small immunocytes : A large pool of small lymphocytes constantly migrates between the spleen, the lymph nodes, and the gut-associated lymphoid tissue (Gowans and Knight, 1964; Goldschneider and McGregor, 1968) but recent evidence contradicts the belief that small lymphocyte recirculation is random. Sheep have been useful in these studies as their size facilitates the cannulation of the efferent lymphatics of peripheral somatic lymph nodes and of the intestine. Small lymphocytes were isolated and radiolabeled, and then injected into the circulation. Small B- and T-cells from the somatic lymph nodes were found to preferentially circulate through other somatic lymph nodes; small lymphocytes from the intestinal lymph tended to reappear in that lymph (Cahill, Poskitt, Frost and Trnka, 1977; Scollay, Hopkins and Hall, 1976).

1.3c Mechanisms of preferential immunocyte migration : Gowans and Knight (1964) suggested that the homing of immunoblasts in the thoracic duct to gut-associated lymphoid tissue might reflect a specific attraction

or retention of cells that had been previously sensitized to antigens prevalent in the intestinal tract. In fact a segment of intestine injected with cholera toxoid tends to select toxoid sensitized immunoblasts to a greater extent than segments of intestine without the antigen (Pierce and Gowans, 1975).

There is, however, evidence that much of the homing to the gut mucosa is antigen independent. When mesenteric lymph is collected from restricted lengths of sheep small gut and the immunoblasts are injected intravenously, they become uniformly distributed along the entire length of the small gut. Also, when killed bacteria are injected locally into the gut wall, specific immunoblasts released later into the lymph show no tendency to localise at the site of initial injection of the antigen. Similarly, lymph borne immunoblasts show the usual patterns of distribution when injected into allogeneic fetal recipients in a sterile environment in utero (Hall, Hopkins and Orlans, 1977).

The selective homing of intestinal immunoblasts has also been shown in the relatively antigen-free environments of germ free recipients (Griscelli, Vassalli and McCluskey, 1969), neonates (Halstead and Hall, 1972), and implants of fetal tissue (Moore and Hall, 1972; Parrott and Ferguson, 1974). In addition, small T-cells from fetal sheep were found in larger numbers in the interfollicular area of the Peyer's

patches and the mesenteric lymph node than in peripheral lymph nodes, after re-infusion intravenously (Pearson, Simpson-Morgan and Morris, 1976).

It has been suggested that IgA is part of the receptor system that mediates the extravasation of immunoblasts in the gut. During migration the B- blasts gradually acquire cytoplasmic immunoglobulin: the cells bearing membrane immunoglobulin will only home to the lamina propria if they have matured to the stage of containing cytoplasmic immunoglobulin (Guy-Grand, Griscelli and Vassalli, 1974).

However, it is unlikely that IgA is part of this postulated receptor system as : many plasma cells secrete IgM and IgG, not IgA (Lee and Lascelles, 1970; Lascelles and McDowell, 1974); and small T-cells as well as T immunoblasts can also home to the gut (Ford, 1975; Sprent, 1976; Cahill, Poskitt, Frost and Trnka, 1977). Whatever the nature of the surface markers that result in the recirculatory bias, it now seems likely that lymphocytes have to be categorized as somatic or secretory immune system cells. Such a dichotomy may account for Hodgkin's disease being primarily a lesion of somatic lymphoid tissue, sparing the gut, and Mediterranean lymphoma often being a lesion of gut associated lymphoid tissue alone (Hall, Hopkins and Orlans, 1977).

1.3d Functions of lymphocyte circulation : The recirculation of small lymphocytes contributes to

primary immune responses by enabling a small depot of antigen to select sensitive cells from a body-wide lymphocyte pool. Experimental evidence for this has been in three areas : general depletion of the recirculating lymphocyte pool (Ford and Gowans, 1967); selective depletion of recirculating antigen-sensitive cells (Sprent, Miller and Mitchell, 1971); and selective enrichment of specific lymphocytes in antigenically stimulated tissues (Atkins and Ford, 1975). Secondary immune responses also benefit, mediated by the dissemination of immunological memory cells (Gowans and Uhr, 1966), and the antigen-specific recruitment of these cells (Rowley, Gowans, Atkins, Ford and Smith, 1972; Thrus and Emerson, 1972).

Immunoblasts which differentiate into effector cells have a brief half-life (Mattioli and Tomasi, 1973), and tend not to recirculate but to terminally migrate (Gowans and Knight, 1964). Their dissemination functions to extend the territory involved in antibody synthesis during the course of a primary response (Hay, Murphy, Morris and Besis, 1972).

1.4 Functional Aspects of the Intestinal Immune Response

IgA was first isolated by Heremans, Heremans and Schultze in 1959. Secretory IgA was later found to be the predominant immunoglobulin of the external secretions (Tomasi and Zigelbaum, 1963), and plasma cells of the IgA class were found to be the most numerous at secretory

surfaces (Tomasi, Tan, Solomon and Prendergast, 1965).

In the human jejunum, IgA plasma cells outnumber those of the IgM class six to one (Crabbe, Carbonara and Heremans, 1965).

The predominance of IgA may have been overemphasized, for the ratio of IgA and IgM plasma cells in the porcine duodenum is near unity (Brown and Bourne, 1976). IgM plasma cells are even more numerous than IgA cells in the intestines of some young animals, including: calves (Porter, Noakes and Allen, 1972; Porter, 1973; Olson and Waxler, 1977); pigs (Allen and Porter, 1973, Porter, 1973); and rabbits (Crandall, Cebra and Crandall, 1967). As well, significant intestinal IgM responses have been recorded in human infants (McNeish, Evans, Gaze and Rodgers, 1975) and rats (Robertson and Cooper, 1973).

Despite much controversy, it appears that sIgA is unable to opsonize for phagocytosis (Zipursky, Brown and Bienenstock, 1973; Steele, Chaicumpa and Rowley, 1974), nor to activate complement via the classical or alternate pathways (Colten and Bienenstock, 1974). There were reports that sIgA could fix complement in the presence of lysozyme (Adinolfi, Glynn, Lindsay and Milne, 1966; Hill and Porter, 1974), but other workers could not find this effect (Steele, Chaicumpa and Rowley, 1974; Heddle, Knop, Steele and Rowley, 1975). Even though components of complement have been found in intestinal secretions (Girard and de Kalbermatten, 1970), these secretions are highly anticomplementary (Freter, 1962).

The major function of sIgA may be merely to bind to

antigens (Steele, Chaicumpa and Rowley, 1975), resulting in the blocking of adherence of pathogenic bacteria to mucosal surfaces (Williams and Gibbons, 1972) and their elimination from the gut (Bloom and Rowley, 1977). Similarly, IgA reduces antigen uptake from the gut (Walker, Isselbacher and Bloch, 1972).

IgG and IgM are secreted locally (Svennerholm and Holmgren, 1977), as well as being transferred from the serum actively (Brandtzaeg, 1973) and passively (Wernet, Breu, Knop and Rowley, 1971; Heddle and Rowley, 1978), and IgM becomes the major secretory immunoglobulin in sIgA deficiency (Lamm, 1976). If sIgA has an advantage over IgG and IgM it is not obvious at present, for even though they are degraded in the gut, their fragments may be active in preventing adhesion of bacteria to the mucosal surface (Steele, Chaicumpa and Rowley, 1975).

The status of other immune functions in the gut is uncertain. IgE plasma cells have been demonstrated in the gut mucosa and the gut associated lymphoid tissue (Tada and Ishizaka, 1970). IgE may degranulate the mast cells of the epithelium (Collan, 1972), facilitating granulocyte migration to the gut lumen. Such increased granulocyte traffic has been induced in pigs by specific immune reactions (Bellamy and Neilsen, 1974), but the significance of this is not clear.

Another unexplored area is cell-mediated immunity in the gut mucosa, which may be important as there is an abundance of T-cells in this site (Guy-Grand, Griscelli

and Vassalli, 1974). It is clear that cell mediated immunity is of little importance in the gut lumen, as intra-epithelial lymphocytes reside in the basal region of the epithelium and do not migrate to the lumen (Darlington and Rodgers, 1966; Meader and Landers, 1967). Small numbers are exfoliated into the lumen (Pink, Croft and Creamer, 1970), but they do not remain viable (Laissue, Chanana, Cottier, Cronkite and Joel, 1976).

Part B Regulation of the Humoral Immune Response

1.5 Introduction

The outcome of an antigenic challenge is the result of complex interactions in the immune system and an understanding of these interactions is vital in order that immunity and tolerance can be induced on a logical basis.

The gut presents peculiar difficulties to our understanding of immune responsiveness, in that it is exposed to enormous quantities of antigen in the form of food. It is advantageous that immune reactions to food antigens be suppressed, but at the same time gut pathogens have to be recognized and effectively eliminated.

Contrary to former belief, macromolecules (Walker, Isselbacher and Bloch, 1972) and even whole cells (Raettig, 1968) can enter the circulation from the gut. However, there is evidence that absorbed antigen may induce a state

of systemic immune unresponsiveness. An example is the serum titre of anti-BSA, which declines with age even though the antigen continues to be absorbed at the same rate (Korenblat, Rothberg, Minden and Farr, 1968). Animal experiments have confirmed the ability of ingested haptens (Chase, 1946) and food proteins (Thomas and Parrott, 1974) to induce such a state of immune unresponsiveness.

It is evident that without adequate controls self-damaging immune reactions to environmental antigens may occur. An example may be the association of infantile IgA deficiency and the development of an atopic state (Taylor *et al*, 1973). More needs to be known before the intestinal and other symptoms of atopic individuals can be effectively controlled by the stimulation of immune regulatory mechanisms.

On the other hand means have to be found to stimulate optimal immune responses against enteric pathogens such as Vibrio cholerae, without inducing a hypo-responsive state with the vaccination. Such a note of caution was sounded by Andre, Bazin and Heremans (1973) and Andre, Heremans, Vaerman and Cambiaso (1975) when they reported the induction of immunological tolerance in mice by feeding sheep erythrocytes.

The control of immune responses is broadly described in terms of central and peripheral mechanisms. In a central mechanism, there is a direct repression of the cells involved in antibody formation. A large number of these

systems have been reported, which is not surprising considering the number of points at which an immune interaction can potentially be disrupted. Peripheral mechanisms include those which are afferent, preventing antigen from stimulating antigen-sensitive cells, and those which are efferent, preventing the products of immune reactions from interacting with the antigen.

Definitions in the field of immunoregulation are ambiguous, the consequence of a poor understanding of a large number of phenomena. In a simple perspective, immune responses can be suppressed by antigen or antibody. Antigen was thought to 'switch off' immunocytes, a relatively long lasting central suppression of the immune response termed 'immunological tolerance' (Dresser and Mitchison, 1968). Antibody could prevent antigen reaching immunocytes, resulting in a shorter term peripheral repression of the immune response termed 'antibody suppression' (Britton and Moller, 1968). In another category it was recognised that some antigens, such as pneumococcal polysaccharide, could suppress by their presence at the lymphocyte cell-membrane, termed 'immune blockade' (Schrader and Nossal, 1974).

However, the situation is no longer straight forward, with reports of antibody working with antigen to suppress the immune response at various levels. Subimmunogenic quantities of antigen induce 'low-zone tolerance' (Dresser and Mitchison, 1968), apparently after complexing with trace amounts of antibody (Feldman and Diener, 1970, 1971, 1972). As well, antibody may be 'suppressive' after

complexing with antigen and acting at a central level (Askonas, McMichael and Roux, 1976).

In this discussion, immunoregulatory mechanisms acting following the exposure to antigen will be considered, and then the part that antibody plays in the feedback control of its own production will be covered.

1.6 Regulation by Central Mechanisms

1.6a Cellular interactions in antibody formation : The diversity of central mechanisms reported can be placed in a framework of what is known about the development of an immune response. The basic interaction is between a B-cell which produces antibody and a T-cell which helps it to do so (Claman and Mosier, 1972; Miller and Mitchell, 1968). Polymerized antigens such as pneumococcal polysaccharide do not require as much T-cell help.

A third cell type, an adherent cell, is required for the initiation of antibody formation after immunization with most antigens (Cozena et al, 1971; Feldman, 1972). It may function by effectively presenting antigen to responding lymphoid cells by concentrating antigen on the cell surface (Feldman, 1973).

It is assumed that in the immune response to hapten-carrier conjugates, T-cells recognize and bind the carrier and present the hapten to the hapten-specific B-cell. Both B-cells and T-cells have

antigen specific receptors (Paul et al, 1970). The T-cell recognition sites may share determinants with immunoglobulins (Eichmann and Rajewsky, 1975) or may be the product of the I region of the major histocompatibility complex (Benacerraf and McDevitt, 1972).

- 1.6b Accessory cell inactivation : One line of work using spleen cells in vitro has shown that only the non-lymphoid accessory cell is inactivated by antibody. The inhibition is apparently related to binding of the Fc portion of 7s antibody to the surface of these cells (Abrahams, Phillips and Miller, 1973). This binding may disrupt antigen focusing (Hoffmann, Kappler, Hirst and Oettgen, 1974), which is considered to be a prerequisite for effective antigen presentation to B cells (Feldman, 1973).
- 1.6c Antigen-antibody complexes : Immune complexes can induce immune unresponsiveness (Feldman and Diener, 1971). The situation may be similar to 'low-zone tolerance', a hypo-reactive state induced by low doses of antigen (Dresser and Mitchison, 1968). It is possible that the antigen only suppresses after combining with the antibody of a few specifically reactive B-cells.

In one mechanism, antibody complexes with antigen on the B-cell and T-cell surfaces, blocking their receptors, and preventing intercellular co-operation (Askonas, McMichael and Roux, 1976). Another group

found that in their system intercellular co-operation was only prevented by immune complexes if the Fc fragment of the antibody was intact. They proposed that multipoint binding of the complexes to cell surface Fc receptors interfered with antigen concentration (Hoffmann, Kappler, Hirst and Oettgen, 1974). Multipoint binding also occurs in the phenomenon of immune blockade, induced by antigens with a high epitope density (Schrader and Nossal, 1974).

1.6d Hapten-carrier complexes : The binding of haptens to autologous proteins can form potent tolerogens. Autologous IgG has been found to be the most effective tolerogenic carrier for DNP, and the formation of antibodies in all classes is suppressed (Borel, 1976). The important observations in this model include : the requirement for intact IgG; the presence of tolerogen on the surface of antigen-binding cells for a longer period of time than the presence of immunogen. Double binding of the Fc portion of IgG and the hapten may produce a stable unit on the lymphocyte membrane providing a tolerogenic signal (Borel, 1976).

1.6e Suppressor T-cells : Suppressor cells form a distinct T-cell subpopulation which carries the Ly 2,3⁺ marker as opposed to helper cells which bear the Lyl⁺ marker (Herzenberg, Okumura, Cantor, Sato, Shen, Boyse and Herzenberg, 1976). The suppression of an immune response can be either antigen specific or non-specific, forming two broad categories into which the

various reports can be divided.

The target of non-specific suppression is apparently the macrophage. These cells promote the immune response by presenting antigen to the responding T- and B-cells (Pierce, Kapp, Wood and Benacerraf, 1974), prolonging lymphoid cell survival (Pierce et al., 1974), and by stimulating lymphoid cell proliferation (Schrader, 1973; Calderon, Kiely, Lefko and Unanue, 1975; Unanue, Kiely and Calderon, 1976). The inactivation of macrophages seemed to be the cause of an anti-mitotic effect in at least one suppression system (Tadakuma and Pierce, 1976). In the converse situation, the activation of macrophages may enable them to inhibit antibody and DNA synthetic responses of lymphocytes to antigens or mitogens. This type of suppression by macrophages may be due to direct cell to cell interaction (Parkhouse and Dutton, 1966) or to soluble factors (Waldman and Gottlieb, 1973; Calderon, Williams and Unanue, 1974). Whether suppressor T-cells do in fact activate macrophages is uncertain, but there is some indication that they have this potential (Tadakuma and Pierce, 1976).

Examples of non-specific suppression include : inhibition of the contact sensitivity reaction of mice to 4-ethoxy-methylene-2-phenyloxazolone by immunization with the unrelated antigen picrylsulfonic acid (Zembala and Asherson, 1974; Asherson and Zembala, 1974); concanavalin A activated T-cell suppression of the AFC response to heterologous

erythrocytes and of the cytotoxic lymphocyte response to alloantigens (Tadakuma and Pierce, 1976); and depression of the response to heterologous erythrocytes induced by T-cells primed with ovalbumin (Thomas, Roberts and Talmage, 1975).

In these systems a soluble suppressive factor is released. The factor can be absorbed by macrophages, but not by the eliciting antigen nor by antisera to immunoglobulins or to histocompatibility antigens (Tadakuma and Pierce, 1976). It is a protein of 48,000-67,000 Daltons, and is relatively stable at 56° (Tadakuma and Pierce, 1976; Thomas, Roberts and Talmage, 1975).

The targets of specific suppression include effector cells and T-helper cells (Kontiainen and Feldman, 1976). The direct inhibition of B-cell activity (Basten, Miller, Sprent and Cheers, 1974; Basten, Miller and Johnson, 1975) can even occur at the stage of antibody synthesis and secretion by active, fully-differentiated high avidity AFC (Warren and Davie, 1977). The more indirect inhibition brought about by diminishing T-help has been studied in greater detail. This suppression is mediated by at least two antigen recognition systems, one possessing immunoglobulin determinants and termed 'IgT' (Feldman, 1974), and the other having determinants specified by the I region of the major histocompatibility complex (Taniguchi, Tada and Tokuhisa, 1976; Herzenberg et al., 1976). In one system the I region product of a

suppressor T-cell acted by binding with a complementary I product on the helper T-cell (Taniguchi, Tada and Tokuhisa, 1976). The suppressive factor was 35,000-55,000 Daltons, quite similar in size to those in the non-specific reactions described above.

1.6f Anti-idiotypic recognition : In the preceeding section it was seen that T-cells could be generated which specifically suppressed the production of an immunoglobulin allotype (Herzenberg, Okumura and Metzler, 1975; Herzenberg et al, 1976). The allotype suppression was regardless of the specificity of the antibody-combining site (idiotype). A parallel is now emerging with the description of idiotype suppression, a process which may be important in the regulation of the humoral immune response.

The initial observations were made by raising anti-idiotypic antibody and then passively transferring it to other animals : the results confirmed the immunoregulatory capacity of anti-idiotypic reactivity (Eichmann, 1974). Eichmann (1975) has also shown that exposure to antibody determinants results in the generation of T-cells which specifically suppress production of immunoglobulin carrying that idiotype. There is now clear evidence that idiotype suppression has a role in regulating the course of an immune response after conventional immunization (Pierce and Klinman, 1977). These workers are investigating the precise mechanism of the suppression, and are considering the involvement

of B-cell receptor blockade (Cosenza and Kohler, 1972) and the generation of idiotype specific T suppressor cells (Eichmann, 1975).

1.6g Central mechanisms and ingested antigen : There are a considerable number of reports that the ingestion of antigens may lead to the induction of tolerance. The reports can be divided into two categories, those involving the feeding of haptens and those involving the feeding of proteins. The feeding of haptens, such as picryl chloride (Pomeranz, 1970), probably induces tolerance by the action of hapten - autologous protein conjugates (Borel, 1976). Proteins may enter the circulation in concentrations which induce low-zone tolerance (Thomas and Parrott, 1974), or form tolerogenic immune complexes (Andre, Heremans, Vaerman and Cambiaso, 1975; Stokes and Swarbrick, 1977).

Information about the central mechanisms of immunoregulation should lead to rational changes in the clinical use of immunotherapy for allergic diseases. An example would be the use of autologous IgG as a carrier for the induction of tolerance to antigenic determinants. This procedure has been successful in suppressing the formation of reaginic antibody in mice to penicillin determinants (Borel, 1976).

1.7 Regulation by Peripheral Mechanisms

1.7a Control of antigen uptake from the gut : The importance of antigen uptake is suggested by the detection

of ingested antigens in the circulation (Rothberg, Kraft, Farr, Kriebel and Goldberg, 1970). Current evidence shows that the antigen load entering the circulation is reduced by immune mechanisms. Examples include experiments in rats in which oral immunization with proteins led to a specific reduction in their absorption. One measurement was made in vitro using everted gut sacs (Walker, Isselbacher and Bloch, 1972); the other was in vivo (Andre, Lambert, Bazin and Heremans, 1974). It is noteworthy in this regard that patients with sIgA deficiency have elevated levels of serum antibodies to ingested antigens (Buckley and Dees, 1969).

The properties of sIgA may well make it the ideal immunoglobulin class to mediate immune elimination of antigen from the gut. It does not opsonize for phagocytosis (Zipersky, Brown and Bienenstock, 1973) nor does it activate complement (Colten and Bienenstock, 1974), thereby reducing the risk of damaging immune reactions in the gut wall. sIgA does function by antigen cross-linking (Steele, Chaicumpa and Rowley, 1975), blocking the adherence of bacteria to mucosal surfaces (Williams and Gibbons, 1972), and by aiding the elimination of antigens, through efficient agglutination (Newcomb and Sutoris, 1974; Knop and Rowley, 1975b; Bellamy, Knop, Steele, Chaicumpa and Rowley, 1975).

prevented from stimulating antigen-sensitive cells in a number of ways. The prevention of antigen uptake is probably the important regulatory method concerning the gut, however other possible mechanisms cannot be ignored.

If an antigen is taken up from the gut there is a possibility that antibody may mask its antigenic determinants. Bacteria coat with s-IgA in vivo (Brantzaeg, Fjellanger and Gjeruldsen, 1968), and they are less immunogenic on injection (Eddie, Schukkind and Robbins, 1971).

Antibody may induce relatively slight changes in the tissue distribution of antigen, resulting in a significant alteration of the amount of effective antigen (Dennert, 1971). Although there have been no examples reported for ingested antigen, a systemic example could be the prevention of Rh sensitization in human volunteers by passive ABO immunization (Stern, Goodman and Berger, 1961).

Another postulated mechanism is that of altered antigen metabolism. This has been proposed for the ability of passive immunization against one antigenic determinant on a molecule to non-specifically suppress the response to other determinants (Henney and Ishizaka, 1968).

- 1.7c Regulation by efferent mechanisms : The ability of IgA to bind to antigens without the production of damaging reactions could well be useful in preventing

antigen/antibody reactions which are harmful. It is a common observation that atopic individuals often have intestinal symptoms, and IgE can be detected in their secretions (Dolovitch, Tomasi and Arbesman, 1970). Secretory IgA has the capacity to block allergic reactions, as has been shown in nasal secretions (Turk, Lichtenstein and Norman, 1970).

Complement dependent bactericidal reactions can be shown to be blocked in the sera of some patients following infection with a gram-negative bacterium, and the same phenomenon can be induced experimentally in animals (Hall, Manion and Zinneman, 1971; Griffiss, 1975). Serum IgA (Griffiss, 1975) and secretory IgA (Eddie, Schukkind and Robbins, 1971) have been shown to bestow this blocking ability which prevents bactericidal reactions.

1.8 Suppression of the Antibody Response by Endogenous Antibody

There is evidence that antibody feedback suppression operates as a normal regulatory mechanism in the immune response. This has two apparent results : antibody avidity generally increases with time after an immunization (Siskind and Benacerraf, 1969) and memory cells are preserved rather than all antibody-precursor cells being exhaustively primed by antigen (Askonas, McMichael and Roux, 1976).

It has been suggested that high affinity antibody suppresses by competing with B-cell receptors of lower

affinity or by removing antigen (Uhr and Moller, 1968). Also, suppression of an ongoing antibody response by passively given antibody is not observed for 2-3 days, which suggests a reduction in antigenic stimulus (Wigzell, 1966). However, as homologous, apart from higher affinity, antihapten antibodies can suppress antihapten clones in an adoptive transfer system, this does not appear to be the entire explanation (Askonas *et al*, 1976).

Feedback regulation of antibody formation has been demonstrated by cyclical antibody responses after immunization. Even cyclical variations in cell-mediated immunity have been shown (Gillespie and Barth, 1974).

Primary immunization of mice with endotoxins from gram-negative bacteria will give rise to a cyclic IgM response at the cellular level (Britton and Moller, 1968). It is thought that the cycles are brought about by : antigenic stimulation; antibody production; antibody masking of antigenic determinants; reduced antigenic stimulation followed by reduced antibody production; antibody catabolism; re-exposure of antigenic determinants and antigenic stimulation once again. Support to this hypothesis is lent by the far slower catabolism of the endotoxin than the IgM (Britton, Wepsic and Moller, 1968).

Cyclicity of humoral responses has also been noted in the marine toad Bufo Marinus, and it too appeared to be regulated by the catabolism of immunoglobulins. Antibody cycles lasted 2-3 weeks in response to an immunization with polymerized flagellin. This cycle length correlated well

with the half-life of IgM, in this system, of 17 days (Azzolina, 1976).

Protein antigens from serum may also result in antibody-response cycles. When rabbits were immunized with heterologous serum protein antigens, IgM and IgG producing cells appeared in cycles (Romball and Weigle, 1973).

It is interesting that one group found a biphasic occurrence of IgM-forming cells in the mouse intestine after oral immunization with sheep erythrocytes (Werner, LeFevre and Raettig, 1971). The possibility that the result was due to lymphocyte migration, or a sequential response to different erythrocyte antigens, was not investigated.

1.9 Suppressive Effects of Passively Given Antibody

Passively given antibodies may inhibit the immune response when injected before, at the same time, or after the antigen. Usually they are most effective when given before the antigen. Passive antibodies injected after antigens such as haemocyanin and sheep erythrocytes are given can effectively suppress antibody formation (Dixon, Jacot-Guillarmod and McConahey, 1967; Moller, 1969). The primary response to these antigens seems to be delayed by some days, presumably whilst metabolic processing to a more antigenic form proceeds.

It is often found that priming for a secondary response, as well as the secondary response itself, is relatively less susceptible to the inhibitory effects of

passively-given antibody than is the primary response (Uhr and Moller, 1968). In other systems, immune memory formation and secondary responses are readily suppressed (Askonas, McMichael and Roux, 1976).

IgG is thought to be more inhibitory than IgM when given passively, but the differences have not been quantitated on a weight-for-weight basis. Also, the IgM is usually taken from an early period in the immune response, and the IgG from a later period. Therefore low avidity IgM has often been compared with higher avidity IgG. As low avidity antibody is less efficient at suppressing the antibody response (Walker and Siskind, 1968), the comparison is biased.

If an antigen dose is too low for optimal stimulation of an antibody response, IgM can augment the response to the optimal level, but not beyond, by effectively localizing the antigen (Henry and Jerne, 1968). Also, low concentrations of IgG2 have been found to augment the response of mouse spleen cells to sheep erythrocytes (Murgita and Vas, 1972).

Part C Cholera : An Introduction1.10 Definition

Cholera is an acute diarrhoeal illness of humans which results from the colonization of the small bowel by Vibrio cholerae.

1.11 Aetiology

Vibrio cholerae is a curved, aerobic, gram-negative bacillus with a single curved polar flagellum. It was first described by Koch in 1883. A second, distinct biotype was first isolated by Gotschlich in the Egyptian quarantine station of El Tor in 1906.

The el tor biotype is distinguished from the classical biotype by its resistance to Mukerjee's type IV cholera phage and to polymyxin B, and by its ability to agglutinate chicken red blood cells.

Each biotype was divided into two serological types ('Inaba' and 'Ogawa') by Kabeshima in 1913. An intermediate serotype ('Hikojima') was discovered by Nobechi in 1923. The three serotypes share a group specific antigen, 'A' (Gardner and Venkatraman, 1935). Antigen 'B' is found in the Ogawa serotype, antigen 'C' in the Inaba serotype, and both antigens in the Hikojima serotype (Pollitzer, 1959).

1.12 Epidemiology

Cholera is endemic in West Bengal and Bangladesh, and is often epidemic throughout south and south-east

Asia. Pandemic spread of the disease has been recorded seven times, the most recent being due to el tor in 1961 to 1966. The major cause of spread is travelling people.

Cholera is primarily a water-borne disease, and in endemic areas there is a vast problem of intermingling sewerage and water supplies. Malnutrition possibly contributes to susceptibility to the disease by reducing secretion of the bactericidal stomach acid (Hornick *et al*, 1971; Music, *et al*, 1971).

Spread of the disease is aided by some convalescent patients who harbour the vibrios in the gallbladder and excrete them for long periods of time (Pierce *et al*, 1970). Another group of vibrio excretors have subclinical cholera (Bart and Mosley, 1970). In one study, infection to case ratios were 36:1 for el tor vibrios against 4:1 for classical vibrios (Bart, Huq, Khan and Mosley, 1970). This difference between the biotypes is thought to be due to the greater persistence of el tor in the intestine, coupled with a lesser toxigenicity (Finkelstein *et al*, 1977).

1.13 Establishment of Infection

It has long been recognized that the attachment of vibrios to the mucosa of the small intestine is an essential step in the establishment of a cholera infection (Freter, 1969). The virulence of many other bacteria is also correlated with attachment to the target tissue. Examples of mechanisms of attachment include : the plasma membrane to bacterial cell wall interaction of Strep.

moniliformis (Hampton and Rosario, 1965); the pili of N. gonorrhoeae (Swanson, 1973); the surface antigen K88 of some E. coli (Jones and Rutter, 1972) and M-protein of Strep. pyogenes (Ellen and Gibbons, 1972); and the synthesis of insoluble dextrans by Strep. mutans (Mukasa and Slade, 1974).

Structures such as fimbriae and pili apparently do not mediate the attachment of Vibrio cholerae to the intestinal mucosa. Nelson, Clements and Finkelstein (1976) showed that there is an interaction between the surface coats of firmly adherent vibrios and the tips of the microvilli. This interaction is apparently mediated by a cell bound haemagglutinin (Finkelstein, Arita, Clements and Nelson, 1977).

Vibrio motility has been correlated with the organism's virulence (Guentzel and Berry, 1975; Schrank and Verwey, 1976). The reason may be the correlation between vibrio motility and the organism's adhesiveness to the gut mucosa (personal communication, S.R. Attridge; Freter and Jones, 1976).

1.14 Pathogenesis

The pathology of cholera is due to the exotoxin produced by live, pathogenic and adherent strains of V. cholerae (Finkelstein, Arita, Clements and Nelson, 1977). The exotoxin binds with membrane receptors of the intestinal epithelial cells, adenyl cyclase is stimulated (Carpenter, 1971, 1972) and a near isotonic fluid is poured into the intestinal tract. There is no demonstr-

able histological damage to gut epithelial cells or to the capillary endothelial cells of the lamina propria (Carpenter, 1971, 1972). The intestinal epithelium retains its structural integrity and cholera vibrios are confined to the lumen of the intestine (Gangarosa et al, 1960; Norris and Majno, 1968; Elliot, Carpenter, Sack and Yardley, 1970).

1.15 Manifestations

Only a minority of those infected with V. cholerae will develop clinical manifestations. After an incubation period of about 6 to 48 hr., these people have an abrupt onset of watery diarrhoea, generally followed by vomiting. Several litres of isotonic fluid may be lost within hours, leading rapidly to profound shock and base deficit acidosis, and later to acute tubular necrosis with uraemia.

1.16. Laboratory diagnosis

Cholera stool is directly plated on bile salt agar and the organisms appear as translucent colonies within 24 hr. V. cholerae appears as distinct large yellow colonies at 24 hr. on thiosulfate-citrate-bile salt-sucrose (TCBS) agar. The latter plating medium is selective, and is used to detect asymptomatic infections. Further classification requires agglutination with type-specific antisera, and with chicken red blood cells to differentiate *el tor*.

1.17 Treatment

Gastro-intestinal losses of fluids, electrolytes and alkali are promptly replaced intravenously, or orally in mild or convalescent cases (Nalin and Cash, 1974). Hypokalaemia contributes significantly to morbidity and mortality in children and potassium should be included in the intravenous fluids administered to them.

The disease terminates in one to six days if there is prompt and adequate fluid replacement. The duration and volume of the diarrhoea can be markedly reduced by oral tetracycline therapy (Greenough *et al*, 1964). With adequate treatment, death rates as high as 60% can be reduced to virtually zero.

Part D Cholera Immunity1.18 Introduction

Koch observed in 1884 that the same individual was rarely attacked twice during a cholera epidemic. A year later Ferran was using live cholera vaccines. However, after all this time it can only be said that some cholera vaccines offer a limited degree of protection against cholera for a limited period of time in some population groups (Finkelstein, 1973).

The U.S.A. has recognised the relative ineffectiveness of cholera vaccines and no longer requires evidence of cholera vaccination of travelers entering the country, even from infected areas. Other factors in this decision were

that the disease is readily treatable; and that it is unlikely to gain hold in a country with high sanitary standards.

Nevertheless, the disease is still devastating in less fortunate countries where application of Western ideas of sanitation is fraught with logistical and cultural difficulties, and where supplies of pyrogen-free intravenous fluids cannot be taken for granted. A single-shot cholera vaccination effective for at least a year would be ideal in the circumstances.

1.19 Local Immunity to Cholera

1.19a Introduction : Davies in 1922 detected copro-antibody in people with bacillary dysentery. Some years later it became apparent that antibodies in the serum do not necessarily bear any direct relationship to the levels at secretory surfaces such as the intestine. An early example was an experimental model of cholera in which levels of copro-antibody were found to be independent of those of serum antibody (Burrows, Elliot and Havens, 1947; Burrows and Havens, 1948). This independence of copro-antibody has been firmly established with the concept of a secretory immune system (Tomasi and Zigelbaum, 1963), and the demonstration of local antibody synthesis against a variety of antigens, including those of Vibrio cholerae (Pierce and Gowans, 1975; Svennerholm and Holmgren, 1977).

1.19b Models of cholera : The development of animal models of cholera has greatly aided the study of cholera pathogenesis and immunity. Important models have been : starved guinea-pigs, morphine-treated (Burrows, Elliot and Havens, 1947) or streptomycin-treated (Freter, 1955); adult rabbit intestinal loops isolated in situ (De and Chatterjee, 1953); infant rabbit model (Dutta and Habbu, 1955); infant mouse model (Ujjiye et al, 1968); and the canine model (Sack, Carpenter, Steenburg and Pierce, 1966b).

1.19c Experimental studies : Some studies with animal models of cholera have shown good protection against V. cholerae with passive local antibody, but not with passive serum antibody. This work was done in the starved streptomycin-treated guinea pig model (Freter, 1965), the rabbit ligated loop model (Freter, 1964) and the infant rabbit model (Freter, 1964; McIntyre and Feeley, 1964). Such a result would be expected with a purely local infection of the intestine, like cholera (Elliot, Carpenter, Sack and Yardley, 1970).

However, some workers have found that passive antibody given parenterally is protective against cholera in the rabbit ligated intestinal loop model and the infant rabbit model (Jenkin and Rowley, 1960; Feeley, 1965), as well as the canine model (Curlin and Carpenter, 1970; Pierce, Kaniecki and Northrup, 1972; Pierce and Reynolds, 1974).

The study by Feeley (1965) may have resolved this

conflict by finding that antisera predominantly 7s rather than 19s in antibody class were protective when given parenterally in the infant rabbit model. Similarly, the passive antibody used by Pierce and Reynolds (1974) was 7s. The difference lay in the ability of 7s antibody to more readily transfer from the serum to the gut than the 19s antibody (Wernet, Breu, Knop and Rowley, 1971). The practical importance of antibody transfer could only be assessed in field and volunteer studies.

1.19d Clinical studies : In general there is no correlation between serum antibody titres and cholera attack rates in volunteers (Cash, Music, Libonati, Craig, Pierce and Hornick, 1974a) or the clinical presentations of cholera in-patients (Finkelstein, 1962; Sack, Barua, Saxena and Carpenter, 1966a). It is the concentration of local antibody that determines the resistance to infection by cholera (Freter, De, Mondal, Shrivastava and Sunderman, 1965). Supporting evidence from volunteer studies clearly shows that following oral challenges with V. cholerae a copro-antibody peak appears which is independent of the serum antibody peak (Freter and Gangarosa, 1963; Waldman *et al.*, 1972).

Some indirect evidence did at one stage imply that serum antibody conferred protection against cholera attack. Increasing serum antibody titres with age were correlated with decreasing cholera attack rates with age, in an endemic area (Mosley, Benenson

and Barui, 1968a). In a similar correlation, increased serum antibody levels after a cholera vaccine field trial were associated with protection against cholera attack (Mosley, Benenson and Barui, 1968b). A later report correlated reduced cholera case rates with annual re-immunization rather than with serum antibody titres per se (Mosley, Aziz, Rachman, Chowdhury, Ahmed and Fahimuddin, 1972). The significant correlation was probably with copro-antibody titres.

1.20 Cholera Vaccines

1.20a Exotoxin : The serotypes of V. cholerae share a single exotoxin (Evans and Richardson, 1968; Finkelstein and LoSpalluto, 1970). Antibodies to it, without significant antibacterial antibody contamination, are protective against cholera in a number of animal models (Curlin, Subong, Craig and Carpenter, 1968; Curlin and Carpenter, 1970; Curlin, Craig, Subong and Carpenter, 1970; Pierce, Kaniecki and Northrup, 1972; Finkelstein, 1970; Kaur, Burrows and Furlong, 1971; Kaur, McGhee and Burrows, 1972).

The relative importance of antitoxic antibodies is, none the less, in doubt. Antibodies to somatic antigens were 15 to 20 fold more protective than anti-toxic antibodies on a weight-for-weight basis in the baby mouse model (Neoh and Rowley, 1972). Also, protection against hybrid vibrios in the rabbit ileal loop model was correlated with antibodies to the somatic O-antigen, not with antibodies to enterotoxin (Sinha and

Bhaskaran, 1973).

Volunteer studies by Levine, Nalin, Craig, Hoover, Bergquist, Waterman, Holley, Libonati, Hornick and Pierce (1977a) have clarified the situation. They reported that : 'Purified cholera toxoid is antigenic in man when given parenterally or orally but fails to provide protection against experimental challenge. In contrast, clinical cholera confers formidable protection against subsequent challenge with the homologous or heterologous serotype. Failure to recover vibrios from the intestinal fluid or stool of rechallenge volunteers suggests that the predominant operative immune mechanisms are anti-bacterial rather than antitoxic in nature.'

- 1.20b Whole cell vaccines : Volunteer studies have shown that the establishment of a cholera infection with virulent organisms can confer 100% protection against rechallenge with vibrios for up to 12 months (Cash, Music, Libonati, Craig, Pierce and Hornick, 1974a). Attenuated organisms which do not colonize the bowel do not confer significant immunity (Cash, Music, Libonati, Schwartz and Hornick, 1974b; Sanyal and Mukerjee, 1969). This implies that the accessory factors which enable V. cholerae to colonize the bowel, such as the adhesive cell-bound haemagglutinin (Finkelstein *et al*, 1977), also aid the development of an immune response.

A killed oral vaccine of V. cholerae has to be

given repeatedly in order to stimulate a good local response (Freter, 1962; Freter and Gangarosa, 1963).

Heat killing may be a poor way of preparing an oral vaccine, due to the loss of labile antigens.

Mechanical disruption with sonication or French pressing may preserve labile antigens, but a way of inactivating the released intracellular enzymes would have to be found. The importance of vaccine preparation is emphasized by the extreme lability of the adhesive haemagglutinin : it is destroyed by heat (56° for 1 minute), alcohol and formalin (Zinnaka, Shinodori and Takeya, 1964).

Killed whole cell vaccines given parenterally offer only incomplete immunity for 6-12 months (Benenson, Mosley, Fahimuddin and Oseasohn, 1968; Mosley, Woodward, Aziz, Rahman, Chowdhury, Ahmed and Feeley, 1970; Azurin, Cruz, Sumpaico, Dizon and Alvero, 1973). They probably do so by boosting the immunity which people acquire by exposure to V. cholerae in endemic areas (Benenson et al., 1968; Svennerholm, Holmgren, Hanson, Lindblad, Qureshi and Rahimtoola, 1977).

1.20c Lipopolysaccharide : The heat stable O antigens A, B and C mentioned previously are contained within the lipopolysaccharide complex, and a major portion of vibriocidal antibodies in an antiserum raised against V. cholerae are specific for LPS determinants (Neoh and Rowley, 1970).

Purified preparations of Ogawa and Inaba LPS have been evaluated in field trials, along with monovalent whole cell vaccines. Inaba whole cell and Inaba LPS vaccines conferred good protection against Inaba infection (Mosley *et al*, 1970) and Ogawa infection (Azurin *et al*, 1973). However, Ogawa vaccines only conferred good protection against Ogawa infection (Azurin *et al*, 1973); the LPS was relatively ineffective as an immunogen against Inaba infection in children, presumably because they were poorly primed compared to adults (Benenson *et al*, 1968); and the whole cell vaccine failed to protect children and adults against Inaba infection (Mosley *et al*, 1970).

The cross-protective ability of Inaba vaccines, as opposed to the lack of this ability by Ogawa vaccines, may be attributed to the group specific A antigen being less immunogenic in the Ogawa serotype than in the Inaba serotype (Watanabe, 1974).

1.20d Protein antigens : Many protein antigens have been described, but their importance in cholera immunity is unknown. The heat stable proteins described by Neoh and Rowley (1970) are common to the Inaba and Ogawa serotypes, and can elicit antibodies protective in animal models (Neoh and Rowley, 1972).

Although the importance of the heat labile H-antigen remains in doubt, an antiserum from which activity against heat-stable antigens had been absorbed out has been found to be protective in the infant mouse

model (Steele, Chaicumpa and Rowley, 1975).

1.21 Resistance to *V. cholerae* : Non-specific Mechanisms

1.21a Saliva : Ingested vibrios first encounter saliva, but it does not appear to be an effective vibriocidal agent. Gorbach, Banwell, Pierce, Chatterjee and Mitra (1970) found living vibrios, at levels up to 10^5 /ml, in samples of saliva collected from cholera patients at least eight hours after the last vomiting.

1.21b Gastric acid : As discussed previously, the gastric acid is a most important vibriocidal barrier. Well nourished people with normal gastric acid are not readily infected with cholera vibrios (Music, Libonati, Wenzel, Snyder, Hornick and Woodward, 1971).

1.21c Intestinal juice : A number of antibacterial factors have been reported to be in the intestinal juice, and these include : lysozyme, lactoferrin, polyamines and enzymes (review in W.H.O. techn. rep. 500, 1972). Their role remains controversial, including suggestions that they may act in concert with antibodies. Some workers have shown IgA to activate complement mediated bacteriolysis in the presence of lysozyme (Adinolfi, Glynn, Lindsay and Milne, 1966; Hill and Porter, 1974), and others have not (Steele, Chaicumpa and Rowley, 1974; Heddle, Knop, Steele and Rowley, 1975).

1.21d Microbial antagonism : The bacterial flora of the small intestine in mice and guinea-pigs has been shown

to prevent infection by V. cholerae (Sasaki *et al.*, 1970; Freter, 1955, 1956). The mechanisms of microbial antagonism are complex, and may involve : bacteriocins; antibody/microbe synergy; bacterial cross-reactive antigens; and modification of the host tissue (Abrams, Bauer and Sprinz, 1963; Shedlofsky and Freter, 1974).

1.21e Peristalsis : Peristalsis of the small intestine has a most important antibacterial action, shown experimentally in monkeys and dogs (Dack and Petran, 1934). In fact experimental shigellosis (Formal, Abrams, Schneider and Sprinz, 1963) and salmonellosis in guinea pigs (Kent, Formal, Le Brec and Takeuchi, 1966) requires inhibition of intestinal motility.

1.22 Resistance to V. cholerae : Antibody Mediated Mechanisms

Peristalsis rapidly removes V. cholerae from the small intestine of infant mice in the presence of antibody (Knop and Rowley, 1975b). Peristalsis may act more efficiently because antibody agglutinates the organisms, or because antibody prevents V. cholerae from adsorbing to the intestinal wall (Freter, 1964, 1969, 1970, 1972; Fubara and Freter, 1973; Bloom and Rowley, 1977). As the attachment of vibrios to the mucosa of the small intestine is an essential step in the establishment of a cholera infection (Freter, 1969), and adhesiveness is an important determinant of virulence (Finkelstein *et al.*, 1977), the antibody mediated enhancement of vibrio elimination from the gut is a significant antibody action.

It has also been suggested that antibody in the small intestine may have a bactericidal action on V. cholerae (Freter, 1970, 1971; Chaicumpa and Rowley, 1972; Knop and Rowley, 1975b,c). The two well known antibody mediated antibacterial effects are complement mediated bacteriolysis and opsonisation leading to killing of the bacteria within phagocytic cells. However, these two effects are not mediated by IgA or Fab, which nevertheless protect infant mice against V. cholerae (Steel, Chaicumpa and Rowley, 1974, 1975). Resolution of this puzzling issue was one of the aims of this thesis.

1.23 Aims of this thesis

The requirements for optimal stimulation of gastrointestinal immunity are poorly understood, and the efficacy of vaccination against many enteric diseases, such as cholera, remains inadequate. The main aim of this thesis will be to compare the systemic and the local immune responses of mice to vaccination with Vibrio cholerae, and to ascertain the requirements for optimal development of these responses. In this work the degree of persistence of a V. cholerae strain in the intestine will be related to its ability to stimulate a local response.

A second aim will be to investigate the development and the control of the mouse's immune response to V. cholerae. This question arose early in the work when explanations were sought for some of the observed characteristics of the immune response.

Other aims will be to examine the growth of V. cholerae

in the gastro-intestinal tract of mice and its elimination, with or without the help of antibody, from the tract. The interest here will centre on oral immunization - what happens to organisms given as an oral vaccine and how local immunity is mediated in a situation apparently inimical to complement fixation, and to opsonization leading to phagocytosis.

CHAPTER 2

CHAPTER 2MATERIALS AND METHODS2.1 Bacterial Strains

Vibrio cholerae 569B (classical Inaba) was originally obtained from Mr. I. Huq of the Cholera Research Laboratories, Dacca.

V. cholerae 569B SR was an avirulent streptomycin resistant (SR) mutant isolated from the parent strain by Dr. S.H. Neoh. $LD_{50} > 6 \times 10^{10}$. Motility < 1%.

V. cholerae 569B IMP was a virulent mutant obtained by Dr. S.H. Neoh using multiple oral passages in infant rabbits and mice. $LD_{50} = 10^7$. Motility < 1%.

V. cholerae 569B IMP SR was selected from V. cholerae 569B IMP grown on nutrient agar culture plates containing 100 µg/ml streptomycin sulphate. $LD_{50} = 10^7$. Motility < 1%.

V. cholerae 017 (el tor Ogawa) was originally supplied by Dr. W.F. Verwey, Department of Microbiology, University of Texas, Galveston, Texas.

V. cholerae 017 SR was the streptomycin resistant mutant isolated from the parent strain by Dr. J. Knop. $LD_{50} = 10^4$. Motility = 20%.

V. cholerae 111 NM SR (classical, Ogawa) was an avirulent, non-motile (NM) strain supplied by Dr. K.

Bhaskaran, Central Drug Research Institute, Lucknow, India.

$LD_{50} > 10^{11}$. Motility 0%.

V. cholerae 111 M SR was a highly motile mutant (100%) obtained by S. Attridge with serial Craigie tube passages.

2.2 Bacterial Maintenance and Growth

The strains were stored lyophilized in skimmed milk at 8°. Ampoules were opened every few months and maintained on nutrient agar stabs at room temperature. Each stab was used up to 8 times to streak nutrient agar plates. 10ml broth cultures were obtained by an inoculation of a few colonies off a plate, and grown at 37° until log phase (2×10^9 vibrios/ml at 4 hours). 1 litre broth cultures were inoculated with 200ml stationary phase cultures grown overnight. The 1 litre cultures reached log phase in 4 hours if shaken in 5 litre flasks at 37°.

2.3 Animals

2.3a Mice : LAC spf mice of both sexes weighing 20-30g were used. They were outbred white mice originally supplied by the Medical Research Council Laboratory Animals Centre (LAC) of the U.K. They were maintained under specific pathogen free (SPF) barrier conditions by the Central Animal House of the Adelaide University, and were six weeks of age at the commencement of any experiment.

2.3b Infant mice : A departmental breeding colony of LAC mice supplied 5-6 day old, 2.3-2.6g infants.

2.3c Goats : Wild goats, captured on Manunda Station, South Australia, were kept in paddocks at the Central Animal House.

2.4 Anti-*V. cholerae* Antisera

Mouse anti-*V. cholerae* 569B and mouse anti-
V. cholerae 017 antisera were kindly supplied by
S.R. Attridge of this department.

Hyperimmune anti-*V. cholerae* 569B IMP SR anti-serum was obtained by immunizing mice over a number of months with intraperitoneal injections three times a fortnight. The number of viable organisms per dose was gradually increased from 10^6 to 10^9 .

Mice were bled from the retro-orbital plexus, the blood clotted at room temperature for 2 hours, and then the clot retracted at 4° for 4 hours. The separated serum was centrifuged free of cells, and either frozen at -20° , or stored at 4° with 0.1% NaN_3 as a preservative.

2.5 Anti-*V. cholerae* Intestinal Juice

Mice were perorally immunized three times in one week with doses of 10^{10} organisms in 0.2ml saline (25% saturated with NaHCO_3). The mice were boosted with 4×10^7 vibrios given intraperitoneally a week after the last peroral immunization. Six days later the mice were killed, their small bowels were removed and the serosal surfaces washed free of blood and then each bowel was flushed out with 1ml of cold

saline. The intestinal washings were homogenized (Ultra-turrax, Janke and Kunkel, Staufen, Germany), centrifuged in the cold to remove debris and then frozen at -20°.

2.6

Oral Challenge of Mice

Log phase cultures of unlabelled bacteria were washed once before use. Labelled bacteria were obtained by overnight culture of bacteria in 50ml of broth containing 400 μ c P³². The labelled bacteria were washed three times before use. Some cultures were treated with antiserum at 37° for 15 minutes. Adult mice were challenged orally with 0.4ml of a standardized bacterial suspension in saline half saturated with NaHCO₃. The oral dose was introduced into the stomach using a round-tipped 19 gauge needle. Infant mice, fasted for three hours, were similarly fed 0.1ml of culture.

2.7

Viable Counts

The mice were killed and dissected at various times after oral challenge. The stomach, small intestine, large intestine and collected faeces were homogenized in a known volume of saline, using an Ultra-turrax mixer. Adult mice were kept on metal grids so that their faeces would fall into saline containing vibriostatic amounts of azide (0.01%). Suitable dilutions of the homogenates were made in

saline, spread onto nutrient-agar plates or nutrient agar plates containing 100 μ g/ml of streptomycin, and incubated overnight prior to counting. From the viable counts an estimate could be made of the numbers of bacteria recovered from the mice and their faeces.

2.8

Radio-active Counts

Duplicate samples of each homogenate (0.1ml) were absorbed onto filter paper discs inserted into planchettes. The radioactivity in each sample was counted using an end-window Nuclear-Chicago counter.

2.9

Sensitization of Sheep Erythrocytes (SRBC)

SRBC were washed three times in isotonic saline and then resuspended to 2.5% and alkali treated LPS was added to a concentration of 50 μ g/ml. Sensitization took place in roller tubes rotating for 90 min. at 37°. Sensitized cells were washed three times before use.

2.10

Guinea-Pig Complement

Guinea-pig serum is a rich source of complement. The animals were bled by cardiac puncture under ether anaesthesia. The blood was clotted at room temperature for 2 hours, and the clot was retracted at 4° for 4 hours. The separated serum was centrifuged free of cells, and stored at -20° for up to 2 months.

2.11

Immunoglobulin Preparation

The author gratefully acknowledges the guidance

of Dr. D.J. Horsfall and the technical expertise of R.B. Willis in the preparation of the immunoglobulin fractions.

2.11a G-200 fractionation of serum : 14ml of mouse anti-569B IMP SR antiserum was chromatographed by gel filtration at 8° on a Sephadex G-200 (Pharmacia) column measuring 930 x 30 mm, with an ascending flow rate of 20ml/hr. The sample was eluted with 0.02M phosphate/0.2M NaCl (pH 7.6). An O.D. profile at 280m μ revealed three protein peaks in the eluate : IgM, IgG and albumin respectively. Fractions in the IgM and IgG peaks were pooled as were fractions of the trough inbetween these two peaks.

2.11b Preparation of IgG subclasses : Mancini tests revealed that the majority of the IgG was in the G-200 'IgG' and 'trough' pools. These samples were chromatographed by anion exchange at 8° on a DEAE-DE52 (Whatman) column measuring 350 x 25 mm with a descending flow rate of 30ml/hr, after dialysis with 0.01M phosphate/0.02M NaCl (pH 8.0). The peaks eluted with this buffer were pooled as were the peaks eluted with 0.02M phosphate/0.3M NaCl. These pools contained a mixture of IgG subclasses, with some IgM and IgA contamination, as detected by the Mancini test.

The IgG pooled samples were sub-fractionated using the method of Ey, Prowse and Jenkin, 1978. In this method samples are run at 8° on Protein A - Sepharose CL-4B (Pharmacia) columns with a bed volume

of 5 ml, at a flow rate of 30ml/hr. The starting buffer (0.14M phosphate, pH 8.0) elutes all immunoglobulin classes other than IgG. The second buffer (0.14M phosphate, pH 6.0) elutes IgG₁; the third buffer (0.15M citrate, pH 5.0) elutes IgG_{2A}; the fourth buffer (0.005M citrate/saline, pH 3.2) elutes IgG_{2B}. This final acid eluate has to be collected into alkaline buffer in the cold.

The fractions containing IgG were pooled into the three subclasses. The small IgG_{2B} pool had to be supplemented by that from 2ml of neat serum fractionated on the column. Mancini testing revealed some IgG sub-class cross contamination, so the IgG₁ and IgG_{2A} pools were re-run on the column three times each, and the IgG_{2B} pool was re-run once.

2.11c Preparation of IgM : The G-200 'IgM' pool was dialysed against 0.02M phosphate/0.05M NaCl (pH 8.0) overnight in the cold. No unbound material came through the DE52 column with this buffer. IgM was eluted with 0.02M phosphate/0.15M NaCl pH 8.0. It was 'Amicon' concentrated using a '10,000' filter to give a final volume of 20ml. Mancini testing showed this fraction to be mainly IgM. It was passed through the Protein-A column to rid the fraction of IgG contamination. The IgM was eluted with starting buffer, leaving little material to be eluted with the final acid buffer. It was also passed through an anti- α -immuno-absorbent column, prepared by Dr. D.J. Horsfall.

2.11d G-200 fractionation of intestinal juice : 20ml samples of mouse anti-569B IMP SR immune intestinal juice were loaded onto the G-200 column, and eluted with 0.02M phosphate/0.2M NaCl (pH 7.6). The first peak of each of the three runs was pooled, giving a volume of 400ml. This was 'Amicon' concentrated using a '10,000' filter to give a final volume of 40ml. The concentrate was relatively pure by Mancini and immuno-electrophoretic testing, containing mainly sIgA. It was passed through the Protein A column to remove any undetected IgG.

2.12 Immunoglobulin Quantitation

2.12a Extinction coefficients : The protein concentrations in mouse immunoglobulin fractions were measured using extinction coefficients described for rabbit immunoglobulins (Eddie, Schukkind and Robbins, 1971). A Hitachi double beam spectrophotometer was used at 280nm.

2.12b Single radial immunodiffusion : SRID is also known as the Mancini technique (Mancini, Carbonara and Heremans, 1965). In the modified technique used, molten agarose mixtures at 45° were run between two plates warmed to 45° on a hot plate. The base plates were wiped with 0.5% silicone in chloroform to provide a non-adherent surface. The test plates were boiled in detergent, rinsed in distilled water, dipped in 0.5% agar (80°) and baked at 85° for 2 hours to provide an adherent surface. The test plates were supported

on the base plates by 1mm thick microscope slide pieces inserted at the corners and the base plates were slid away after the agarose had set. Wells were punched in the agarose with a perspex former and a brass punch, and they were filled with 3 μ l of antigen solution. The plates were incubated at room temperature in a humid chamber for 2-3 days.

The agarose mixtures used were : 7.5ml of 2% agarose (A grade, Calbiochem); 7.5ml of 0.1M sodium barbital/HCl buffer (pH 8.2); 0.15ml of SP54 (Benechemie, Munich, Germany) to reduce background staining; goat anti-mouse α , μ (0.1ml), γ 1, γ 2a or γ 2b (0.2ml) heavy chain antiserum (Meloy Laboratories, Springfield, Va.).

The Mancini plates were developed using the following sequence : 5 washes in 0.15M saline/0.1% azide over 2-3 days; drying at 85° for 2 hours; 10 minutes of staining in 1-2% Xylene Brilliant Cyanine G (Sandoz) in methanol:distilled water:acetic acid solvent, 5:5:1; 5 minutes of de-staining in three washes of the same solvent.

Precipitin ring diameters were measured with an eye gauge. The area inside the ring is proportional to the quantity of antigen added to the well, and so results were obtained by comparison with standard preparations of mouse Ig (Meloy Laboratories, Springfield, Va.).

2.13 Antibody Assays

2.13a Haemagglutination assay : The HA assay was performed as described by Steele, Chaicumpa and Rowley, 1974. The direct assay was done in perspex microtitre trays (Cooke Eng. Co., Alexandria, Va.) and test samples were serially diluted two-fold in the wells. Sensitized SRBC (0.5%) were dropped into each well, and the trays incubated at 37° for 1 hour. The endpoints were read after storage overnight at 4°. The HA titre was the highest dilution showing a different settling pattern from that of the control.

The antiglobulin enhanced HA assay (Coombs test) extended the results by indicating the predominant immunoglobulin class of the haemagglutinating antibody. Serial two-fold dilutions of the samples beyond the direct HA endpoint were performed in tubes. An equal volume (0.3ml) of sensitized SRBC (0.5%) was added to each tube, which were then incubated for 15 minutes at 37°. The suspensions were washed three times in saline and resuspended to 0.3 ml. The then antibody coated SRBC were dropped into microtitre tray wells, and haemagglutination induced with the addition of optimal amounts of anti-heavy chain antisera (Meloy anti μ 1/2,500; anti γ_1 , γ_{2a} , γ_{2b} , α 1/500).

Haemagglutination assays of immune intestinal juice were performed using glutaraldehyde fixed SRBC in order to prevent non-specific lysis. A 1% suspension of washed cells was fixed with 0.1%

glutaraldehyde, kept on ice for 30 min, washed three times in saline and finally washed three times in distilled water.

2.13b Vibriocidal assay : This vibriocidal assay was described by Rowley, 1968. Test samples were serially diluted two-fold in peptone-saline (0.1% proteose-peptone, Difco, in isotonic saline). Each dilution received an equal volume (0.5ml) of 1/10 guinea-pig serum in peptone-saline containing 10^4 viable organisms. After 60 minutes of incubation at 30° , each dilution was dropped in triplicate onto nutrient agar plates. The vibriocidal titre was the dilution which gave a 50% reduction in the control level of organisms. This was determined by interpolation from a plot of percentage surviving organisms against log antibody dilution.

Samples were also assayed after mercapto-ethanol reduction. This was achieved by adding equal volumes of 0.2M 2-mercapto-ethanol (in phosphate buffered saline, pH 7.6) to each tube and incubating at 37° for 60 minutes.

2.13c Radio-immuno-assay : The RIA was modified for the anti-vibrio antibody system by Dr. D.J. Horsfall, who also kindly supplied the radio-labelled antibodies.

Each serum sample was serially diluted two-fold in 0.1ml aliquots of P.B.S. pH 7.4 with 0.5g/l bovine serum albumin (CSL BSA-Cohn Fraction 5). Glutaralde-

hyde-fixed organisms (0.05ml of $10^{10}/\text{ml}$) were added to each tube, and kept at 8° for 60 minutes. The antibody sensitized organisms were then washed free of excess antibody with two lots of cold diluent and centrifugation in the cold. Each pellet was resuspended in the $100\mu\text{l}$ of fluid remaining in the tube after the final centrifugation.

The anti-vibrio antibody coating the organisms was labelled with 0.025ml goat anti-mouse light chain or goat anti-mouse IgA radio-iodinated antiserum (6000 cpm; 10ng specific antibody). The tubes were kept at room temperature for 30 minutes and excess label was washed off with two lots of cold diluent and centrifugation in the cold.

The pellets were counted in a gamma counter (Packard Autogamma : I^{131} 50-1,000, 30% gain; I^{125} 50-250, 70% gain). The endpoint titre was the dilution at which radio-label counts bound were twice the background count of controls with no added anti-vibrio antibody.

Samples with a low concentration of antibody, in particular intestinal juice, could also be assayed. Antibody in aliquots of 1ml was absorbed with 5×10^8 glutaraldehyde fixed organisms, and the test was completed as described above.

- 2.13d Baby mouse protection test (BMPT) : This was described by Chaicumpa and Rowley, 1972, and Steele, Chaicumpa and Rowley, 1974.

- (i) LD₅₀ of the vibrios was determined by feeding infant mice serial ten-fold dilutions of the organisms. The LD₅₀ dose was calculated from a graph of cumulative % mortality against challenge dose, for the 48 hr time-point.
- (ii) PD₅₀ of the antiserum was determined by feeding 10-20 LD₅₀ of the organisms sensitized by five-fold serial dilutions of the test sample. The 50% survival end-point was interpolated from a plot of cumulated % survivors against antibody dilution. The antibody activity was expressed as PD₅₀ doses/ml of sample.

2.14 Antibody Class Purity

2.14a Immuno-electrophoresis : IEP is one method used to determine if one or more antibody classes are present in a preparation. Pre-coated glass-slides were covered by a 1mm layer of 1% agarose (A grade, Calbiochem) in 0.05M sodium barbital/HCl buffer (pH 8.2). Samples were placed in wells cut in the agar and were electrophoresed by 12-15 volts/cm for 1 hour.

After electrophoresis, antisera were placed in precut troughs parallel to the line of electrophoresis. The slides were kept at room temperature in a humid chamber for 48 hours, and were then prepared and stained as previously described.

2.14b Double radial immunodiffusion : This Ouchterlony method is another sensitive way to determine if a preparation of an antibody class is uncontaminated by another class. Once again glass slides were pre-coated and then agarose coated. A central well was punched, and five equidistant wells were punched around its perimeter. The wells were filled with antigen (antibody classes) and antibody (Meloy goat anti-mouse heavy chain antisera). They were kept at room temperature in a humid chamber for 48 hours, and were prepared and stained as previously described.

2.15 Lipopolysaccharide Extraction

The technique of hot phenol extraction was described by Westphal, Luderitz and Bister, in 1952. Log phase broth cultures of V. cholerae were centrifuged and washed in saline. The pellets were resuspended in distilled water/0.01M EDTA up to a wet weight concentration of 10mg/ml. An equal volume of 90% phenol was added to the suspension and the mixture heated to 65-68°. The temperature was maintained for 30 minutes when the two liquid phases merged into one. The mixture was then cooled to room temperature, protein entering the phenol phase and LPS entering the aqueous phase. The phenol phase was re-extracted and the two aqueous layers were mixed, centrifuged and dialized against distilled water until free of phenol.

The aqueous volume was reduced on a rotary evaporator, and the LPS was precipitated with five volumes of cold

ethanol and some sodium acetate as an adjunct. The precipitate was spun down and treated with nucleases and 5mM MgCl₂ at room temperature for 60'. Insoluble matter was removed by a low speed centrifugation, and the LPS brought down by ultra-centrifugation (100,000g for 120'). The LPS was then extracted a second time with phenol at 65° to reduce the contaminating protein to less than 0.5%.

2.16 Protein estimation : The purity of the LPS was assessed with the protein estimation method of Lowry, a modified Folin-Ciocalteu reaction reported by Williams and Chase, 1968.

2.17 Dry weight estimation : The LPS samples were dried under a heat lamp until three consecutive weights were in agreement. The LPS concentration of the preparation could be determined from the estimates of the dry weight and the percentage of protein contamination.

2.18 Haemagglutination inhibition assay : HAI assay was also used to estimate LPS concentrations. The antigen sample was serially diluted two-fold in saline in a micro-titre tray. Four haemagglutinating units of specific antibody were added to every well, and the tray incubated at 37° for 45 minutes. A drop of LPS sensitized SRBC was added to each well and the tray incubated at 37° for 45 minutes once more. Unbound LPS in the test sample reduces the amount of haemagglutinating antibody available to act on the LPS sensitized SRBC and so the amount of antigen can be determined by comparing the test inhibition with that of a known standard.

- 2.19 Alkali-treatment of LPS : A known quantity of LPS in a pellet was dissolved in distilled water to a concentration of 1mg/ml and NaOH was added until it was 0.02M. The alkaline mixture was kept at room temperature for six hours before the pH was restored to 7.4. The LPS was then stored at 4°, preserved with 0.1% NaN₃.
- 2.20 Glutaraldehyde-fixing of Vibrios : Log-phase broth cultures of V. cholerae were centrifuged and washed three times in saline, and resuspended to 10⁹/ml in PBS pH 7.4. Glutaraldehyde was added to a final concentration of 0.1% and the mixture stirred for 15 minutes on ice. After five washes in PBS, the glutaraldehyde fixed organisms were resuspended to 10¹⁰/ml.
- 2.21 Lymphocyte Preparations
- 2.21a Spleen : Mouse spleens were cut into three pieces and were gently disrupted into Hanks Balanced Salt Solution (HBSS) using a glass tissue homogenizer. A single cell suspension was obtained by filtering through a coarse and a fine nickel steel mesh. The cells were washed once in HBSS to remove debris, and were resuspended to 1.0ml.
- Lymphocytes were counted in white cell counting fluid using an improved Neubauer chamber and the percentage of viable cells was estimated by the trypan-blue dye exclusion test. Viable counts usually ranged from 0.5-2.0 x 10⁸ cells/spleen, with greater than 90% viability.

2.21b Small intestine mucosa : The technique of Robertson and Cooper, 1972 was modified. Each bowel was flushed with 10ml of saline, placed on paper, slit longitudinally with a scalpel, and spread open. A scalpel was used to scrape off the mucosa down to the muscularis mucosae. It was put into 10ml HBSS and dispersed by agitation. The suspension was blown through a 350 mesh stainless steel filter, and the residue washed through with 5ml of medium. The cells were then washed twice and resuspended in 1ml of HBSS. Viable lymphocytes were counted in 0.2% trypan blue : stained dead lymphocytes were not counted amongst the stained epithelial cells.

The only reported attempt to enumerate mucosal lymphocytes of the small intestine has been by planimetric examination of tissue sections (Brantzaeg and Baklien, 1976). Making the assumption that the mouse has similar numbers of lymphocytes per surface area of small intestinal mucosa to that of the human, then 5×10^7 lymphocytes may be expected per small intestine. Therefore the 10^7 lymphocytes actually obtained may represent a 20% recovery.

This high lymphocyte recovery was at the expense of purity. For every lymphocyte there were at least 20 epithelial cells, in agreement with reported data (Douglas and Weetman, 1975). Nevertheless, microscopic examination confirmed that at the centre of every plaque on the Jerne plates there was a single lymphocyte. Non-specific lysis in the plates was

not a problem, as long as the intestinal cells were brought down with the minimum of centrifugation so that the maximum amount of debris could be removed with the supernatant. This procedure also prevented autolysis of the isolated cells.

Bull and Bookman (1977) isolated human intestinal mucosal lymphoid cells, free from epithelial cells, by an enzymatic technique. This lengthy procedure was not used in the present work because the intestinal mucosa of the mouse is less fibrous than that of the human, and samples were easier to prepare. Furthermore, large numbers of samples had to be prepared daily and experience showed that the assay did not require pure lymphocyte preparations.

2.22 Plaque-forming Cell Assays

2.22a Haemolysis in agar: The Jerne technique was reported by Jerne and Nordin, 1963 - a thin layer modification was employed. 2ml aliquots of 0.5-0.7% agarose in HBSS were maintained at 45° in a water bath. 0.1ml of sensitized SRBC (2×10^9 /ml) and 0.1ml of a lymphocyte dilution were pipetted into the agarose just prior to spreading it onto a culture plate. Each tube was rapidly mixed by rolling between the hands and its contents spread by rocking across the plate. Spreading was facilitated by the presence of 0.5% BSA in the mixture. After setting, the plates were incubated upside-down at 37° in a humid atmosphere for 1 hour.

Direct plaques were developed by the addition of 2ml of 1/20 guinea-pig serum in isotonic saline. The plates were developed for 45 minutes at 37° before the liquid was tipped off, and the plaques counted and marked off with a grease pencil.

IgA plaques were developed by the addition of 2ml of 1/200 goat anti-mouse IgA antiserum kindly supplied by Dr. D.J. Horsfall.* Tests showed that this was an optimal developing concentration. After 45 minutes at 37° the liquid was poured off and once again 2ml of fresh 1/20 guinea-pig serum was added and the plates incubated for 45 minutes at 37°. All the plaques which developed in addition to the direct ones already marked were the indirect IgA plaques.

Background PFC against SRBC were negligible, and numbered only one or two per spleen.

2.22b Vibriolysis in agar : The vibriolytic technique was described by Kateley, Patel and Friedman, 1974. 2ml

* Footnote : Goat anti-mouse IgA antiserum was essentially mono-specific for IgA. Immuno-electrophoresis showed a strong line against IgA without staining. After staining a very faint line was visible against IgG₁, and the other immunoglobulin classes were not detected at all.

It was produced by purifying secretory immuno-globulins from mouse intestinal juice using a Sephadex-G200 column. Mancini testing showed that of the Ig, 95% was in the IgA class and 5% was in the IgG₁ class. Goats were immunized once a week with 1-2mg of Ig given i.m. with complete Freunds adjuvant. The goats were bled after six immunizations. Later sera were not used as they contained significant anti-light chain activity.

aliquots of 0.5-0.7% agarose in HBSS were maintained at 45° in a water bath; 0.1ml of the lymphocyte dilution and 5×10^8 vibrios in 0.1ml saline were pipetted into the agarose. The tubes were mixed and their contents spread onto nutrient agar culture plates. The plates were incubated at room temperature for 1 hour and then at 37° for one hour. Direct plaques were developed by reincubation with 2ml of 1/20 guinea-pig serum for 2 hours at 37°. Indirect plaques were developed by incubation with 2ml of 1/200 goat anti-mouse IgA antiserum for 1 hour at 37°, before the addition of complement. Direct and indirect plaques had to be enumerated on different plates in this technique.

2.23

Statistics

Mathematical significance was calculated using Students 't' test. As the numbers of PFC for groups of mice show log normal distribution, a log transformation of the data had to be performed for use in the 't' test (Sell, Park and Nordin, 1970). As well, PFC assay results had to be plotted on a log scale using geometric means.

Population variances were compared in a two-sided test using the F distribution. The 't' test was applied where the variances were comparable (Lapin, 1975). The method of least squares was used when regression analysis was required.

CHAPTER 3

CHAPTER 3RESISTANCE TO VIBRIO CHOLERAE IN THE
GASTRO-INTESTINAL TRACT OF THE MOUSE

3.1

Introduction

Like most other adult animals, mice are not susceptible to infection by Vibrio cholerae (Ujiiye *et al*, 1968) due to the action of the non-specific defences of the gastro-intestinal tract. The first important defence is the gastric acid, which tends to prevent cholera infection in well nourished people (Music *et al*, 1971). When the bacteria reach the intestine they are subject to removal by peristalsis which is also a most important anti-bacterial mechanism (Dack and Petran, 1934). This point is illustrated by the use of morphine in guinea-pigs which inhibits the motility of their intestine and allows induction of an infection with V. cholerae (Burrows, Elliot and Havens, 1947). Other defences of the intestine include : microbial antagonism (Sasaki *et al*, 1970), which is reduced by streptomycin in another guinea-pig model of cholera (Freter, 1955); the bactericidal properties of intestinal juice (W.H.O. techn. rep. 500, 1972); and a bactericidal action by the mucosa of the small intestine has been proposed (Knop and Rowley, 1975a).

Work reported in this chapter examines the passage of Vibrio cholerae through the gastro-intestinal tract of mice and how this is influenced by differences between bacterial strains. It was hoped that this study would provide background data for the work on oral immunization reported in later

chapters. In particular, it enabled the examination of a proposed correlation between the ability of a V. cholerae strain to persist in the intestine and its immunogenicity when given by the oral route (Cash *et al*, 1974b).

Finally in this chapter a study was made of the action of antibody on Vibrio cholerae in the gut, which is believed to be a specific antibacterial defense. Antibody mediated antibacterial mechanisms can be studied in the infant mouse because it is susceptible to a cholera-like infection (Ujiiye *et al*, 1968), probably due to the immaturity of the intestine (Schaedler, Dubos and Costello, 1965). As well, the experimentally induced illness may be inhibited by the oral administration of antibody concurrently with the oral challenge with the organisms (Ujiiye and Kobari, 1970).

Bacterial agglutination could be mediated by antibody in the small intestine, and may be important when animals are challenged orally with large numbers of organisms (Bellamy, Knop, Steele, Chaicumpa and Rowley, 1975; Schrank and Verwey, 1976). The bacterial agglutinates are then moved to the colon by peristalsis (Knop and Rowley, 1975b). There is also considerable evidence that antibody prevents the attachment of vibrios to the small bowel mucosa, and this also enhances their removal by peristalsis (Freter, 1964, 1969, 1970, 1972; Fubara and Freter, 1973; Bellamy *et al*, 1975; Bloom and Rowley, 1977).

There have been suggestions that antibody may mediate the killing of V. cholerae in the small intestine (Freter, 1970, 1971; Chaicumpa and Rowley, 1972; Knop and Rowley, 1975b,c).

However, the conditions in the small intestine destroy complement (Freter, 1962) and there is little evidence of phagocyte activity at the mucosal surface apart from one study in pigs (Bellamy and Nielsen, 1974). Therefore it seems improbable that the two well known bactericidal actions of antibody, complement mediated bacteriolysis, and opsonization leading to phagocytosis, can be effective in the gut. This brings into question the nature, or the very existence of the postulated bactericidal mechanism, an issue which is examined in this chapter.

3.2 Recovery of *Vibrio cholerae* from the G.I.T. after an Oral Challenge : Adult Mice

A detailed study was made of the recovery of *Vibrio cholerae* from adult mice after an oral challenge. Mice were fed a 0.4ml suspension containing 2×10^8 *v. cholerae* 569B SR, introduced into the stomach using a round-tipped 19 gauge needle. When vibrio suspensions were given, only 1-5% of the viable count could be recovered from the gastro-intestinal tract one minute later. The suspending fluid was then half-saturated with sodium bicarbonate, which was found to maximize recovery of organisms from the GIT. The mice were killed and their gastro-intestinal tracts removed at various times after feeding them bacteria. The stomach, small bowel and large bowel were homogenized separately, and suitable dilutions of the homogenates were spread onto nutrient agar plates. These plates incorporated streptomycin to suppress the growth of other organisms present in the gut. During the experiment

TABLE 3.1

Recovery of V. cholerae 569B SR from adult mice after an oral challenge of 2×10^8 organisms. Each time point represents accumulated results from 10 groups of 5 mice

Sample	% Recovery of Inoculum with Time (minutes)				
	1	12	60	120	180
Stomach	35	4	0.5	0.1	0.01
Small bowel	16	51	17	0.1	0.02
Large bowel	0.1	0.6	26	25	1
Faeces	-	-	0.3	6	20
Total	51 (Range 35-64)	56	44	31	21 (Range 7-43)

the mice were kept on metal grids so that their faeces were collected directly into saline, and then viable counts were performed on the faeces after homogenization. The saline for faecal collection contained 0.01% sodium azide, which prevented vibrio growth but did not kill the organisms.

Within one minute only half the organisms fed to mice could be recovered from the gut (Table 3.1). Presumably stomach acid and enzymes could still kill a large proportion of ingested vibrios despite the alkalinity of the inoculum. Most of the organisms were in the small intestine after 12 minutes, and they had moved to the large intestine in considerable numbers by 60 minutes. After 180 minutes few vibrios were recovered from the gut, with excretion of large numbers in the faeces.

Apart from the bactericidal action of the stomach, the major antibacterial process in the gut was obviously removal by peristalsis. However, of the vibrios which had entered the small intestine alive, over half were killed in the three hours in which the organisms were virtually eliminated from the gut by excretion.

3.3 Vibriocidal Actions of the Intestine in Adult Mice

3.3a 'Natural' antibodies in the intestine : V. cholerae

569B SR fed to adult mice were being killed at a steady rate after they had left the stomach, and the killing might have been attributed to 'natural' antibodies in the intestine. The existence of 'natural' antibody is illustrated by the presence of

PFC directed against sheep red cells found in the spleens of mice not stimulated with this antigen, but possibly stimulated with cross-reacting antigens (Haskill, Byrt and Marbrook, 1970).

The intestinal juice of unimmunized SPF mice has a low haemagglutinating titre (up to 1:8) against LPS coated red cells. Similarly, normal intestinal juice often has an anti-V. cholerae antibody titre by radio-immuno-assay. However if LPS-SRBC are washed after their incubation with normal intestinal juice, an HA titre is no longer obtained. A similar procedure with immune serum does not reduce the HA titre. Therefore the haemagglutination produced by normal mouse intestinal juice is non-specific.

Animals not exposed to V. cholerae do not have antibody forming cells against this antigen in the lymphoid organs (Veldkamp, van der Gaag and Willers, 1973; Sajid, McAlack, Cerny and Friedman, 1971). The LAC mice used were examined to confirm this finding. Suspensions of spleen and small bowel lymphocytes were assayed for PFC with the Jerne technique. On average 2 PFC were found per spleen, but these were directed to the SRBC substrate in the assay, not to the LPS coating. No PFC were found in the unstimulated gut. The assays were repeated using the vibriolysis-in-agar technique to detect antibodies to all V. cholerae antigens, not just the LPS. Once again no PFC were found in the unstimu-

lated animal.

In conclusion the vibrio killing in the gut of adult mice could not be attributed to 'natural' antibody, which is not demonstrable in this system.

3.3b Vibrio death by dehydration : V. cholerae is extremely sensitive to dehydration, as proven by the following experiment. Mice, in two groups of 20, were each fed 3×10^7 V. cholerae 569 SR. One group was kept on a metal grid and their faeces fell directly into saline/0.01% NaN_3 ; the other group was kept on tissue paper and their faeces were transferred within five minutes to saline/0.01% NaN_3 . 180 minutes after vibrio feeding the average gastro-intestinal recoveries were less than 0.1% in each group. However, faecal recovery was 1% with the tissue paper collection, and 30% for the direct collection into saline.

Not only do faeces dehydrate after excretion, but also beforehand due to the water conservation function of the colon. Water deprived animals conserve more water by increasing its extraction from the colon (Guyton, 1971). This fact was used to test a theory that vibrios were being killed by dehydration in the colon.

A group of 20 control mice were compared with a group of 20 mice deprived of water for 18 hours. The mice were fed 2×10^8 V. cholerae 569B SR, and they were killed 180 minutes later. The average gastro-

intestinal vibrio recovery was less than 1% but there was a faecal recovery of 28% for the watered mice which was in contrast with the 4% recovery for the water deprived mice ($p < 0.01$).

'It was concluded that after V. cholerae were fed to adult mice losses of organisms could be accounted for by killing in the stomach, dehydration in the colon, and elimination with the faeces. A bactericidal mechanism on the mucosa of the small intestine (Knop and Rowley, 1975a) was not excluded, as a process of minor magnitude could have been overlooked with the techniques used.

3.4 Recovery of Vibrio cholerae from the G.I.T. after an Oral Challenge : Infant Mice

Infant mice are susceptible to infection by V. cholerae whereas adult mice are not (Ujjiye et al, 1968). The recovery of V. cholerae from infant mice after oral challenge was a sensitive model in which to examine the association of the organisms with the small bowel.

The mice were fed 0.1ml of a bacterial suspension introduced into the stomach with a round-tipped 25 gauge needle. At various times after feeding the mice were killed and their gastro-intestinal tracts were removed. The gut was divided into upper and lower portions, stomach with small intestine, and large intestine. The upper and lower portions were homogenized separately, and suitable dilutions of the homogenates were spread on streptomycin-nutrient agar plates.

In a series of 20 experiments infant mice were fed inocula of V. cholerae 569B SR ranging from 10^4 to 2×10^7 organisms. The recovery of organisms from the upper portion of the gut revealed a constant pattern, with an initial decline in vibrio numbers followed by a phase of vibrio growth. There was a day to day variation in the experiments, with the minimum recovery of 1-8% falling between 150 to 360 minutes after feeding. This variation prevented a useful graphing of the combined data, but individual examples of the experiments can be seen in Table 3.2

It was not known whether the decline in numbers of V. cholerae in the stomach and small intestine was the result of bacterial death or the peristaltic elimination of bacteria from the upper gut. A balance sheet of GIT and faecal recoveries could not be drawn up to answer this question because there was no reproducible pattern in the recovery of organisms from the large bowel. When vibrio numbers were at their minimum in the upper gut, recoveries in the large bowel ranged from 7-361%, apparently reflecting a balance of vibrio death, multiplication and faecal elimination. Faecal losses could not be taken into account because the feat was not accomplished of collecting minute semi-liquid excreta spread around by wriggling infant mice.

Attempts were made to prevent faecal loss of vibrios by sealing the ani of the infant mice with an acrylic contact glue, CO-APT Behringwerke. In a series of 10

experiments inocula ranging from 10^4 to 2×10^7 V. cholerae 017 SR were fed to baby mice. Recovery of viable organisms from the stomach and small intestine fell to a minimum of 2-7% between 150 and 310 minutes after feeding, and at this time the range of colon recoveries was 13-24%. The results were similar to those obtained with V. cholerae 569B SR (vide supra). By contrast, similar feeding of V. cholerae 017 SR to infant mice with glued ani gave large bowel recoveries over the same time scale averaging only 0.7%. The recovery of viable organisms from the remainder of the gastro-intestinal tract of these animals fell to 6%, indicating that the upper part of the tract was functioning normally. It was apparent that this anal treatment resulted in killing of vibrios in the colon, so this approach was abandoned.

As in adult mice, there has been a suggestion that there is a bactericidal mechanism in the intestines of baby mice (Chaicumpa and Rowley, 1972). Once again it has not been possible to positively affirm or deny the presence of such a bactericidal mechanism.

3.5 Growth of Vibrio cholerae in the Small Intestine of Adult Mice

Adult mice are not infected by V. cholerae, and only small numbers of the organisms remain in the small bowel three hours after oral inoculation (Table 3.1). Despite this, two V. cholerae strains of different virulence were tested for survival and growth in the small intestine. Virulence was assessed in the infant mouse lethality test :

FIGURE 3.1

RECOVERY OF V. CHOLERAE FROM THE MUCOSA OF THE SMALL
INTESTINE WITH TIME AFTER THE ORAL INOCULATION OF
ADULT MICE

Mice were fed an inoculum of 10^{11} organisms, with NaHCO_3 to neutralize the gastric acid. Mice were killed at the five time points indicated, and viable counts of V. cholerae on the washed small bowel were obtained. Each time point represents the geometric mean of the results from six mice.

— 569B IMP SR

..... 111 NM SR

Figure 3.1

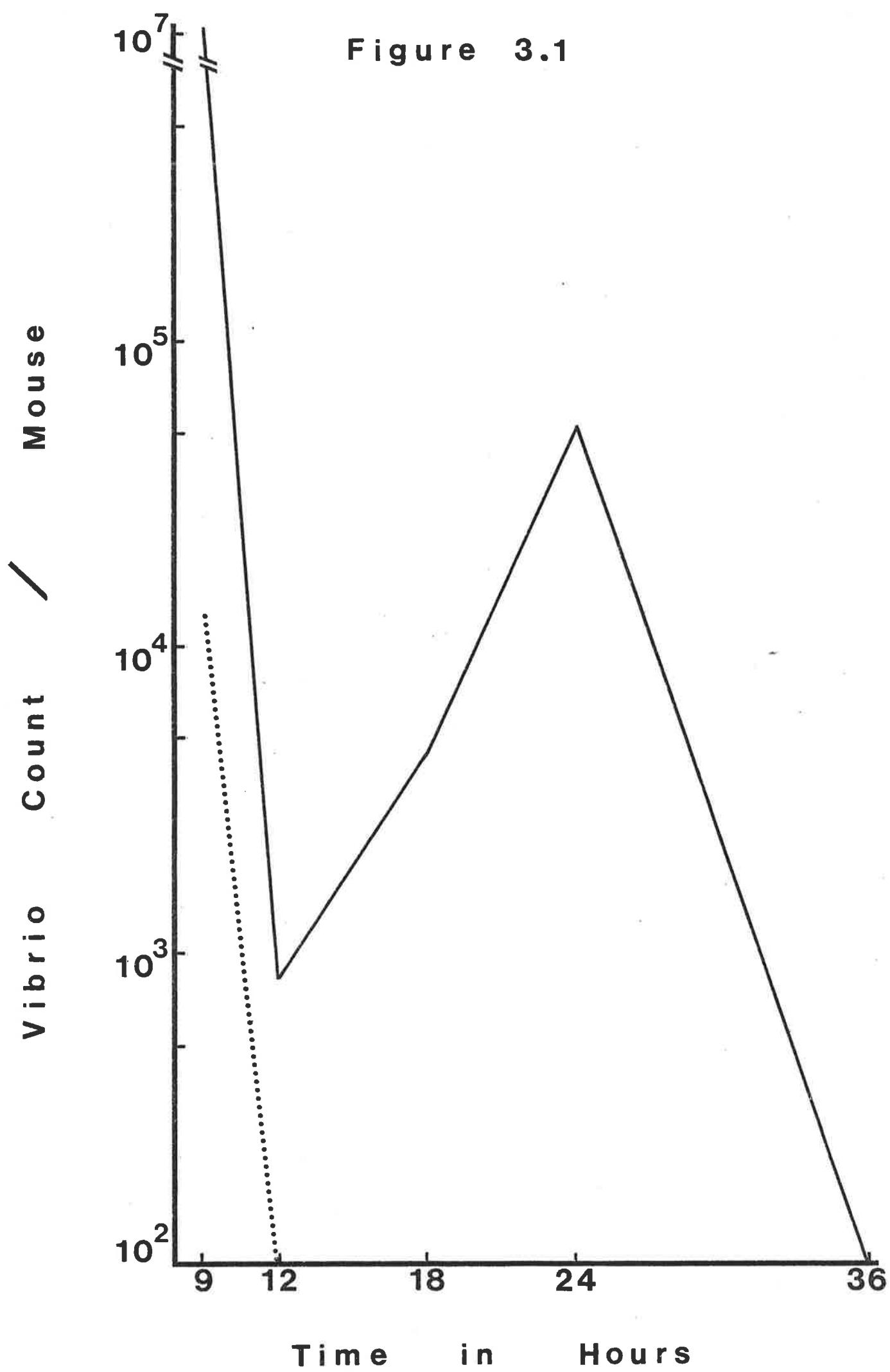


FIGURE 3.2

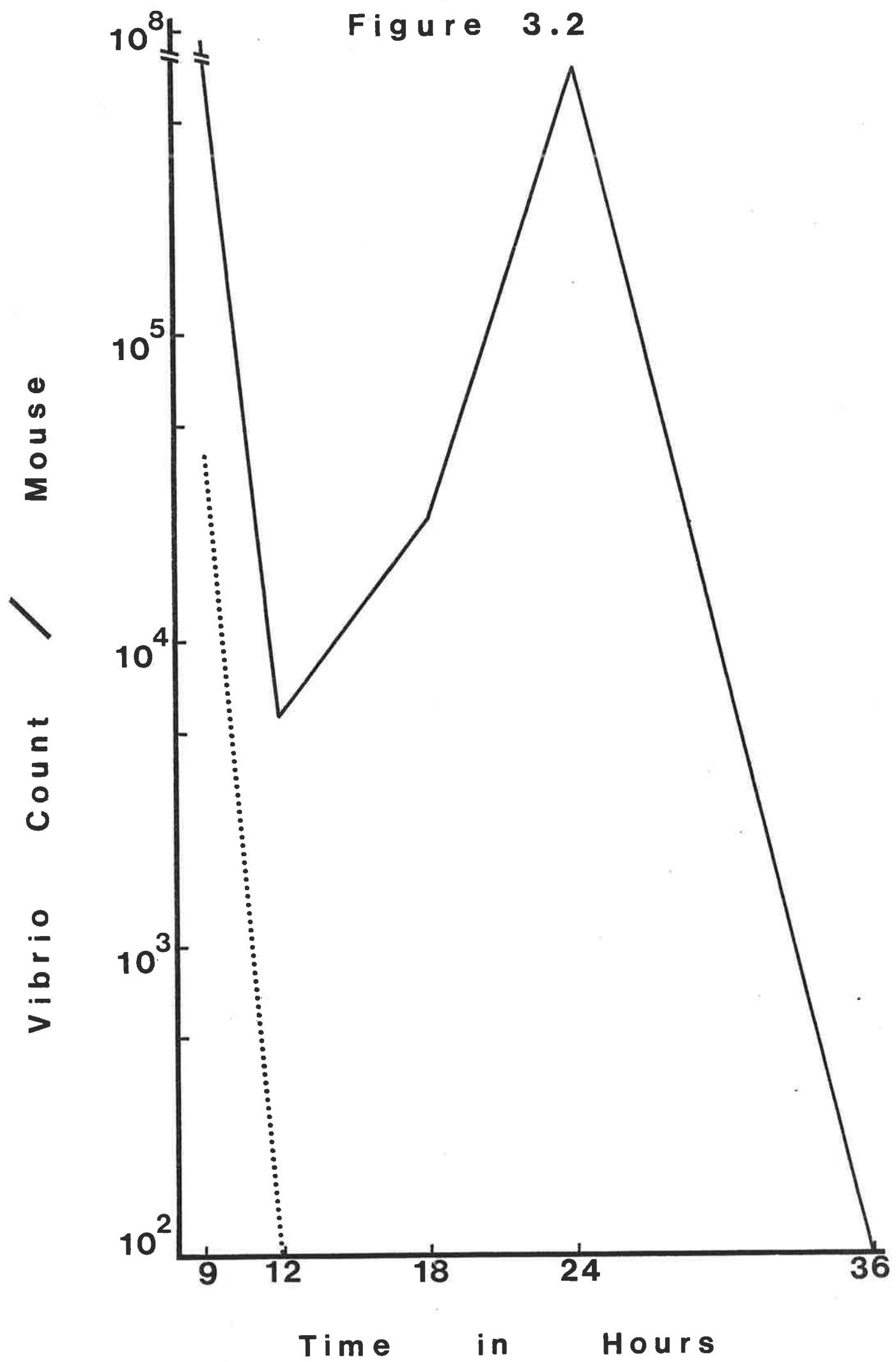
RECOVERY OF V. CHOLERAE FROM THE LUMEN OF THE SMALL
INTESTINE WITH TIME AFTER THE ORAL INOCULATION
OF ADULT MICE

Mice were fed an inoculum of 10^{11} organisms, with NaHCO_3 to neutralise the gastric acid. Mice were killed at the five time points indicated, and viable counts of v. cholerae in the small bowel wash out fluid were obtained. Each time point represents the geometric mean of the results from six mice.

— 569B IMP SR

..... 111 NM SR

Figure 3.2



the virulent 569B IMP SR strain had an LD₅₀ of 10⁷, and the avirulent 111 NM SR strain was not lethal for the mice (LD₅₀ > 10¹¹).

Mice were each given an oral inoculum of 10¹¹ organisms and were killed at various times after feeding. The small intestine was removed and washed out with 10ml of saline : bacteria which remained in the washed bowel were said to be 'mucosal', and those in the wash out fluid were 'lumenal'.

12 hours after feeding, 569B IMP SR had declined to number fewer than 10⁴/bowel, but 111 NM SR was no longer even detectable in the small bowel (< 10²/bowel). The virulent strain did, however, show a period of growth between 12 and 24 hours, before its numbers declined below the detectable limit by 36 hours. For both strains the numbers of organisms adherent to the mucosa paralleled the numbers washed free from the lumen (Figs. 3.1, 3.2).

The superior survival and growth of the virulent 569B IMP SR strain could have been due to either of two virulence characteristics, toxigenicity or mucosal adherence (Finkelstein, Arita, Clements and Nelson, 1977). If the action of toxin on the bowel enhanced conditions for vibrio growth, then both strains given together in an oral inoculum would be expected to grow in the intestine.

Mice were fed a combined inoculum of 10¹¹ 111 NM SR and 10¹¹ 569B IMP. The strains were distinguished by obtaining viable counts on nutrient agar plates with and without incorporated streptomycin. Normal bowel flora also

grew on the nutrient plates without streptomycin, but the colonies were usually easily distinguished from the large translucent vibrio colonies. Colonies were tested for agglutination with an anti-V. cholerae antiserum in cases of doubt. The results were similar to those displayed in Figs. 3.1 and 3.2 even though the two V. cholerae strains were given as a combined dose to mice. It was concluded that the difference in strain growth in the bowel was due to a difference in mucosal adhesiveness, a conclusion which has been supported by work in vitro (S.R. Attridge, personal communication).

3.6

Growth of Vibrio cholerae in the Small Intestine of Infant Mice

The work with V. cholerae survival and growth in the small intestine (Section 3.5) was extended to include infant mice. Once again the virulent 569B IMP SR strain was compared with the avirulent 111 NM SR strain. The mice were killed at various times after being fed 2×10^8 organisms. The small bowel was removed and then washed out with 10ml of saline, and viable counts were obtained for the washed bowel and the wash out fluid.

In Figs. 3.3 and 3.4 a profound difference is seen between the two V. cholerae strains in their survival in the small intestine. 111 NM SR was steadily eliminated from the bowel and was undetectable 18 hours after being fed to infant mice, in contrast to the maintenance of the 569B IMP SR population. Recovery of 569B IMP SR from the mucosa increased progressively with time while numbers in the bowel

FIGURE 3.3

RECOVERY OF V. CHOLERAE FROM THE MUCOSA OF THE SMALL
INTESTINE WITH TIME AFTER THE ORAL INOCULATION
OF INFANT MICE

Mice were fed an inoculum of 2×10^8 organisms.

They were killed at the six time points indicated and viable counts of V. cholerae on the washed small bowel were obtained. Each time point represents the geometric mean of the results from three mice.

— 569B IMP SR
..... 111 NM SR

Figure 3.3

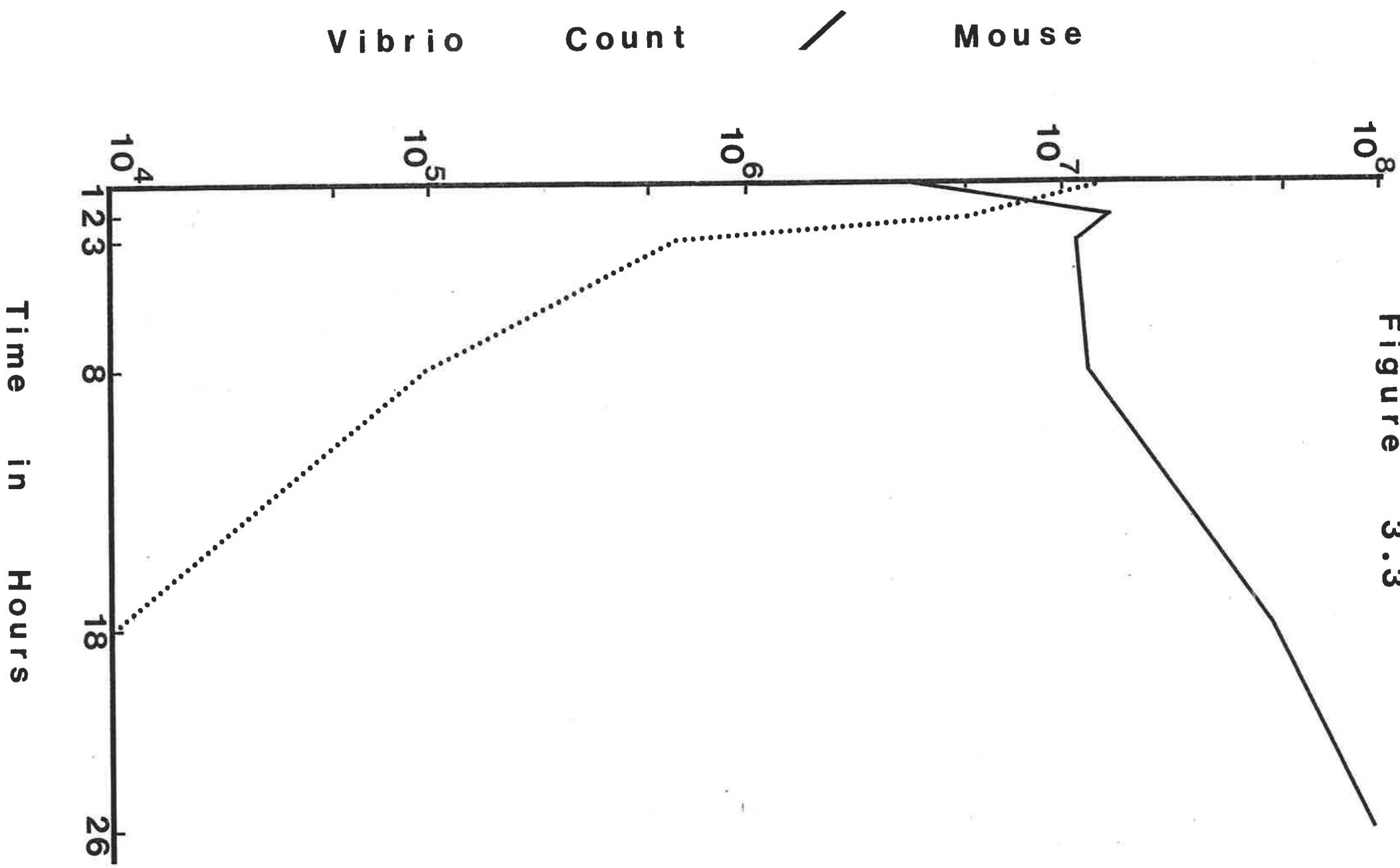


FIGURE 3.4

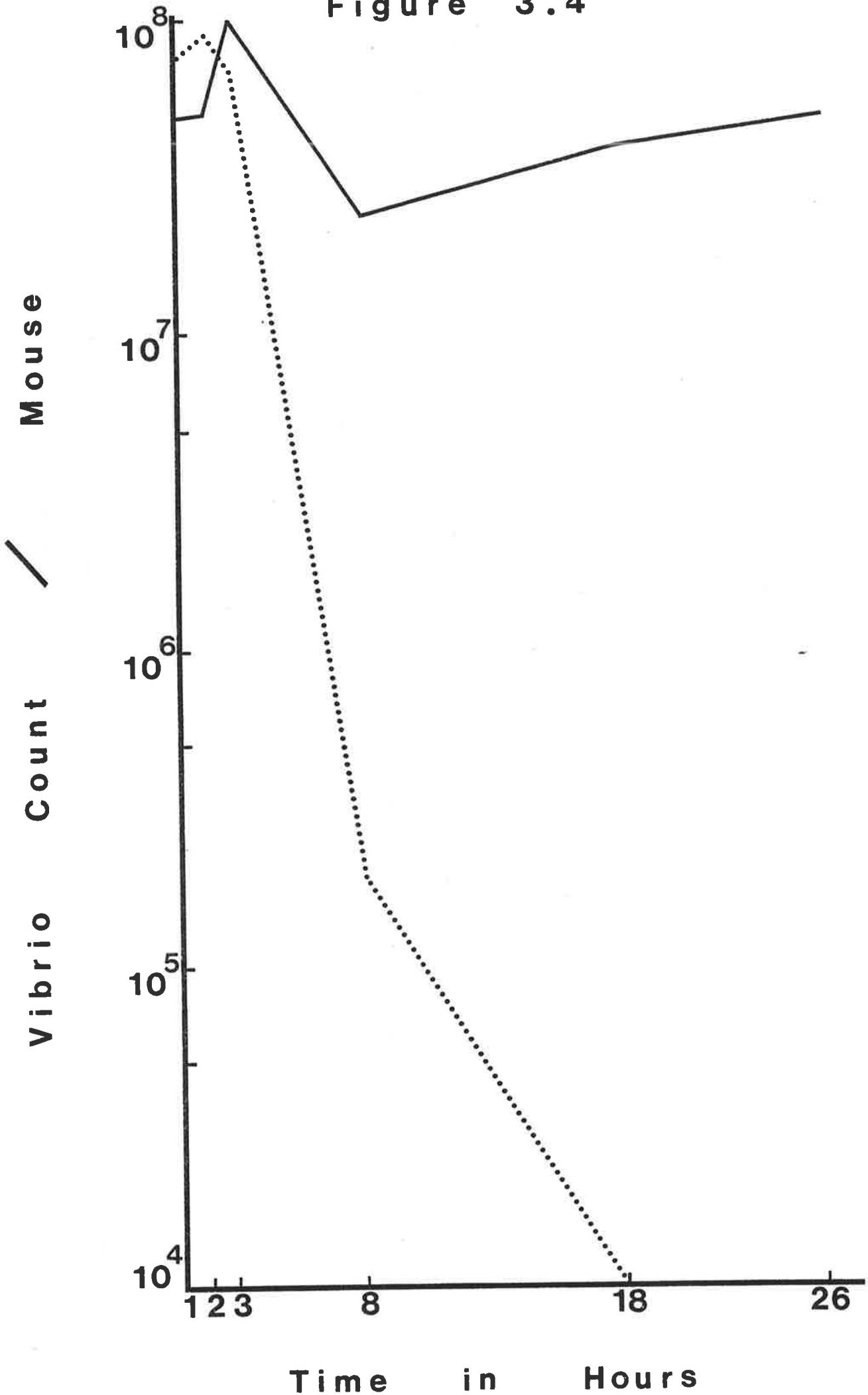
RECOVERY OF V. CHOLERAE FROM THE LUMEN OF THE SMALL
INTESTINE WITH TIME AFTER THE ORAL INOCULATION
OF INFANT MICE

Mice were fed an inoculum of 2×10^8 organisms. They were killed at the six time points indicated, and viable counts of V. cholerae in the small bowel wash out fluid were obtained. Each time point represents the geometric mean of the results from three mice.

— 569B IMP SR

..... 111 NM SR

Figure 3.4



lumen remained virtually static, emphasizing mucosal adherence as a virulence determinant in this organism.

To eliminate cholera toxin as the factor enhancing the growth of 569B IMP SR, the two strains were given as a combined oral inoculum in order to determine the effect on 111 NM SR growth. Each infant mouse was given 2×10^8 111 NM SR and 2×10^8 569B IMP. As in the similar experiment with adult mice, viable counts were performed on nutrient agar plates with and without streptomycin to differentiate between the two strains.

The recoveries of 569B IMP and 111 NM SR on the mucosa and in the lumen remained similar to those shown in Figs. 3.3 and 3.4, once again discounting the possibility that 569B IMP SR grew well in the intestine because it secreted toxin.

3.7

The Protective Action of Antibody in the Infant Mouse Cholera Model

The illness induced in infant mice by challenge with V. cholerae (Ujjiye et al, 1968) may be inhibited by previous or concurrent administration of antibody given orally (Ujjiye and Kobari, 1970; Pitkin and Actor, 1971; Neoh and Rowley, 1972). This model was used to investigate the action of antibody on V. cholerae in the gastro-intestinal tract.

The agglutination of organisms is thought to be a significant action of antibody in the gut (Bellamy et al, 1975), and this was avoided in the following experiments in order to study other antibody actions. Two strains of V. cholerae

TABLE 3.2

Recovery of V. cholerae from infant mice
after oral challenge

Inoculum	Sample	% Recovery of Inoculum with Time (minutes)							
		30	60	90	120	180	360	540	1200
10^4 <u>V. cholerae</u> 017 SR	CS	44	24	62	205	2	25	5	4860
	CL	0	0.6	6	68	112	62	558	310
	*AS	44	17	1	6	0	0	0.2	2
	AL	0.06	0	0	1	8	6	22	0.2
2×10^7 <u>V. cholerae</u> 569B	CS		94	67	59	22	2	26	266
	CL		6	5	2	39	16	6	24
	**AS		43	57	37	14	0	0	1
	AL		2	5	6	10	16	4	0

Each time represents a group of 5 mice. C = control, A = antibody treated, S = stomach and small bowel, L = large bowel.

* 017 SR sensitized at a concentration of 4x agglutinating titre.

** 569B sensitized at a concentration of $\frac{1}{4}$ agglutinating titre.

were used, the virulent 569B IMP strain ($LD_{50}=10^7$), and the even more virulent 017 SR strain ($LD_{50}=10^4$). 0.1ml of a bacterial suspension containing either 2×10^7 569B IMP or 10^4 017 SR was fed to baby mice. Some mice were fed bacteria which had been sensitized with antibody, without the formation of agglutinates detectable with phase-contrast microscopy. The low concentration of 017 SR prevented agglutination even though the antiserum was used at a titre four times higher than that required to agglutinate 10^8 vibrios/ml. 569B IMP suspensions were sensitized with anti-serum diluted to a quarter of its agglutinating titre.

The mice were killed at various times after being fed the vibrios and recovery of organisms from the upper gut (stomach and small intestine) and the lower gut (large intestine) was determined by viable counts on the homogenates. The results in the control mice given organisms untreated with antibody were similar to previous findings (Section 3.4). In these control animals vibrio numbers declined in the upper gut to reach a minimum of 2% after 180 to 360 minutes (Table 3.2). After this time the numbers recovered from the upper gut rapidly increased. Large numbers of 017 SR were recorded in the large bowel, but there were fewer 569B IMP recovered from this site probably due to the diarrhoea which was observed in these mice. Antibody treatment significantly reduced ($p < 0.01$) the numbers of both strains in the upper gut, and 017 SR in the large bowel.

Table 3.2 gives a clear indication of the basis of the baby mouse protection test. Mice of a particular weight

(2.3-2.6g) and age (5-6 days) are able to eliminate from the small intestine all but 2% of the V. cholerae fed to them, and antibody is able to prevent the lethal growth of the residual organisms. In tests with infant mice of different ages, older animals (3.3-3.6g) eliminated all the ingested organisms from the upper gut without the aid of antibody, and younger animals (1.3-1.6g) could not begin to reduce vibrio numbers in the upper gut even with the aid of antibody (personal observations).

The question remained as to the nature of the antibody mediated anti-bacterial mechanism. There can be little doubt that it is the adherence of V. cholerae to the mucosa of the small bowel that enables it to resist removal by peristalsis and to multiply in this site (Figs. 3.1-4). It can also be seen from the same data that a greater proportion of a V. cholerae inoculum is found to adhere to the mucosa in infant mice than in adult mice, possibly due to the immaturity of the gut flora in infant mice (Schaedler, Dubos and Costello, 1965). Therefore it is reasonable to suggest that antibody protects 2.3-2.6g infant mice from lethal cholera infection by preventing the organisms from attaching to the mucosa. This view was put forward by Steele, Chaicumpa and Rowley (1974, 1975) to explain the protective activity of IgA and F(ab)₂, which they found would bind to vibrios, but would not kill them by complement fixation or by opsonization. As discussed in Section 3.1, it would be hard to understand how these two well known bactericidal actions of antibody could occur in the gut in any case.

3.8

The Action of Antibody at the Mucosa of the Small Intestine

There was evidence that antibody prevents V. cholerae from attaching to the mucosa of the small intestine (see Section 3.7). The experiment shown in Table 3.2 was repeated to add further weight to this explanation, but vibrio recovery was determined on the washed small bowel and on the washout fluid. Infant mice were fed 2×10^7 V. cholerae 569B IMP and their small bowels were washed out 1-4 hours later using 10ml of saline per bowel. The recovery of vibrios adherent to the mucosa averaged a remarkably constant $4.4\% \pm 3.2\%$ in 55 mice sampled in this period. However, the recovery of vibrios from the bowel lumen displayed a familiar pattern, with a decline to $1.2\% \pm 0.9\%$ between 150-290 minutes preceding a rapid upsurge in numbers. Average recovery in the lumen for the 55 mice was $48\% \pm 44\%$. This experiment confirmed the role of vibrio adherence to the small bowel mucosa in the pathogenesis of cholera in the infant mouse.

In a further experiment, the action of antibody on mucosally adherent vibrios was examined. 25 control mice were fed 2×10^7 V. cholerae 569B IMP. Another 25 mice were fed the same number of organisms treated with a subagglutinating titre of antibody. The mice were killed 60-220 minutes after feeding, and viable counts were obtained to determine the vibrio recovery for the washed small bowel and the washout fluid. The recovery of vibrios from the mucosa was significantly ($p < 0.01$) decreased by antibody

treatment ($1\% \pm 2\%$) compared with the controls ($5\% \pm 7\%$).

Differences in the recoveries from the wash-out fluid were not significant ($p > 0.50$): $48\% \pm 62\%$ controls, $43\% \pm 51\%$ antibody treated.

These experiments strongly suggest that a major antibody mediated anti-bacterial mechanism in the small bowel is to block the adherence of bacteria to the mucosa. An alternative explanation could be that antibody mediates the killing of bacteria attached to the mucosa, but there is a lack of supporting evidence.

3.9 Use of a P^{32} Radiolabel to Detect V. cholerae Death in the Intestine of Infant Mice

In the last section the weight of evidence was against an antibody mediated killing of V. cholerae in the small intestine of infant mice. However, in the past a contrary view has been supported by data gained from the use of P^{32} -radiolabelled V. cholerae (Knop and Rowley, 1975b,c).

$2 \times 10^7 P^{32}$ labelled V. cholerae 569B IMP were fed to infant mice. At various times after feeding the mice were killed and dissected. Homogenates of upper and lower bowel were spread on nutrient agar plates for a viable count of vibrios, and were absorbed onto filter paper discs glued to planchettes for a radio-active count of vibrios, alive and dead. In this way bacteria viable at the time of oral challenge and killed prior to sampling were distinguished from vibrios alive at the time of sampling.

Antibody treatment reduced the recovery of viable

TABLE 3.3

Recovery of an inoculum of 2×10^7 P³² labelled V. cholerae

569B from infant mice after an oral challenge

Sample	Count by	% Recovery of Inoculum with Time (minutes)						
		180	210	240	270	300	330	600
C Stomach & small bowel	Viability	6	1	4	3	5	4	28
C large bowel		21	6	8	4	2	0.4	3
A Stomach & small bowel	Viability	1	0.9	0.3	0.2	0.09	0.09	0.3
A large bowel		12	19	5	4	47	2	1
C Stomach & small bowel	Isotopic label	17	9	8	10	11	10	6
C large bowel		53	29	43	43	114	31	63
A Stomach & small bowel	Isotopic label	9	10	5	8	5	7	7
A large bowel		47	83	40	65	30	30	61
C Stomach & small bowel ratio P ³² /viable nos.		3	9	2	3	2	2	0.2
A Stomach & small bowel ratio P ³² /viable nos.		9	11	16	40	56	78	23

Each time represents a group of 5 mice. C = control animals given unsensitized V. cholerae. A = animals given antibody-sensitized bacteria.

organisms from the small bowel ($p=0.01$), but it did not significantly ($p=0.05$) reduce the recovery of radiolabel in this site (Table 3.3). This antibody action can be expressed as a marked rise in the specific activity (ratio of radiolabel to viable counts) of the organisms in the small bowel, which could be interpreted as vibrio killing.

There are trends in the Table 3.3 data which obscure the interpretation. The major flaw concerns the growth of vibrios in the small bowel of the control mice, which would reduce the specific activity recorded. By comparison, a lack of growth of vibrios in the antibody treated animals would show as a higher specific activity, leading to an erroneous conclusion of vibrio killing. It is also of doubtful validity to compare small reductions in viable count against such a high background of radiolabel activity.

In conclusion, there are formidable problems in determining if there is an antibody mediated process which kills small numbers of vibrios in the small intestine, and this type of experiment does not provide sound evidence.

3.10

Summary

Adult mice rapidly cleared ingested V. cholerae from the gut. Peristalsis appeared to be the major antibacterial action in the small intestine, and there was no definite evidence of a bactericidal mechanism at the mucosal surface. Very few of the organisms remained in the small intestine after three hours, although the virulent 569B IMP SR strain persisted for some hours longer than the avirulent 111 NM SR strain. The difference in strain persistence was

related to the ability of the 569B IMP SR strain to adhere to the mucosa. This information was used in immunization studies in order to confirm a suspected correlation between V. cholerae growth in the intestine and the local immune response (Chapters 5 and 6).

The final part of this chapter investigated antibody mediated antibacterial mechanisms in the small intestine of infant mice. The crucial factor leading to a fatal V. cholerae infection in baby mice was found to be the attachment of the organisms to the mucosa of the small intestine. Conversely, the major effect of antibody, apart from bacterial agglutination, was to reduce the numbers of organisms adherent to the mucosa. The evidence strongly suggested that antibody only prevented the binding of V. cholerae to the mucosa, although an antibody mediated bactericidal mechanism at the mucosal surface could not be entirely dismissed.

CHAPTER 4

CHAPTER 4REGULATION IN THE MOUSE OF THE SYNTHESIS
OF ANTIBODY AGAINST THE LIPOPOLYSACCHARIDE
OF VIBRIO CHOLERAE4.1 Introduction

The number of antibody forming cells detected in the spleen after an immunization, their immunoglobulin class, and the kinetics of their appearance and decline, all depend upon the antigen and the mouse strain involved (Kateley, Patel and Friedman, 1974). For this reason the parameters of an immune response should be defined for the particular immunization system under examination. In this Chapter the parameters of the response of LAC outbred mice to a single intravenous dose of Vibrio cholerae are examined by the enumeration of spleen AFC specific for the lipopolysaccharide (LPS) of the organism.

The typical primary immune response to the LPS of Gram negative bacteria is of the IgM class, and examples include Salmonella (Landy, Sanderson and Jackson, 1965); E. coli (Britton and Moller, 1968); Shigella (Friedman, 1973) and V. cholerae (Kateley, Patel and Friedman, 1974). However the strain of the animal is also a factor as BALBc mice also respond to V. cholerae with AFC in the IgG subclasses (Kataley et al, 1974). By contrast, sheep erythrocytes stimulate the formation of IgM, IgG and IgA, in equal proportions in the rat (Robertson and Cooper, 1973) and with a predominance of IgM in the mouse (Sell, Park and

Nordin, 1970). Other variables include the time of onset of a response, which for instance is much longer for V. cholerae than for E. coli (Kateley et al, 1974; Britton and Moller, 1968), and the amplitude of the response which may differ greatly between mouse strains (Friedman, 1964).

It is usually found that IgM AFC appear in the spleen before those of other classes in a primary response (Sell, Park and Nordin, 1970; Robertson and Cooper, 1973; Kateley, Patel and Friedman, 1974), and this has been open to various interpretations. It has been suggested that IgA forming cells in the spleen have migrated from the lamina propria of the intestine (Crabbe, Nash, Bazin, Eyssen and Heremans, 1969; Andre, Bazin and Heremans, 1973), but this explanation seems unlikely after consideration of the response kinetics (Robertson and Cooper, 1973). There was a report that a transfer of Peyer's patch cells to irradiated allogeneic rabbits resulted in a proliferation of IgA plasma cells not only in the intestine but also in the spleen (Craig and Cebra, 1971). However, the proliferation in the spleen was apparently due to the allogeneic situation (Rudzic, Perey and Bienenstock, 1975). The overwhelming evidence suggests that there are two distinct lymphocyte pools, systemic and secretory (Section 1.3), and that the delayed formation of IgG and IgA is due to a 'switch' from IgM synthesis (review by Lamm, 1976).

The formation of antibody in cycles has been observed in many immunization systems and this is seen as evidence

of the operation of immunoregulatory mechanisms (Section 1.8). The prolonged cyclic appearance of IgM antibodies following the parenteral immunization of mice with E. coli has been thoroughly examined (Britton and Moller, 1968; Britton, Wepsic and Moller, 1968). They concluded that the cycles were a result of a feedback mechanism in which antibodies suppress their own synthesis by binding antigen molecules in vivo. Other regulatory mechanisms also accompany immune responses, and they include anti-idiotype reactivity (Pierce and Klinman, 1977) and suppressor T-cell activity (Warren and Davie, 1977). The presence of a regulatory process affecting the immune response of mice to V. cholerae can also be inferred from the kinetic data to be detailed in this Chapter.

4.2 Parameters of the Immune Response of Mice to Vibrio

cholerae

4.2a Development : LAC outbred mice were given V. cholerae as an intravenous dose of 10^7 organisms on day 0. Each day the spleens of three mice were assayed for IgM and IgA antibody forming cells, using the Jerne technique. Sufficient quantities of an anti-mouse γ heavy chain antiserum were not available, and so numbers of IgG AFC could not be determined.

In Figures 4.1 and 2 it can be seen that the IgM response developed in two phases. In the initial phase there was a small IgM peak between days 1-6. This peak was a constant feature the dozen times the experiment was performed, and appeared after either

FIGURE 4.1

KINETICS OF THE IMMUNE RESPONSE OF MICE TO V. CHOLERAES

569B IMP SR : SPLEEN PFC/ 10^7 VIABLE LYMPHOID CELLS

Mice were given 10^7 organisms i.v. on day 0.

Each day three mice were selected at random, and the results were expressed as a geometric mean.

— IgM PFC

..... IgA PFC

Figure 4.1

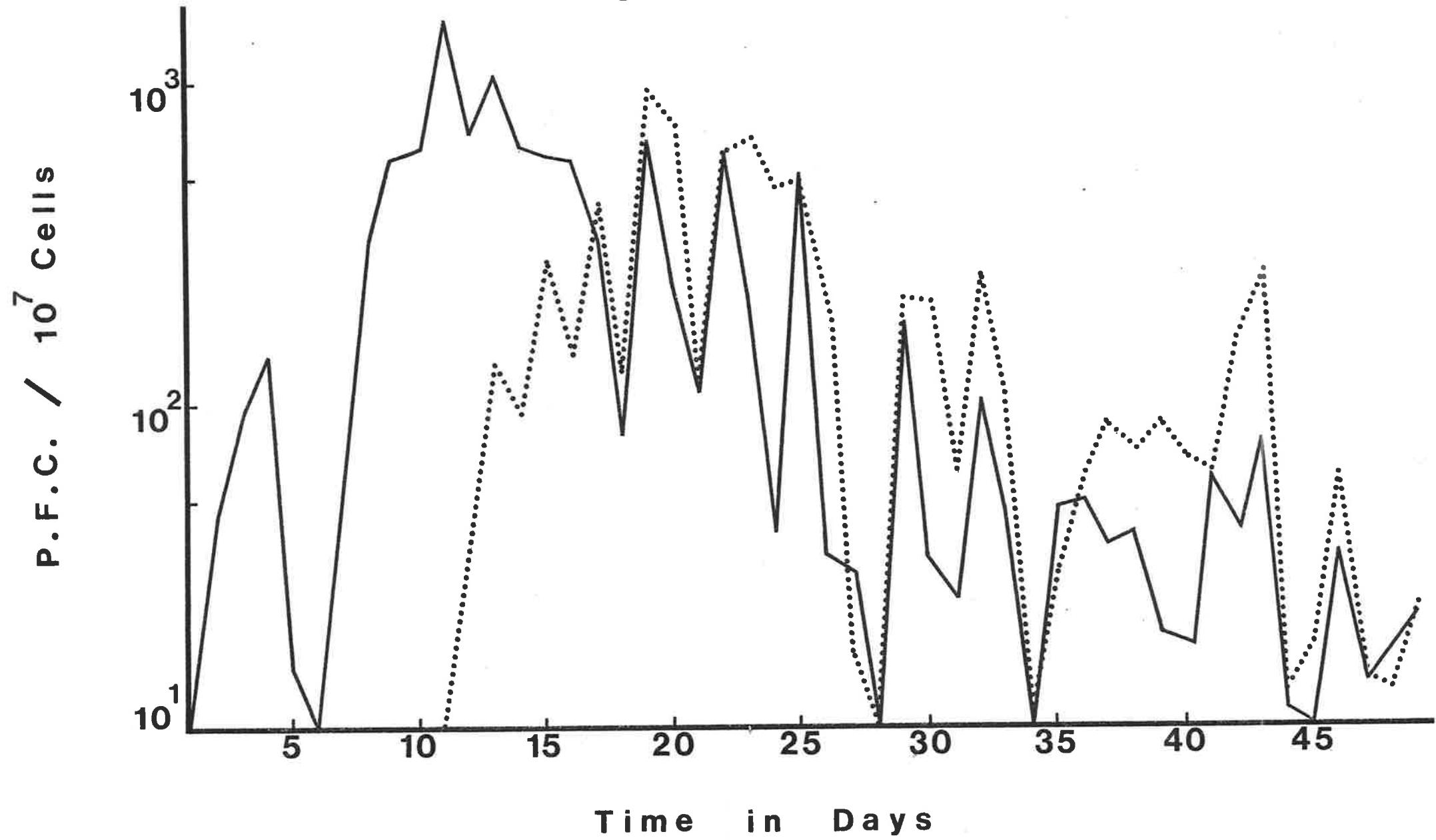


FIGURE 4.2

KINETICS OF THE IMMUNE RESPONSE OF MICE TO V. CHOLERAE

111 NM SR : SPLEEN PFC/10⁷ VIABLE LYMPHOID CELLS

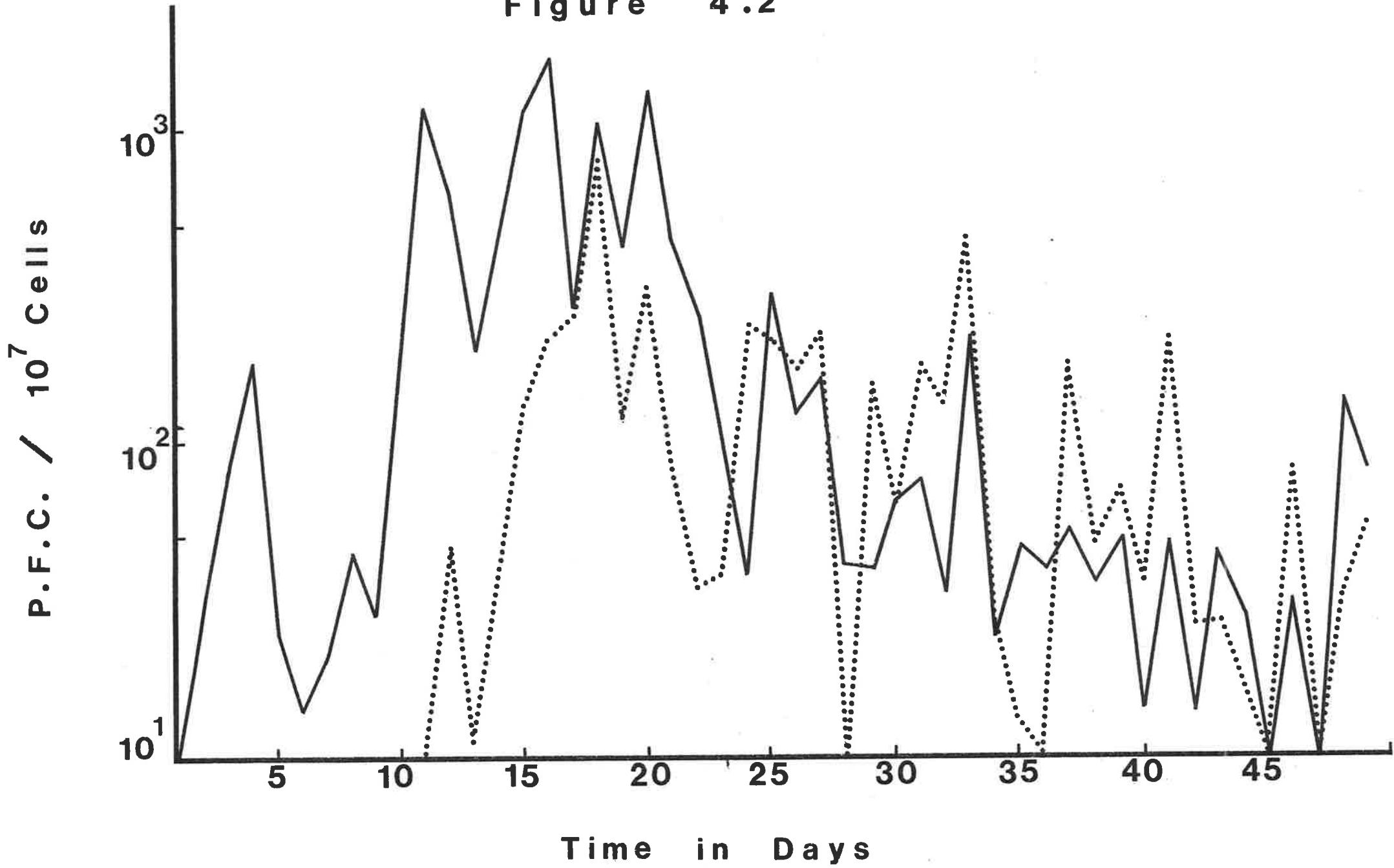
Mice were given 10^7 organisms i.v. on day 0.

Each day three mice were selected at random, and the results were expressed as a geometric mean.

— IgM PFC

..... IgA PFC

Figure 4.2



the 569B IMP SR strain or the 111 NM SR strain was given. It did not result in the formation of serum antibody detectable by the baby mouse protection test, haemagglutination or by radio-immuno-assay.

The second phase of the IgM response was the larger of the two, and it rose to reach a peak on day 11 after which it was sustained for some days (Figs. 4.1,2). The response of mice to E. coli LPS (Britton and Moller, 1968) and to sheep erythrocytes (Sell, Park and Nordin, 1970) reaches a maximum much earlier, at about day 3. However, a similar lag in the production of antibodies to V. cholerae LPS has been reported by Kateley, Patel and Friedman, 1974.

The small initial IgM phase has not been noted in other systems, including the immunization of mice with V. cholerae (Kateley *et al*, 1974). Britton and Moller (1968) suggested that some response cycles may be due to a sequential immune stimulation by different parts of a complex antigen such as LPS, although they found no evidence of this. An analogy may be the immunization of goats with mouse secretory IgA : the initial response is to the γ heavy chain, followed later by a response to the light chain (see Materials and Methods). However, it is more likely that the two 'cycles' represent a depression of the response on days 6 and 7 after immunization, an unexplained phenomenon frequently encountered in later work (see Chapters 5 and 6).

4.2b Amplitude : The amplitude of the response to V. cholerae LPS was in accordance with the data for other antigens. It reached a maximum of approximately 10^3 PFC/ 10^7 viable lymphoid cells, similar to a primary response to E. coli (Britton and Moller, 1968), sheep erythrocytes (Robertson and Cooper, 1973) and to previous data for V. cholerae (Kateley et al, 1974).

4.2c Antibody class composition : Predominantly IgM responses are usually found against the lipopolysaccharide of Gram negative bacteria (Section 4.1), although V. cholerae evokes an IgG response of equal magnitude in BALBc mice (Kateley et al, 1974). In the present system large numbers of AFC of the IgA class appear in the spleen (Figs. 4.1,2), more like the response seen with an immunization with sheep erythrocytes (Robertson and Cooper, 1973).

It is possible that the IgA plasma cells migrated to the spleen from the lamina propria of the intestine (Crabbe et al, 1969). However no IgA AFC were detected in the lamina propria up to day 14 (Fig. 5.3), and so there would have to have been complete migration of large numbers of cells for the explanation to be valid.

An experiment was designed to determine if the large IgA response was a characteristic of LAC mice. The mice were immunized with the O8 antigen of E. coli, given intravenously and in combination with oral

doses. In no immunization schedule with this antigen was the ratio of IgA:IgM AFC in the spleen greater than 1:40. This result suggested that it was a combination of factors concerning the antigen and the type of mouse which determined the high IgA response to the V. cholerae LPS. It is difficult to extrapolate a result from one mouse strain to the next, much less to humans, as this point illustrates.

On day 15 there were substantial numbers ($\approx 10^3/10^7$ viable lymphoid cells) of plasma cells forming IgG2A antibody in the spleen. The result was obtained using a commercial goat anti-mouse γ 2A antiserum, which was not employed routinely on economic grounds.

4.2d Sequence of antibody class formation : IgA plasma cells increased to a maximum in the spleen days after the IgM maximum (Figs. 4.1,2), and the delay was probably due to the time taken to switch from IgM to IgA production (Lamm, 1976). This developmental sequence has been reported frequently, an example being the immunization of mice and rats with sheep red cells (Sell, Park and Nordin, 1970; Robertson and Cooper, 1973). Once the priming for IgA production has been completed, IgA and IgM are produced synchronously late in the primary response (Figs. 4.1,2) and in the secondary response (Figs. 5.1,2; Sell et al, 1970).

4.2e Decline of the response : The response of mice to an immunization with V. cholerae fell rapidly once it had reached its maximum (Figs. 4.1,2). Small numbers of AFC were in the spleen as late as 49 days after immunization, which is about the time that LPS can no longer be found in a biologically active form in vivo (Britton, Wepsic and Moller, 1968). However, the decline was rapid and virtually exponential, whereas the response to E. coli declines in a slower fashion (Britton and Moller, 1968). This implied that an active process was terminating the response to V. cholerae. It has been proposed that most immune responses are terminated by suppressor T-cells (Thomas, Roberts and Talmage, 1975), which may account for the findings in the system described here.

4.2f Cyclic variations in the response : Superimposed upon the declining response to V. cholerae were significant ($p < 0.01$) fluctuations in the numbers of antibody forming cells in the spleen. The result was more defined after vaccination with the 569B IMP SR strain (Fig. 4.1). Here the response came to an end with the two cycles on days 28-34 and 34-44, with the synchronous appearance ($p < 0.01$) of IgM and IgA plasma cells in the spleen. The IgA response to 111 NM SR also ended in the formation of two cycles, but the IgM fluctuations were not significant statistically (Fig. 4.2). The results suggested that

the response was brought to an end by a feedback mechanism. It was interesting that the decline in the response to 569B IMP SR was heralded by significant fluctuations ($p < 0.01$) in the IgM class between days 18-26. This perturbation may have been a sign of the sudden imposition of a regulatory process on the system.

As outbred mice were used it was surprising that a cyclic phenomenon was observed, especially six weeks after immunization, but it was reproducible. However, the cycles were not similar to those described following immunization of mice with E. coli (Britton and Moller, 1968), rabbits with heterologous serum proteins (Romball and Weigle, 1973), and marine toads with polymerized flagellin (Azzolina, 1976). The immune response cycles in these systems were attributed to feedback suppression by antibody, and were approximately equal in amplitude and duration unlike the ones in this study. The more rapid reduction in the anti-V. cholerae response suggested the action of a more active process than antibody suppression, such as suppression by T-cells. T-helper and T-suppressor cells are generated together (Thomas, Roberts and Talmage, 1975), and it is perhaps not coincidental that the response declined soon after the rise of IgA AFC which are known to develop with T-help (Jacobsen, Caporale and Thorbecke, 1974; Mond, Caporale and Thorbecke, 1974).



4.2g Synchronous antibody cycles : In Fig. 4.1 it can be seen that IgM and IgA are produced in synchronous cycles late in the primary response to V. cholerae 569B IMP SR. There is obviously an active process which brings production of the two antibody classes into phase. Romball and Weigle (1973) reported the production of synchronous IgM and IgG cycles in the immune response of rabbits to heterologous serum proteins, and attributed it to antibody feedback suppression. As the antibody classes have different half-lives in the serum (Fahey and Sell, 1965) the suppression could not be due to a simple masking of antigenic determinants as proposed by Britton and Moller, 1968. It could be due to a special property of one of the antibody classes, but there is no evidence of this. In addition, mice can respond to sheep erythrocytes with synchronous production of IgA and IgG1,2A,2B, but without the formation of IgM cycles (Sell, Park and Nordin, 1970). Such data is not consistent with an antibody feedback mechanism, but could well be explained by the selective action of suppressor T-cells.

4.3 Suppression of the Immune Response to Vibrio cholerae

4.3a Passive administration of purified antibody fractions : The cyclic variation in the immune response of mice to E. coli is apparently due to feedback antibody suppression (Britton and Moller, 1968), and a similar

mechanism might also account for the fluctuations in the response to V. cholerae. Britton and Moller were able to profoundly suppress the numbers of E. coli-specific PFC in the spleens of mice by giving the animals a passive immunization with specific antibody. In a similar experiment, equivalent amounts of antibody were given intravenously to mice during the immune response to V. cholerae.

Highly immune serum and intestinal juice were raised in mice against V. cholerae 569B IMP SR, and antibody in immunoglobulin (Ig) class fractions was isolated from them (see Materials and Methods). Relatively pure Ig fractions were sought as the antibody classes differ in their ability to suppress immune responses (Henry and Jerne, 1968; Murgita and Vas, 1972). Ig class cross-contamination was not detected (< 5%) in the IgG2A, IgG2B and IgM fractions, using the Mancini technique. The IgG1 fraction contained 8% IgG2A, and the IgA was contaminated with 7% IgM.

It was necessary to make quantitative comparisons of the anti-V. cholerae antibody content of the Ig fractions. Such comparisons are difficult because the antibody classes vary greatly in their efficiency of haemagglutination, complement fixation and opsonization (Steele, Chaicumpa and Rowley, 1974). Steele and co-workers found that all classes of antibody were equally protective by weight against lethal cholera

FIGURE 4.3SUPPRESSION OF THE IMMUNE RESPONSE TO V. CHOLERAE569B IMP SR BY THE ADMINISTRATION OF IMMUNESERA : IgM PFC IN THE SPLEEN/10⁷ VIABLELYMPHOID CELLS

Mice were given 10^7 organisms i.v. on day 0. Immunoglobulin fractions were administered i.v. on day 0 (50 PD_{50} units of antibody), day 6 (50 PD_{50} units) and day 12 (250 PD_{50} units).

A	IgG1
B	IgG2A
C	IgG2B

Each day three mice were selected at random from every group, and the results were expressed as a geometric mean.

..... Mice immunized with 569B IMP SR

— Mice immunized with 569B IMP SR
and given immune sera

Figure 4.3

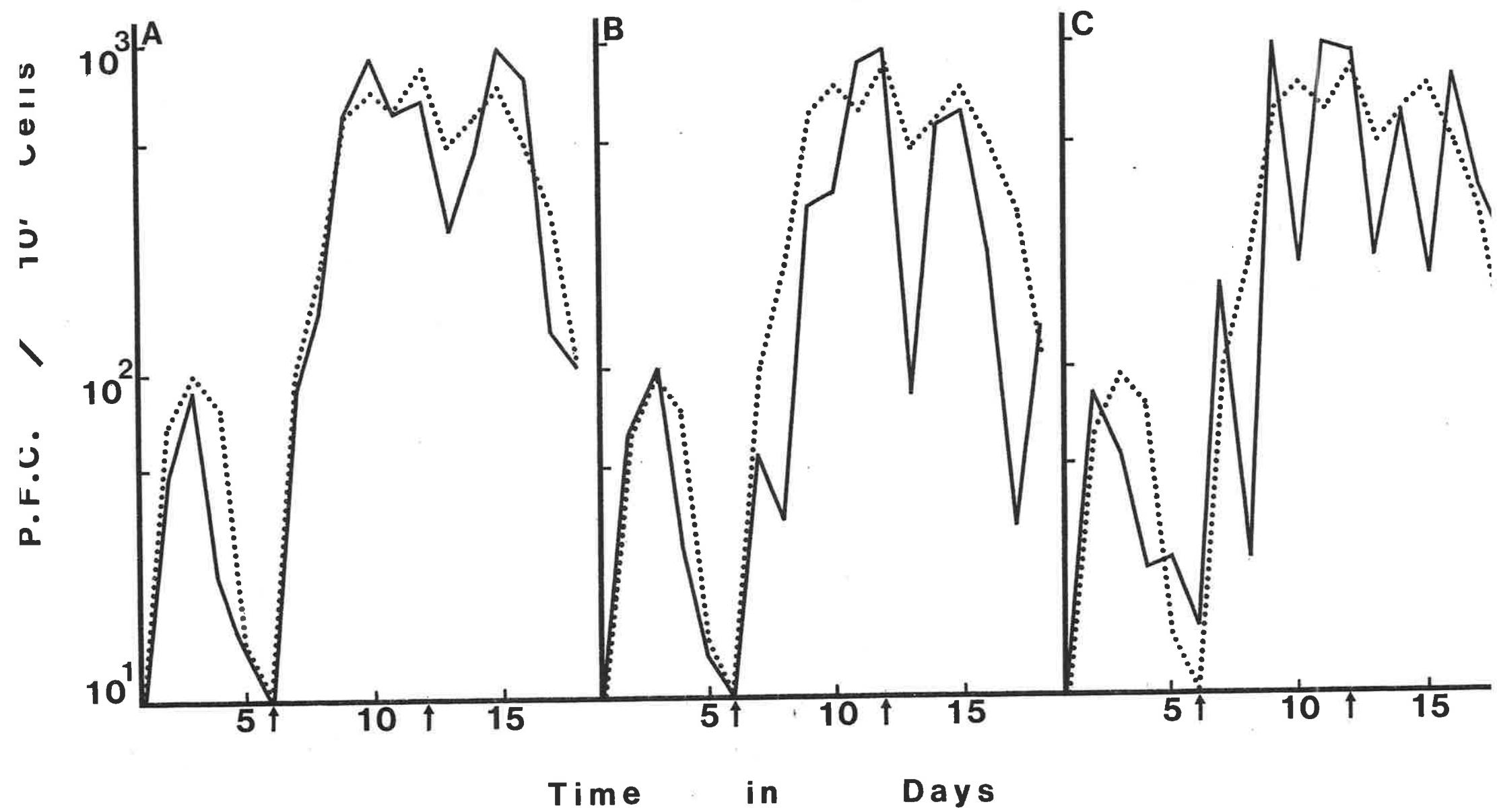


FIGURE 4.4SUPPRESSION OF THE IMMUNE RESPONSE TO V. CHOLERAE 569BIMP SR BY THE ADMINISTRATION OF IMMUNE SERA : IGMPFC IN THE SPLEEN/10⁷ VIABLE LYMPHOID CELLS

Mice were given 10^7 organisms i.v. on day 0.

A. IgA administered i.v. on day 0 (50 PD_{50} units of antibody), day 6 (50 PD_{50} units) and day 12 (250 PD_{50} units).

B. IgM administered i.v. on day 0 (50 PD_{50} units of antibody), day 6 (50 PD_{50} units) and day 12 (250 PD_{50} units).

C. Serum pooled from mice late in the response to v. cholerae 569B when AFC numbers in the spleen were at a minimum (days 27, 28, 34, 44, 45). The serum was freed of particles by ultracentrifugation, and 0.2ml diluted 1:3 was given i.v. on day 12 (0 PD_{50} units).

..... Mice immunized with 569B IMP SR

— Mice immunized with 569B IMP SR and given immunoglobulin fractions

----- Mice immunized with 569B IMP SR and given pooled serum

Figure 4.4

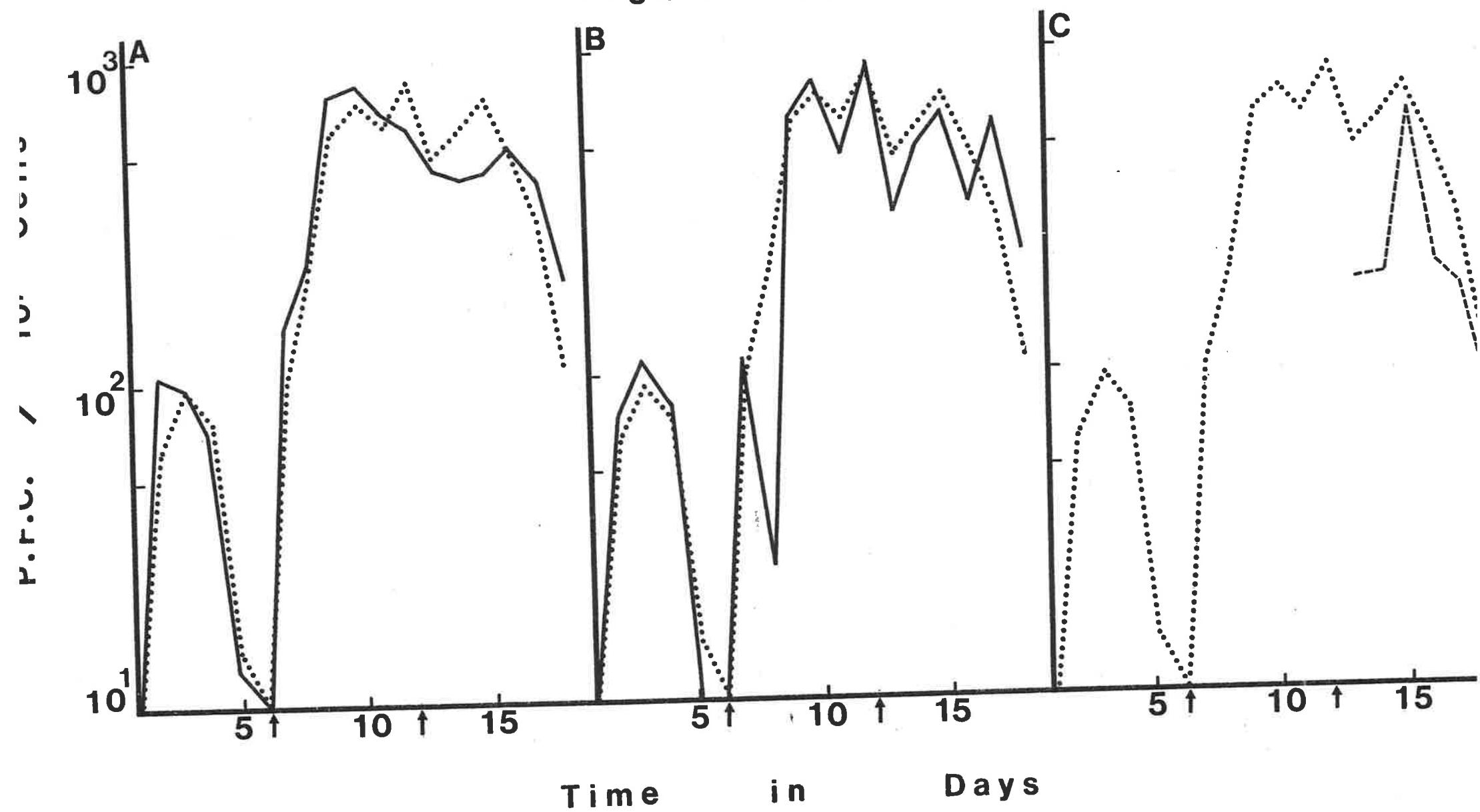


FIGURE 4.5SUPPRESSION OF THE IMMUNE RESPONSE TO V. CHOLERAE 569BIMP SR BY THE ADMINISTRATION OF IMMUNE SERA : IgMPFC IN THE SPLEEN/ 10^7 VIABLE LYMPHOID CELLS

Mice were given 10^7 organisms i.v. on day 0.

A. Control.

— Serum pooled from mice early in the response to V. cholerae 569B IMP SR (days 3,4). 0.2ml given i.v. on days 0 and 6 (0 PD_{50} units of antibody).

---- Serum pooled from mice late in the response to V. cholerae 569B IMP SR at the peak of a cyclic fluctuation (days 36-42). 0.2ml of serum diluted 1:3 was given i.v. on day 12 (0 PD_{50} units).

B. Control.

— Serum pooled from mice at the height of the response to V. cholerae 569B IMP SR (days 8-12). 50 PD_{50} units of antibody given on days 0 and 6.

---- Serum from mice late in the response (days 36-42) freed of particles by ultracentrifugation. 0.2ml diluted 1:3 was given i.v. on day 12 (0 PD_{50} units).

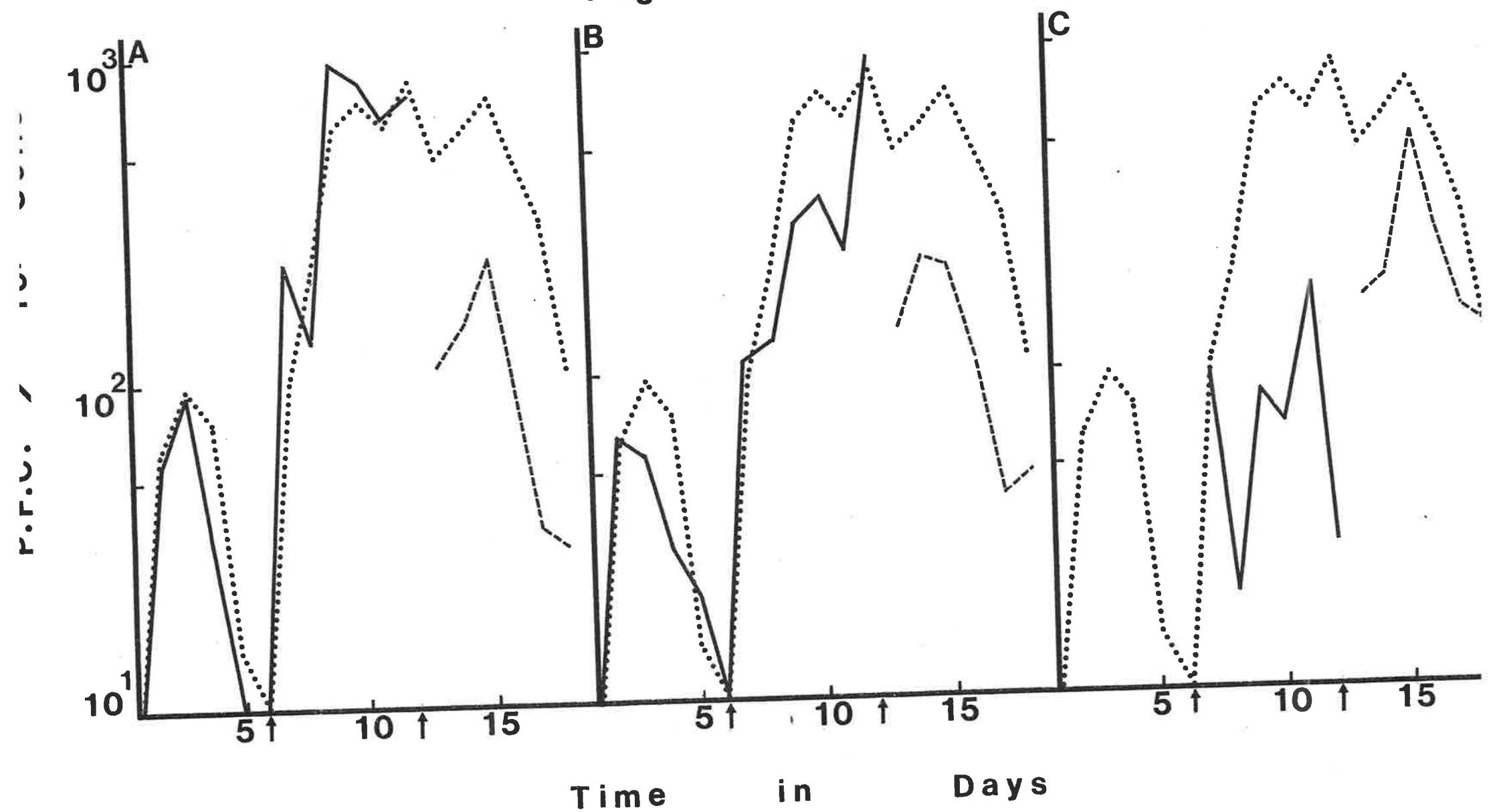
C. Control.

— Serum pooled from mice late in the response to V. cholerae 569 IMP SR when AFC numbers in the spleen were at a minimum (days 27,28,34, 44,45).

0.2ml given i.v. on day 6 (0 PD_{50} units).

---- 0.2ml serum diluted 1:3 given i.v. on day 12.

Figure 4.5



infection in baby mice, thus enabling quantitative comparisons.

The Ig fractions were assayed in the baby mouse protection test and aliquots were standardized to contain equivalent numbers of PD₅₀ units (i.e. equivalent weights of specific antibody). The mice were immunized i.v. on day 0 with 10⁷ V. cholerae 569B IMP SR; 50 PD₅₀ units of Ig were also given with the immunization, except for the control group. Passive immunization was repeated on day 6 (50 PD₅₀ units), and at the height of the response on day 12 (250 PD₅₀ units).

Passive administration of specific antibodies in the Ig classes G₁, G_{2A}, G_{2B}, A and M made remarkably little difference to the numbers of IgM antibody forming cells which were assayed in the spleen, when compared with controls immunized with V. cholerae alone (Figs. 4.3A-C, 4.4A,B). In none of the six day periods following the three passive immunizations was there a significant depression in the numbers of IgM AFC ($p > 0.02$). There were significant depressions on individual days induced by : IgG_{2A} on day 4 ($p < 0.02$) and day 17 ($p < 0.01$); IgG_{2B} and IgM on day 8 ($p < 0.02$). It could be concluded that the response to V. cholerae in these mice was not readily suppressed by specific antibody, despite the system being similar to the one used by Britton and Moller (1968). They could suppress the immune response of mice to E. coli by giving 0.2ml doses of an immune

serum diluted 1:5. It should be noted that 12 days after the mice were immunized with V. cholerae the levels of antibody in the serum rose to a maximum of 1300 PD₅₀ units/ml, i.e. 50 units in 0.2ml of serum diluted 1:5.

4.3b Passive administration of immune sera : The immune response of mice to V. cholerae was not readily suppressed by large i.v. doses of specific antibody. Therefore it was unlikely that the cyclic variations in the response seen in Fig. 4.1 were due to antibody feedback suppression. Serum taken from mice at various times after vaccination with V. cholerae was then examined for the presence of a non-antibody suppressive factor. This serum was from mice whose response to V. cholerae 569B IMP SR can be seen in Fig. 4.1, and it was pooled in four portions corresponding to the following response phases : early (days 3,4); maximum (days 8-12); late peak (days 36-42); late trough (days, 27,28,34,44,45). The mice were immunized with 10⁷ V. cholerae 569B IMP SR on day 0, and were given 0.2ml of immune serum i.v. on days 0, 6 or 12. The response of the mice treated with serum was compared to the response of controls immunized with V. cholerae alone.

Mice were given a 0.2ml dose of 'late trough' serum on day 6. There was a significant ($p < 0.01$) suppression of the numbers of IgM PFC in the spleen during the six day period observed (Fig. 4.5C). The

effect was not related to specific antibody, as there were fewer than 2 PD₅₀ units per dose. It was also in contrast to the lack of effect that was found when early and maximum response phase sera were used (Figs. 4.5A,B). The 'early' serum was concentrated to half its original volume by dehydration in dialysis tubing coated with polyethylene-glycol, but was not protective in the baby mouse test. The 'maximum' serum was diluted 1:5 so that each 0.2 ml dose delivered 50 PD₅₀ units of antibody. The sera were given on days 0 and 6 but they caused no suppression of statistical significance.

It could be concluded that the declining phase of the response of mice to V. cholerae was associated with a suppressive factor in the serum. This factor did not appear early in the response or at its height, and it was apparently unrelated to specific antibody.

4.3c Suppression associated with antibody cycles : There was a factor in the serum which could explain the rapid termination of the response to V. cholerae, and it seemed possible that it could also account for the cyclic variations which were observed. The possibility was tested by comparing the suppressive effects of two pools of late response sera : one taken at the time of the peak of a cycle and the other at the time of the troughs inbetween the peaks. The sera were given i.v. on day 12, in 0.2ml doses

diluted 1:3.

The 'late peak' serum depressed the numbers of IgM AFC assayed in the spleen ($p < 0.01$) during the entire six day period under examination (Fig. 4.5A). The 'late trough' serum was less effective ($p < 0.01$), and only induced a depression ($p < 0.01$) for the first two days (Fig. 4.5C). The data are consistent with the theory that the appearance of antibody forming cells in cycles in the spleen reflects feedback suppression by serum factors. The result stressed once again that specific antibody was not the suppressive factor as each dose of these sera contained less than 1 PD₅₀ unit of antibody.

The depression of the response induced between days 12 and 18 with late response serum (Fig. 4.5A) enabled the effect on IgA plasma cells to be examined. The numbers of IgA AFC in the spleens of the immunized controls reached $10^2/10^7$ viable lymphoid cells on day 14, as expected from the data shown in Fig. 4.1. The 'late peak' serum given to mice on day 12 significantly ($p < 0.01$) reduced the IgA AFC count by an average of 83% on days 14-18 (IgM data only shown in Fig. 4.5A). Therefore the formation of IgA cycles could also be explained in terms of a suppressive factor in the serum.

4.4

Nature of the Suppressive Factor in the Serum

4.4a Particles in the serum : The suppressive factor in the serum could have been particulate, like antigen-antibody complexes. Immune complexes are known to repress immunocyte function in vitro (Askonas, McMichael and Roux, 1976; Hoffman, Kappler, Hirst and Oettgen, 1974) and in vivo (Andre, Heremans, Vaerman and Cambiaso, 1975). Another possibility was lipopolysaccharide, which persists in a biologically active form for long periods of time in the body (Britton, Wepsic and Moller, 1968). LPS activates C3 in vivo (Lachmann and Nichol, 1973), and C3 depletion has been implicated in the inhibition of immune responses (Pepys, 1974) and even in the formation of a cyclic response (Nielsen and White, 1974).

To test these possibilities the suppressive sera were ultracentrifuged at 106,000g for 60 minutes, and the supernatants were tested for any loss of their ability to depress the immune response to v. cholerae. Particles were brought down out of 1ml of serum that completely consumed 64 CH₅₀ units of complement in a consumption assay. However, the supernatants suppressed the response (Figs. 4.4C, 4.5B) just as well ($p > 0.02$) as the whole sera (Figs. 4.5A,C). Therefore the suppressive factor was probably soluble. It was also more than 5,000 Daltons as it was not lost with dialysis to

remove azide from the serum.

4.4b Molecular weight of the factor : An attempt was made to determine the size of the suppressive factor. The serum was passed through a '50,000 Amicon' filter, and the filtrate (MW < 50,000) was compared with the residue (MW > 50,000). The filtrate retained its suppressive activity, whereas the residue was inactive ($p < 0.01$). A number of suppressive factors of around 50,000 Daltons have been described and they seem to be the products of suppressor T-cells (Section 1.6e).

4.4c Antigen recognition by the factor : Suppressive factors have been described which are able to recognize antigen either through immunoglobulin-like determinants (Feldman, 1974) or with a product of the I region of the H2 complex (Taniguchi *et al*, 1976; Herzenberg *et al*, 1976). However, it is not necessarily possible to absorb these factors out of a serum by the use of specific antigen, as they may already be in the form of complexes with antigen (Feldman, 1974). None the less the serum was three times absorbed with 10^{10} V. cholerae per ml and then 'millipore' filtered. Within two days of giving the immunized mice absorbed serum i.v., the numbers of IgM AFC in the spleen increased five fold, which would suggest that LPS had contaminated the absorbed serum. No conclusions could be drawn as to the capacity of the suppressive factor for recognizing

antigen.

4.4d Specificity of the suppression : Suppressive T-cell factors can either act specifically (Herzenberg *et al*, 1976) or non-specifically (Tadakuma and Pierce, 1976). The late response serum was tested to make this important distinction. Mice were immunized with 2×10^9 sheep red cells i.v. on day 0. On day 3 a test group was given 0.2ml of serum i.v. Three mice were taken from both the test and the control groups on days 4,5 and 6, and their spleens were assayed for anti-SRBC antibody forming cells. The average count of IgM AFC per 10^7 cells in the controls was 2,960 which was virtually the same as the average of 2,843 for the group treated with serum. Therefore the suppressive factor in the serum was specific in its effect on the response to V. cholerae.

4.5 Summary

Mice were immunized with V. cholerae and the kinetics of the response were studied by Jerne assay of antibody forming cells in the spleen. The count of IgM AFC took 12 days to reach a maximum, but this relatively slow development may be characteristic for the response to V. cholerae (Kateley *et al*, 1974). There was a temporary depression in the IgM count on days 6 and 7 which remained unexplained. It was similar to a suppressive effect noted to occur a few days after a booster vaccination with the same organism (Chapters 5 and 6).

The IgM peak was followed by an IgA peak of equal magnitude. The delayed formation of an IgA response is a routine observation and is apparently due to a switch from IgM to IgA synthesis (Lamm, 1976). However, Gram negative organisms do not usually evoke a systemic IgA response (Kateley *et al*, 1974). The result seemed to be a peculiarity of this immunization system because the highest ratio of IgA : IgM AFC in the spleen found for the response to the O8 antigen of E. coli was 1:40.

The rise of the IgA response was soon followed by a rapid, fluctuating decline in the numbers of spleen AFC. This decline was associated with the appearance of a suppressive factor in the serum which also varied with the AFC fluctuations. The serum factor was not specific antibody and was not particulate. It had a molecular weight between 5,000 and 50,000 Daltons, and its suppressive effect was specific for the response to V. cholerae. It is proposed that it was a specific suppressor T-cell factor. Such factors, specific and non-specific, may participate in the termination of most immune responses (Thomas, Roberts and Talmage, 1975).

CHAPTER 5

CHAPTER 5PRIMING MICE FOR AN IMMUNE RESPONSETO *VIBRIO CHOLERAE*5.1 Introduction

To a large extent cholera immunization has been a trial and error process, but now some of the underlying principles have emerged. Central to this understanding is the fact that cholera is an infection confined to the intestine (Carpenter, 1972), and that it is the concentration of local antibody that determines resistance to the disease (Freter, De, Mondal, Shrivastava and Sunderman, 1965). It is now known that cholera immunization must stimulate the formation of local antibodies, for although systemic antibodies may enter the intestine (Wernet, Breu, Knop and Rowley, 1971; Brantzaeg, 1973; Pierce and Reynolds, 1974) their importance in resistance to the human infection is doubtful (Cash, Music, Libonati, Craig, Pierce and Hornick, 1974a).

Parenteral vaccines have been the mainstay of cholera vaccine field trials, and their use has met with some limited success (Benenson, Mosley, Fahimuddin and Oseasohn, 1968). It is reasonably clear that the parenteral vaccinations have boosted the levels of pre-existing local immunity (Benenson *et al.*, 1968; Svennerholm, Holmgren, Hanson, Lindblad, Quereshi and Rahimtoola, 1977). Therefore future improvements in cholera immunization may involve increasing the degree of gastro-intestinal priming.

It is a common observation that repeated local

application of antigen is often required to evoke an immune response in the intestine (Robertson and Cebra, 1976). Volunteer studies with Vibrio cholerae concur with this observation, for it takes a protracted course with a killed oral vaccine to stimulate a local response (Freter, 1962; Freter and Gangarosa, 1963). Similarly, live oral vaccines have been successful, but only in cases where they have actually caused a cholera infection (Cash *et al*, 1974a). Therefore it is hard to see any advantage in this approach, as people living in cholera areas are already primed at regular intervals with oral challenges of V. cholerae.

A different approach holds some promise, and consists of a parenteral immunization to prime for a response to a local boost (Ogra, Wallace, Umana, Ogra, Grant and Morag, 1974). This schedule has been used to stimulate significant local responses in rats to cholera toxoid (Pierce and Gowans, 1975). Whether the technique will find general application remains to be seen, for there are signs that cholera toxoid may be a special situation. This is shown by the requirement that the toxoid must contain the membrane binding B-subunit for it to be an effective local immunogen (Pierce, 1978).

In this chapter a number of immunization schedules using live V. cholerae vaccines are investigated in mice. The work involved the use of two antibody assays in conjunction, the Jerne technique to enumerate the numbers of plaque forming cells (PFC) in the spleen and the mucosa of the small intestine, and the HA assay on the serum and the intestinal juice. An advantage in using these two assays

was their comparability, as they both used LPS coated SRBC to bind antibody. Also, it is known that the majority of the antibodies produced in a response to V. cholerae are specific for its LPS determinants (Neoh and Rowley, 1970). The PFC assay was a sensitive means of measuring the development of an immune response, while the HA assay gave a rapid determination of the actual antibody levels achieved.

Another objective of work detailed in this chapter was to determine if the persistence and multiplication of a V. cholerae strain in the small intestine influenced the immune response. While the answer may seem obvious, the main evidence concerned is circumstantial. For instance, the failure of oral vaccines of attenuated V. cholerae to stimulate local immunity has been associated with the failure of the organisms to establish and multiply in the small intestine (Cash *et al*, 1974b). However, it is not known if these strains were capable of stimulating an immune response when given parenterally either.

This question was examined by testing two strains of V. cholerae in parallel in the immunization studies. The two strains were 569B IMP SR and 111 NM SR, which were noted in Chapter 3 to differ in their capacity to adhere to, and multiply on, the mucosa of the small intestine.

5.2 A Single Parenteral Immunization

Mice were given 10^7 V. cholerae in a single i.v. dose, which stimulated the appearance of antibody forming cells (AFC) in the spleen (Figs. 5.1,2) but few in the mucosa of the small intestine (Fig. 5.3). The haemagglutination assay

FIGURE 5.1

TWO INTRAVENOUS IMMUNIZATIONS WITH *V. CHOLERAE* 569B IMP SR
SPACED 14 DAYS APART : SPLEEN PFC/ 10^7 VIABLE LYMPHOID CELLS

Mice were given 10^7 vibrios i.v. on days 0 and 14 (indicated by †). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM PFC
..... IgA PFC

Figure 5.1

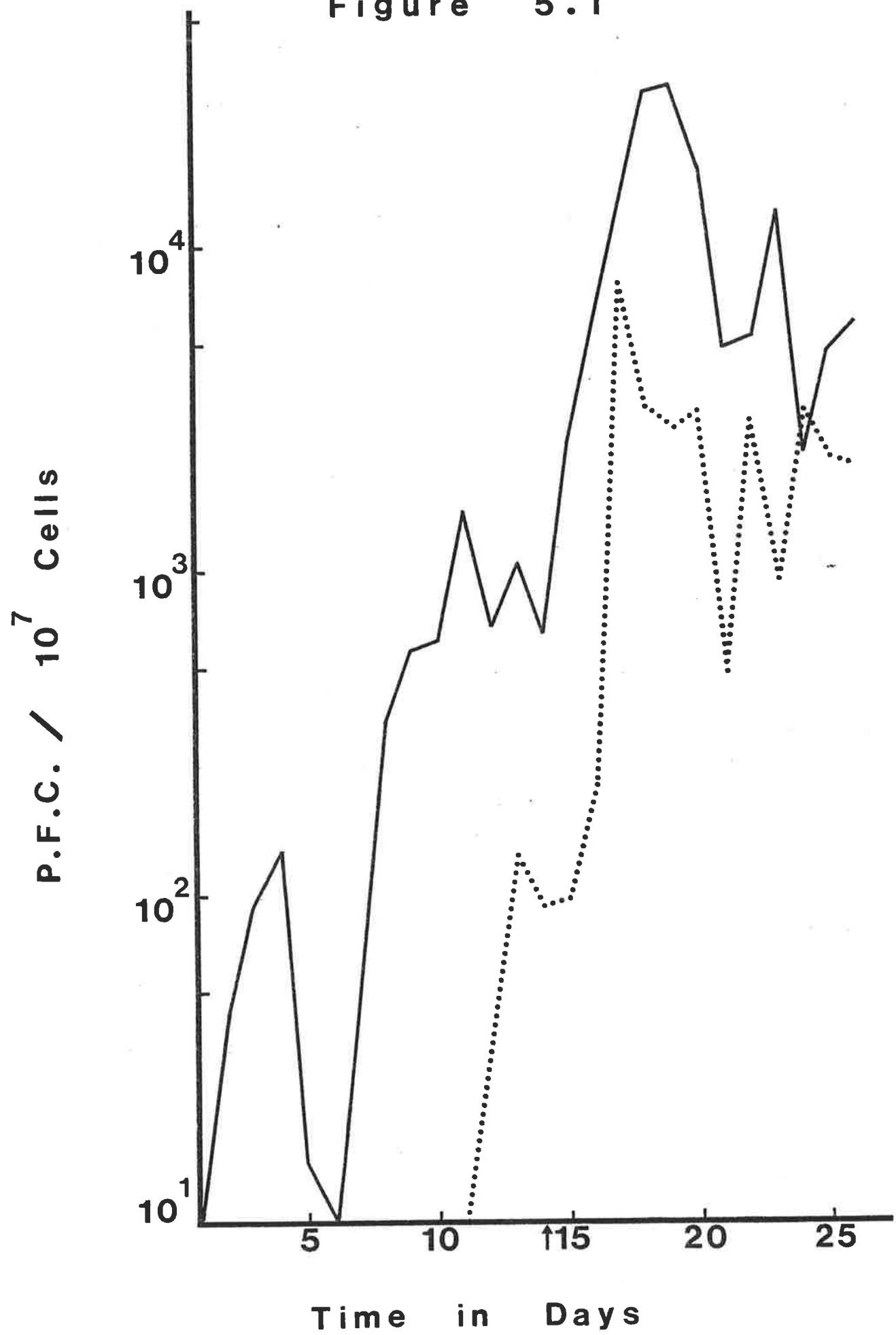


FIGURE 5.2

TWO INTRAVENOUS IMMUNIZATIONS WITH V. CHOLERAE 111 NM SR
SPACED 14 DAYS APART : SPLEEN PFC/10⁷ VIABLE LYMPHOID CELLS

Mice were given 10^7 vibrios i.v. on days 0 and 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM PFC

..... IgA PFC

Figure 5.2

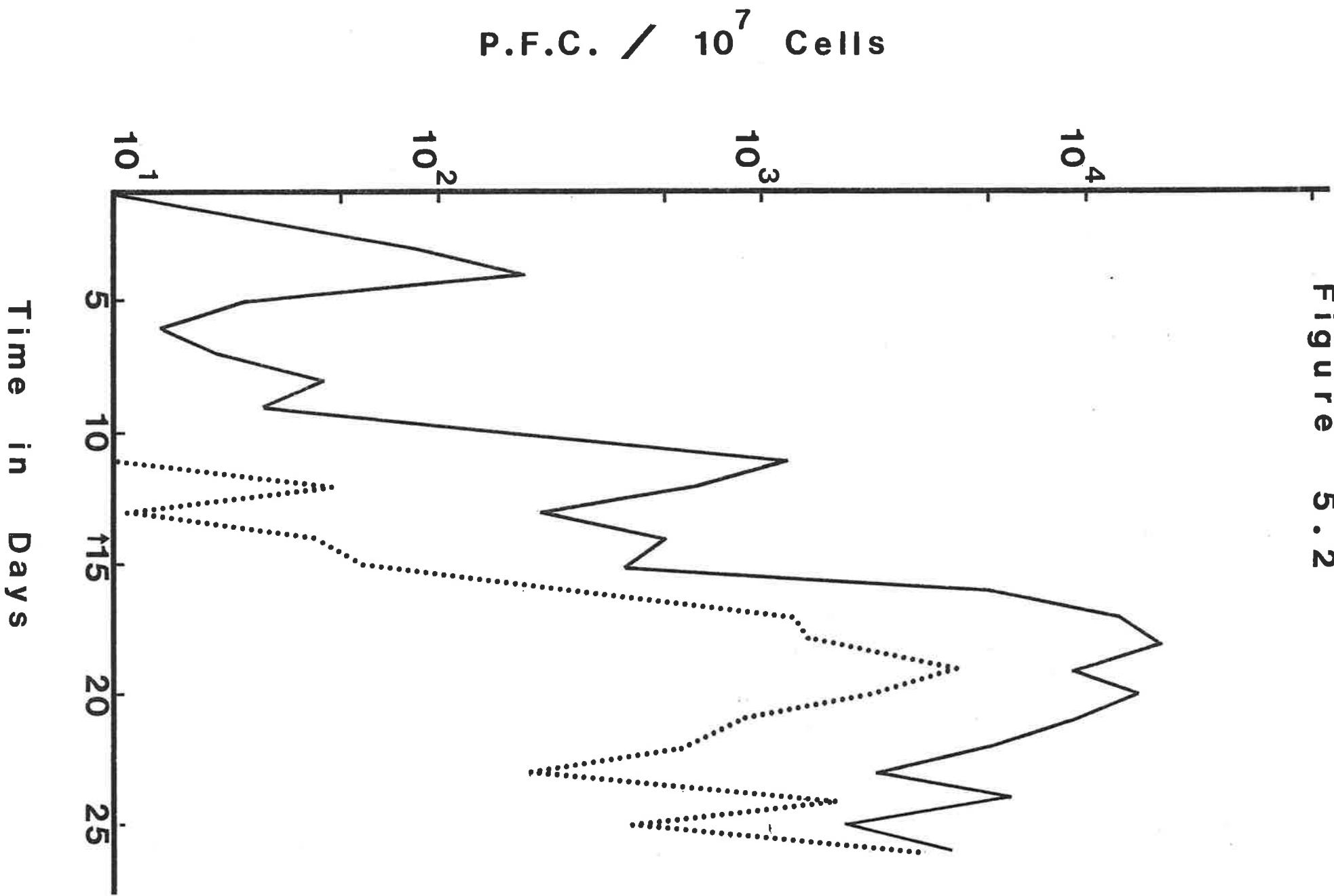


FIGURE 5.3

TWO INTRAVENOUS IMMUNIZATIONS WITH V. CHOLERAE SPACED

14 DAYS APART : PFC IN THE MUCOSA OF THE
INTESTINE/10⁷ VIABLE LYMPHOID CELLS

A. 111 NM SR

B. 569B IMP SR

Mice were given 10^7 vibrios i.v. on days 0 and 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM PFC

..... IgA PFC

Figure 5.3

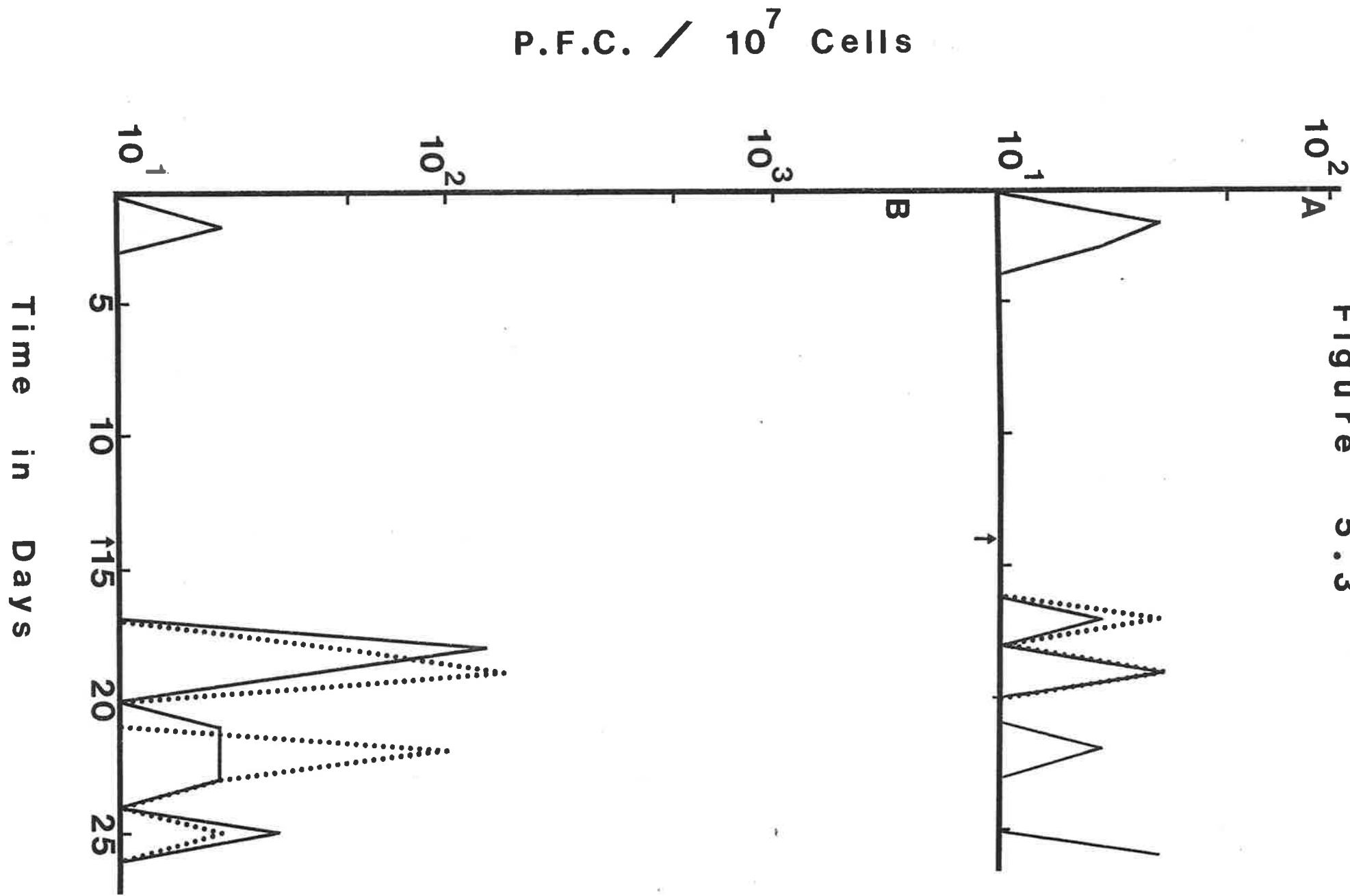


FIGURE 5.4

TWO INTRAVENOUS IMMUNIZATIONS WITH *V. CHOLERAE* 569B IMP SR
SPACED 14 DAYS APART : HAEMAGGLUTINATING UNITS OF
ANTIBODY PER MOUSE

Mice were given 10^7 vibrios i.v. on days 0 and 14 (indicated by †). Three mice were sampled at random every day. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

— Serum
 Intestinal juice (no antibody detected by direct HA in this fluid for this experiment).

Coombs enhancement of the direct HA titre, expressed in additional numbers of two-fold dilutions.

	IgM	IgA	IgG2A
Serum	3	2	2
Intestinal juice	0	2	0

Figure 5.4

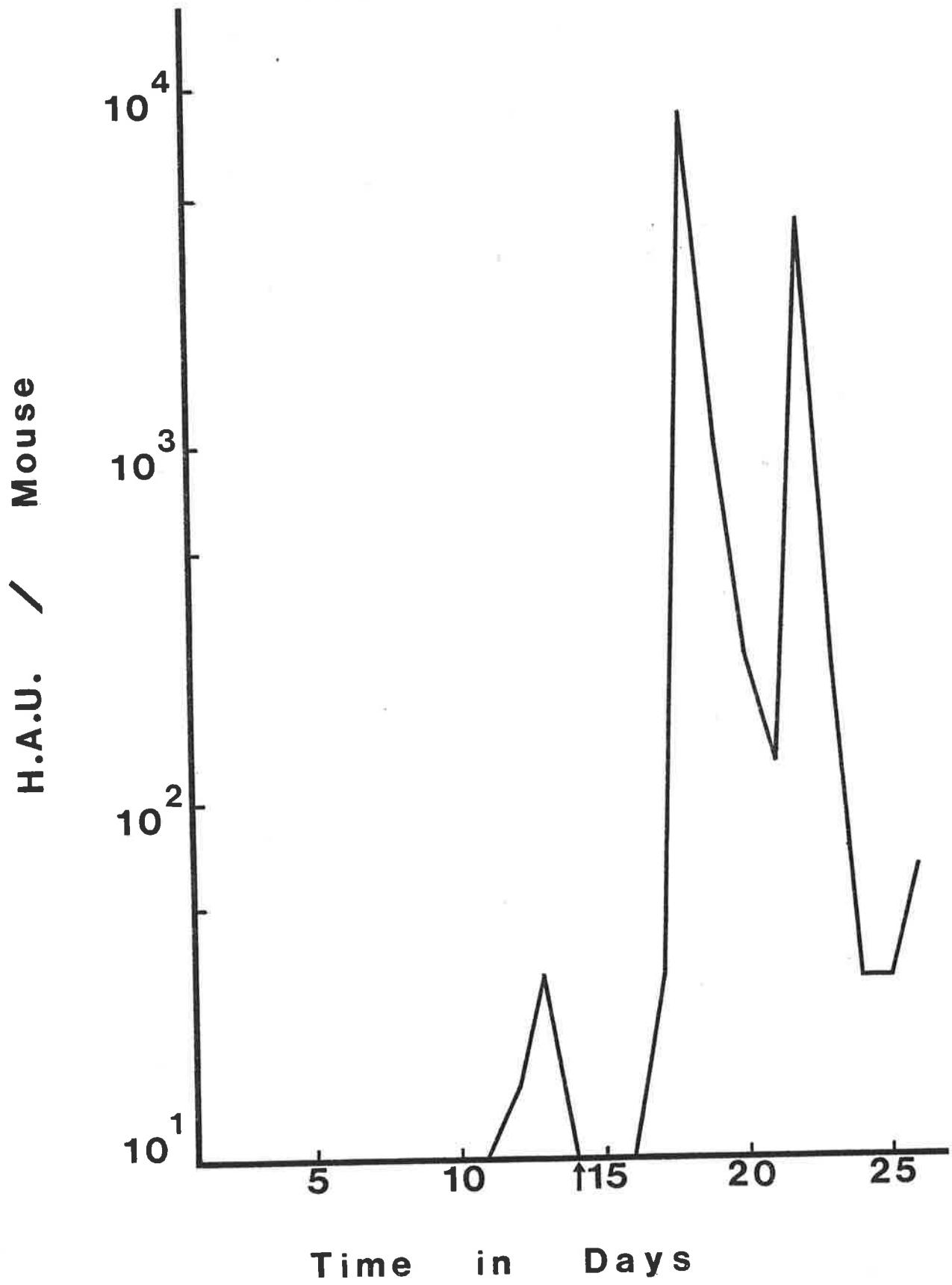


FIGURE 5.5TWO INTRAVENOUS IMMUNIZATIONS WITH V. CHOLERAE 111 NM SRSPACED 14 DAYS APART : HAEMAGGLUTINATING UNITS OFANTIBODY PER MOUSE

Mice were given 10^7 vibrios i.v. on days 0 and 14 (indicated by ↑). Three mice were sampled at random every day. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

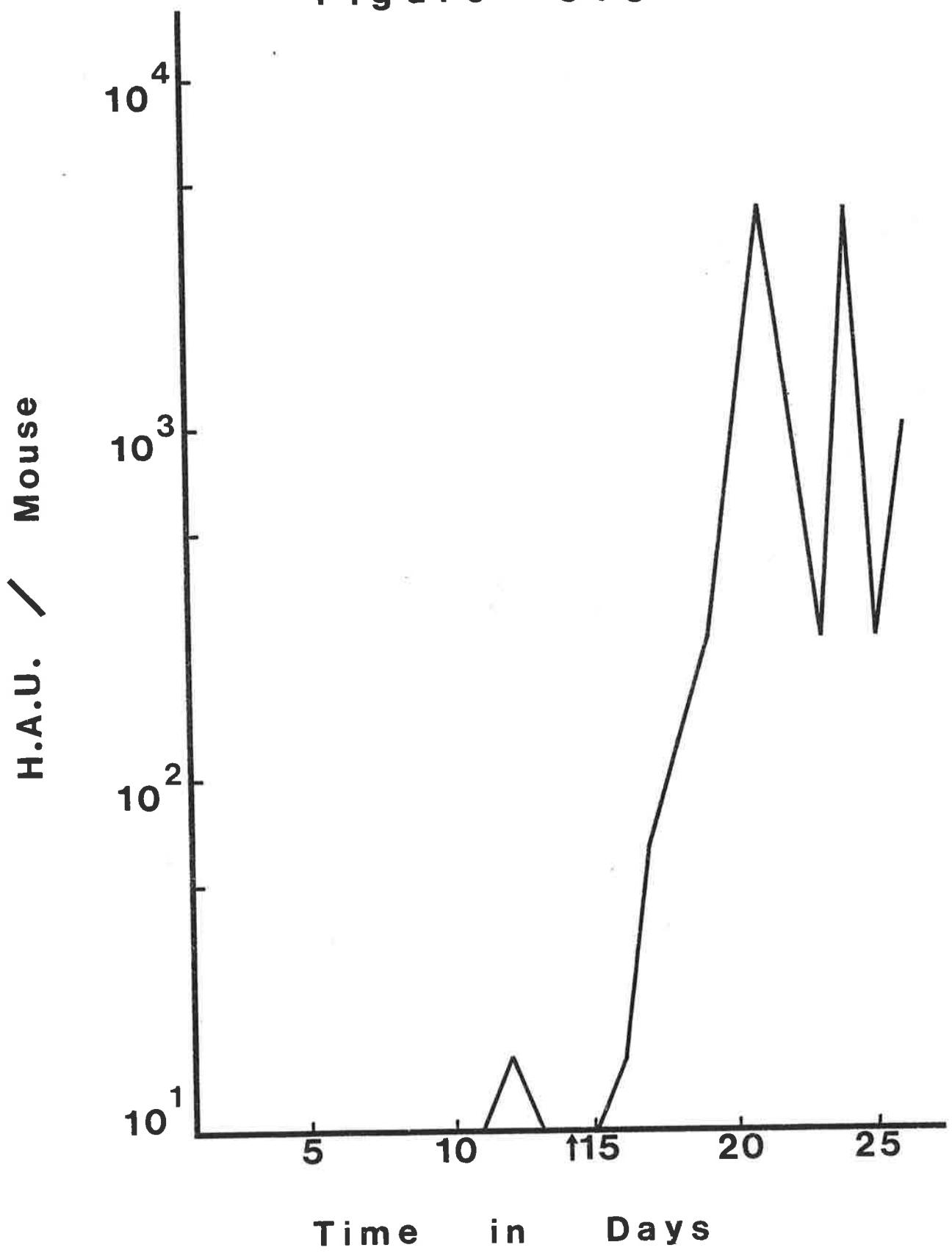
— Serum

..... Intestinal juice (no antibody detected by direct HA in this fluid for this experiment).

Coombs enhancement of the direct HA titre, expressed in additional numbers of two-fold dilutions.

	IgM	IgA	IgG2A
Serum	4	3	2
Intestinal juice	0	2	0

F i g u r e 5 . 5



for antibody was less sensitive : it barely detected antibody in the serum (Figs. 5.4,5) and showed none in the intestinal juice (Figs. 5.4,5).

5.3 A Parenteral Priming and Boosting

The experiment in Section 5.2 was extended by giving the mice a booster dose of 10^7 V. cholerae i.v. This followed the primary immunization by 14 days, a time known to allow IgM AFC numbers to reach a maximum in the spleen (Fig. 4.1,2). The booster stimulated a large increase in AFC numbers in the spleen, which reached levels ($> 10^4/10^7$ viable cells) near the maximum that could be obtained with any immunization schedule (Figs. 5.1,2).

High haemagglutination titres were achieved in the serum, a reflection of the large spleen response. Coombs enhancement of the direct HA assay revealed more IgM than IgA antibody, also in agreement with the data from the Jerne assay (Figs. 5.4,5). However, the antibody peak was split in two by a deep depression which was not evident in the numbers of antibody forming cells. One could only conclude that antibody was still being produced at a high rate even though the titre of HA antibody in the serum was temporarily reduced.

This phenomenon was not easily explained. It was reproducible and could last for more than two consecutive days, as shown in Fig. 5.4. It will be seen again in other experiments, and often occurred within six days of an i.v. boost with V. cholerae. One explanation could be the binding of antibody by a sudden release of LPS, possibly from macro-

phages which have a very short life in the mouse (Gorczynski, Miller and Phillips, 1971). However the binding of 10^4 units of antibody would require the LPS of 10^{11} organisms (Steele, Chaicumpa and Rowley, 1974), and this makes the explanation unlikely. The effect might also be due to the toxic properties of LPS (Chedid and Audibert, 1977; Rosenstreich, Glode and Mergenhagen, 1977) but how this would be mediated is speculative.

Contrasting with the strong stimulation of the spleen, PFC numbers remained at a low level in the intestine (Fig. 5.3) and antibodies were not found in the intestinal juice by haemagglutination (Figs. 5.4,5).

The immune responses to the two V. cholerae strains were virtually identical (Figs. 5.1-5), which confirmed that they were equally immunogenic despite any other difference.

5.4 Delayed Parenteral Boosting

After an i.v. immunization of mice with V. cholerae, the IgA PFC peak in the spleen arrives some days after the IgM PFC peak (Figs. 4.1,2). Therefore the timing of an i.v. boost may well affect the relative proportions of the IgA and the IgM responses. This possibility was tested by delaying the i.v. boost with 10^7 organisms until 49 days after the primary dose. At this time both IgA and IgM AFC numbers had risen to a maximum and had declined (Figs. 4.1,2).

The delay in i.v. boosting from day 14 (Figs. 5.1,2) to day 49 (Fig. 5.6) significantly ($p < 0.01$) increased IgA AFC in the spleen, and at the same time decreased the IgM AFC response to 111 NM SR. The net result was an IgA

FIGURE 5.6

TWO INTRAVENOUS IMMUNIZATIONS WITH *V. CHOLERAE* SPACED

49 DAYS APART : SPLEEN PFC/10⁷ VIABLE

LYMPHOID CELLS

A. 569B IMP SR

B. 111 NM SR

Mice were given 10^7 vibrios i.v. on days 0 and 49.
Three mice were sampled at random every day and a geometric
mean of the results was obtained.

— IgM

..... IgA

Figure 5.6

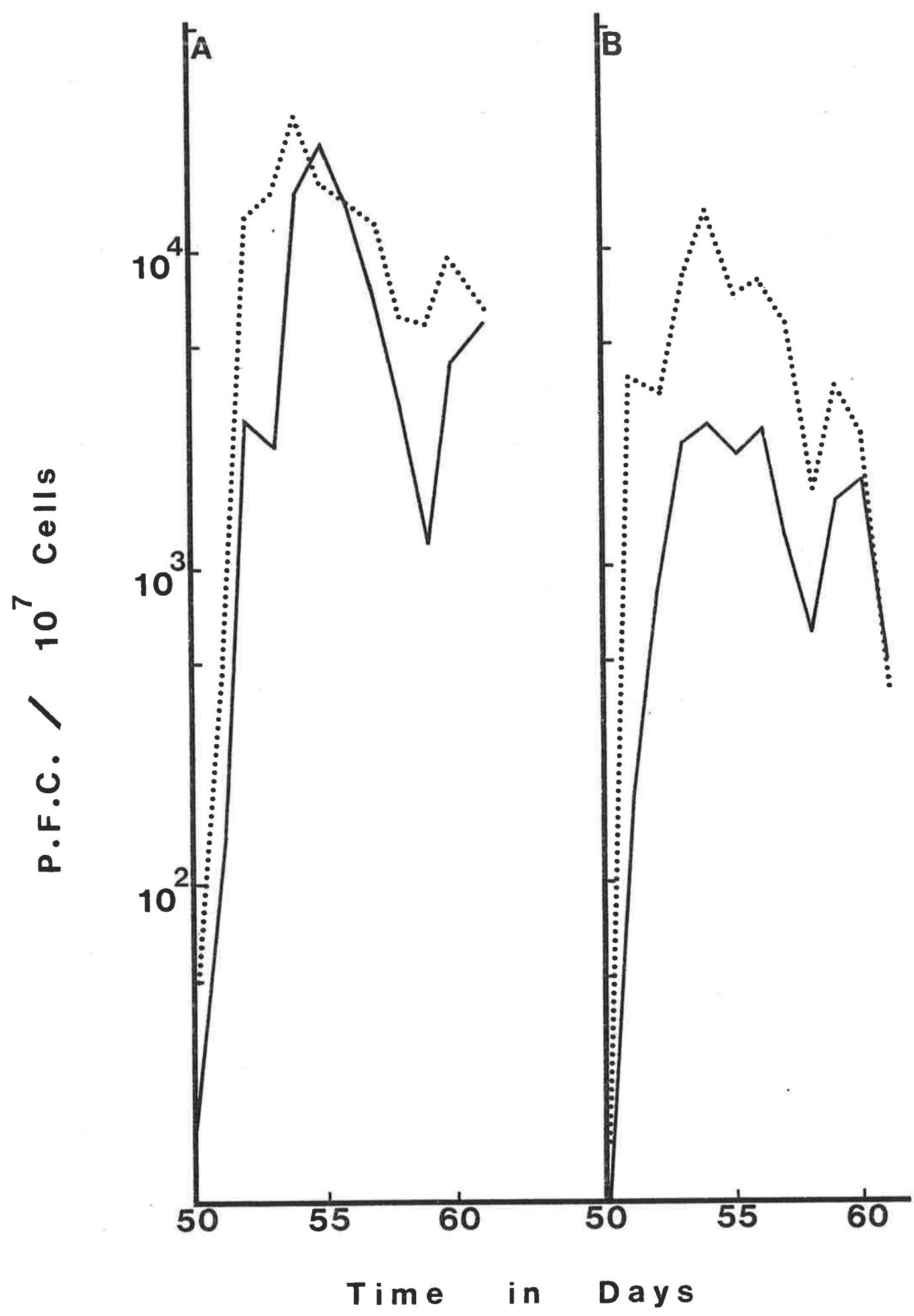


FIGURE 5.7TWO INTRAVENOUS IMMUNIZATIONS WITH V. CHOLERAE SPACED49 DAYS APART : HAEMAGGLUTINATING UNITS OF ANTIBODYPER MOUSE

A. 569B IMP SR.

B. 111 NM SR.

Mice were given 10^7 vibrios i.v. on days 0 and 49.

Three mice were sampled at random every day. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

— Serum

..... Intestinal juice (no antibody detected by direct HA in this fluid for this experiment)

Coombs enhancement of the direct HA titre expressed in additional numbers of two-fold dilutions.

		IgM	IgA	IgG2A
A	Serum	3	3	1
	Intestinal juice	0	2	0
B	Serum	2	4	0
	Intestinal juice	0	2	0

Figure 5.7

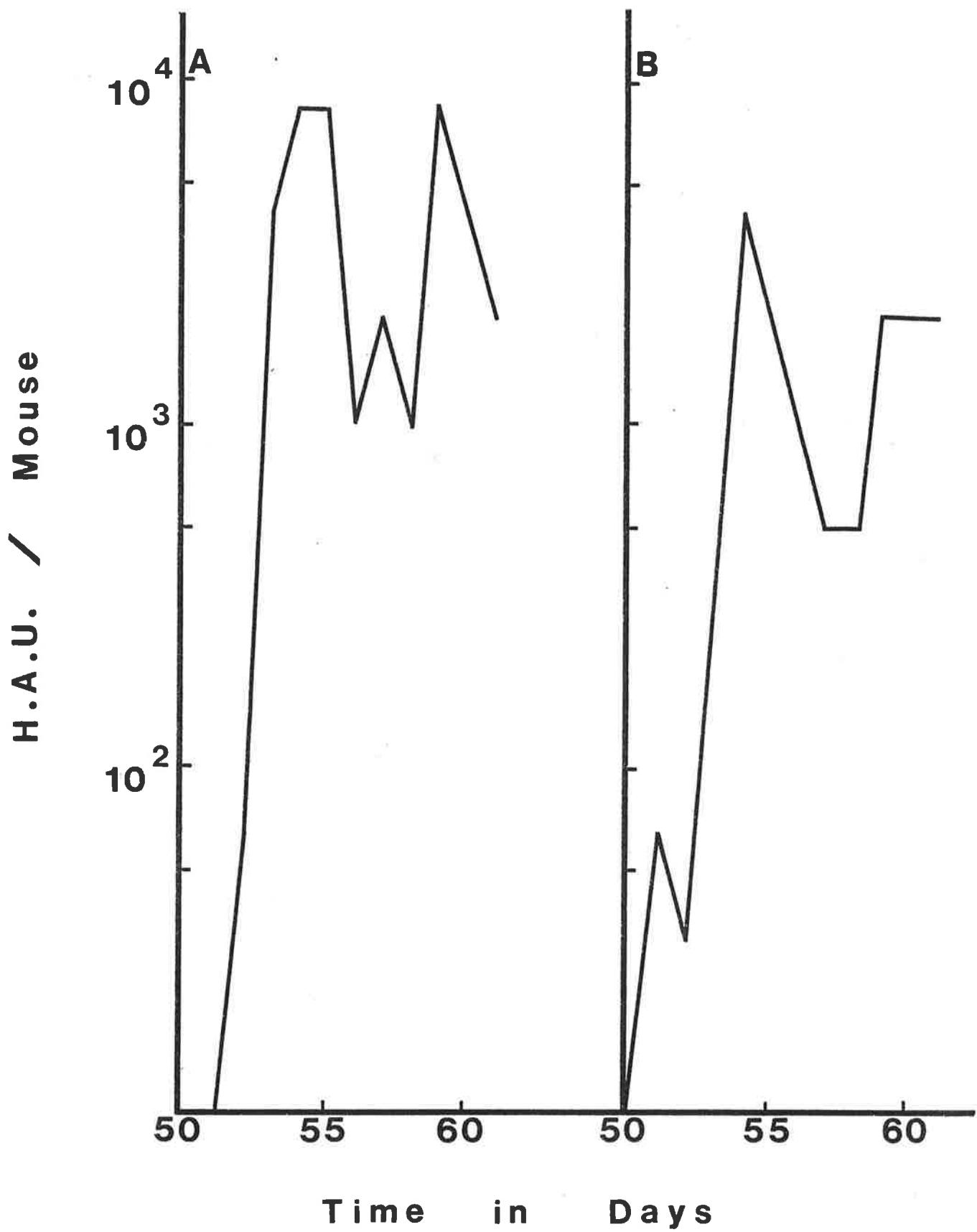


FIGURE 5.8

MULTIPLE INTRAVENOUS IMMUNIZATIONS WITH V. CHOLERAE

569B IMP SR : SPLEEN PFC/10⁷ VIABLE LYMPHOID CELLS

Mice were given four doses of 10^6 vibrios i.v. at twice-weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.8

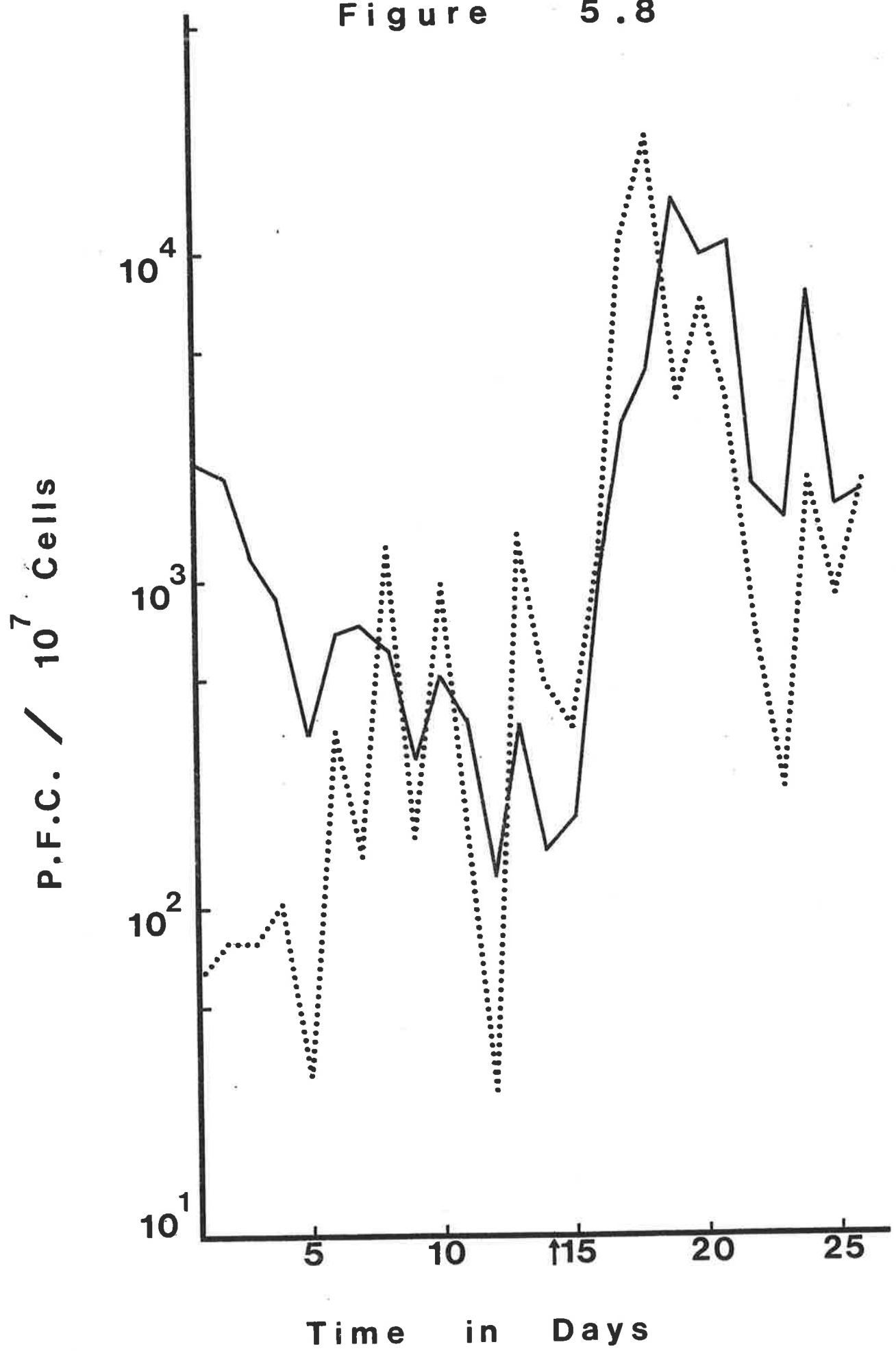


FIGURE 5.9

MULTIPLE INTRAVENOUS IMMUNIZATIONS WITH V. CHOLERAE
569B IMP SR : PFC IN THE MUCOSA OF THE INTESTINE/10⁷

VIABLE LYMPHOID CELLS

Mice were given four doses of 10^6 vibrios i.v. at twice-weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.9

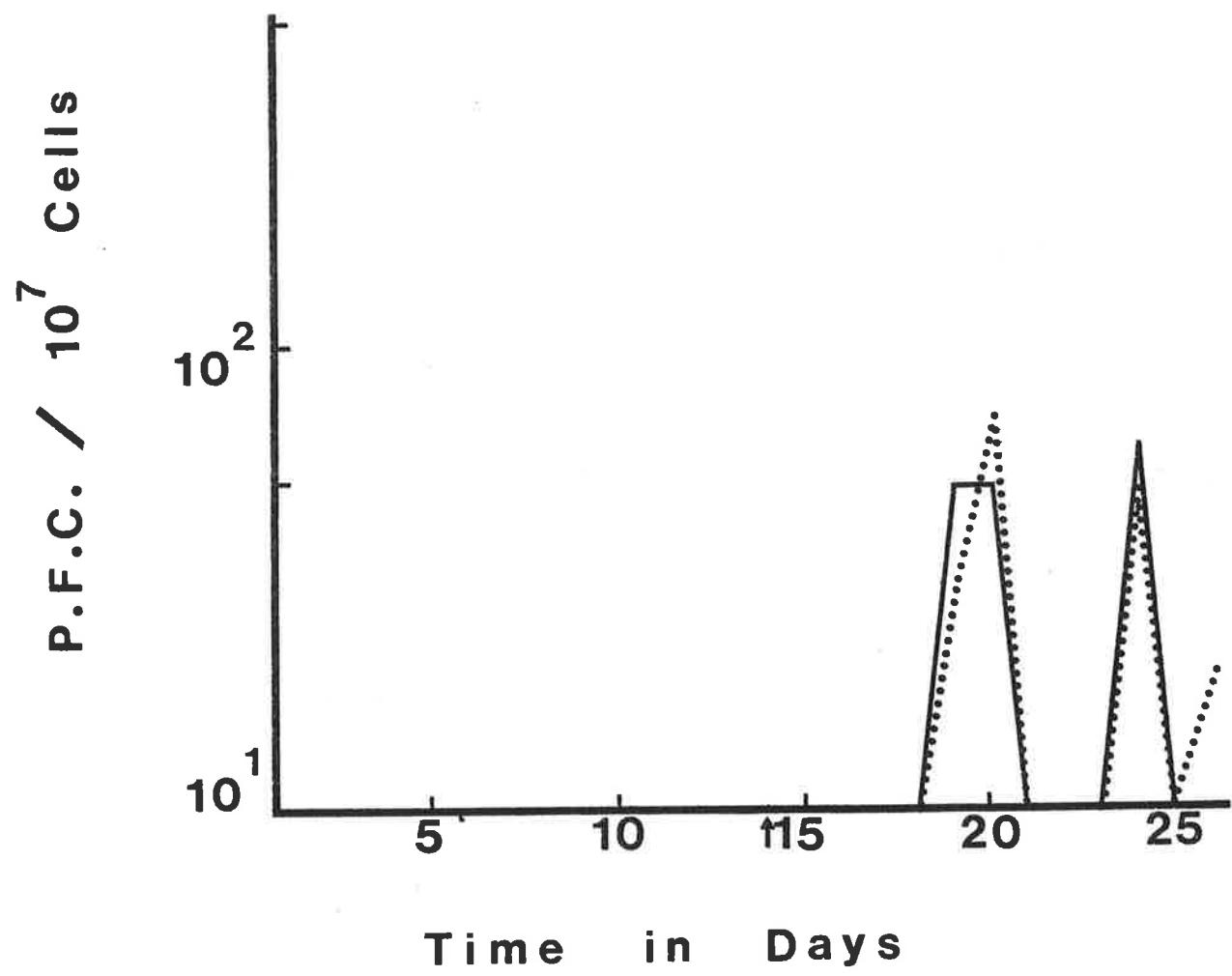


FIGURE 5.10MULTIPLE INTRAVENOUS IMMUNIZATIONS WITH V. CHOLERAE 569BIMP SR : HAEMAGGLUTINATING UNITS OF ANTIBODYPER MOUSE

Mice were given four doses of 10^6 vibrios i.v. at twice-weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by †). Three mice were sampled at random every day. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

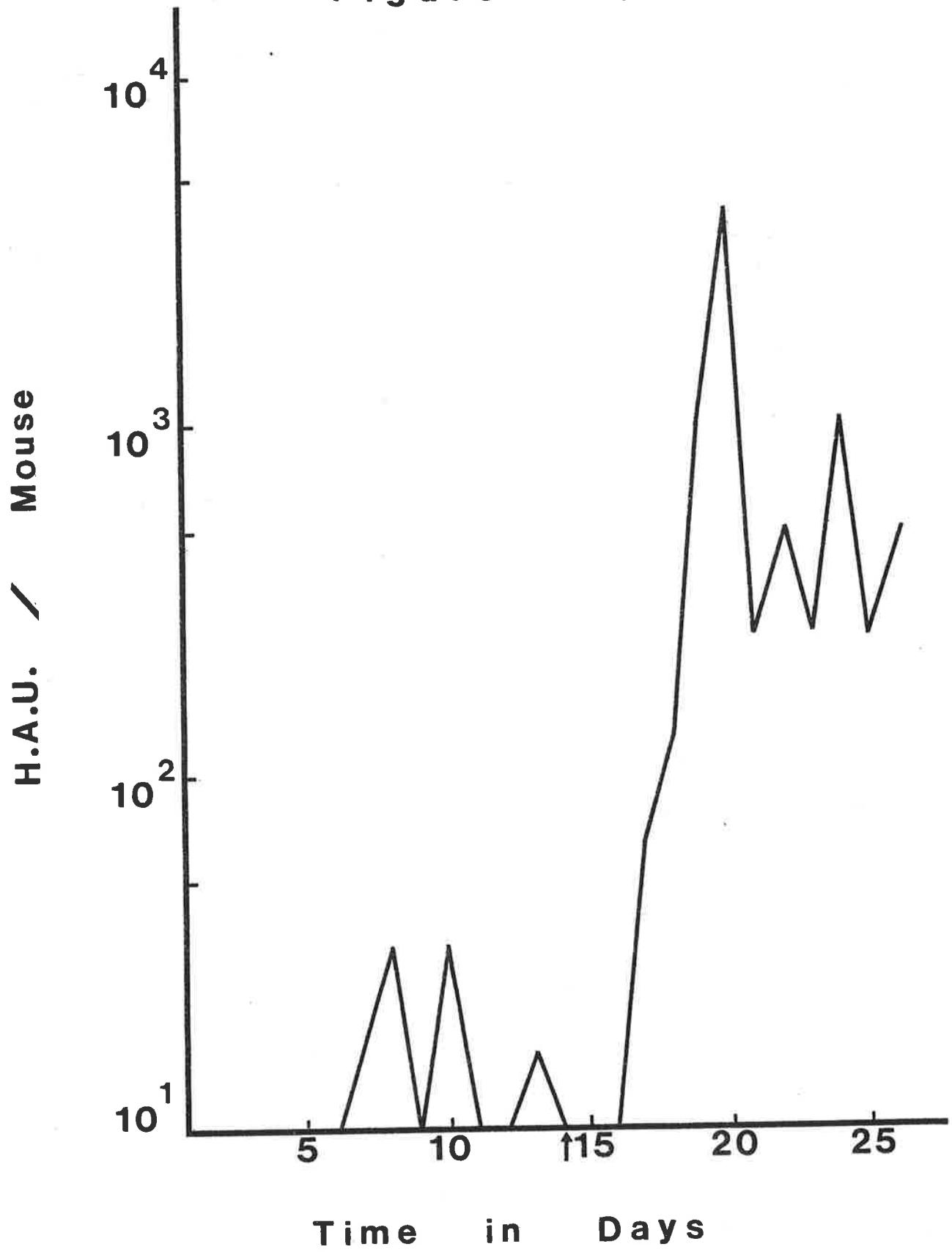
— Serum

..... Intestinal juice (no antibody detected by direct HA in this fluid for this experiment)

Coombs enhancement of the direct HA titre, expressed in additional numbers of two-fold dilutions.

	IgM	IgA	IgG2A
Serum	4	3	1
Intestinal juice	0	2	0

Figure 5.10



response equal to, or even greater than, the IgM response.

As previously discussed (Section 4.2a) large IgA responses to Gram negative bacteria are unusual. However there was confirmation of this result from assay of the antibody. Very high HA titres were recorded in the serum, but in keeping with the Jerne data, Coombs enhancement confirmed that there was either more IgA or an amount equal to the IgM (Fig. 5.7). Once again, the antibody peak in the serum was divided by a depression.

It was concluded that if an i.v. boost was given before the full development of the relatively slow IgA priming, then the IgA secondary response was less than maximal.

5.5 Multiple Parenteral Priming with a Parenteral Boost

Parenteral immunization with V. cholerae stimulated the appearance of large numbers of antibody forming cells in the spleen but not in the mucosa of the small intestine (Section 5.3). An immune response in the intestine could be effectively evoked by an immunization schedule which included multiple oral doses (see Section 5.9) and so it was queried if multiple parenteral doses would do the same.

10^6 V. cholerae 569B IMP SR were given i.v. to mice four times at twice-weekly intervals. 14 days after the last priming dose the mice were given 10^7 organisms in an i.v. booster dose. It can be seen from Fig. 5.8 that spleen AFC reached large numbers in this programme, similar to the numbers achieved by an i.v. priming dose followed 49 days later by an i.v. booster (Fig. 5.6). However, few AFC

appeared in the mucosa of the small intestine (Fig. 5.9) and no antibodies were detected by haemagglutination in the intestinal juice (Fig. 5.10).

The experiment reinforced the view that parenteral immunization alone is inadequate to evoke more than a minimal response by the gut associated lymphoid tissue (Pierce and Gowans, 1975).

5.6 Oral Immunization Schedules

Mice were orally immunized with doses of 10^{10} *V. cholerae* 569B IMP SR to see if better immune responses in the intestine could be obtained in this way. No PFC were found in either the spleen or the small intestine after a single oral dose. This was perhaps not surprising considering the rapidity with which adult mice eliminate the bulk of an oral challenge of *V. cholerae* from the gastrointestinal tract (Table 3.1).

Mice were then given two oral doses, 14 days apart. Once again no PFC were detected in the intestine, but after 12 days a very small peak of IgM ($19 \text{ PFC}/10^7$ viable cells) appeared in the spleen.

The course of oral immunization was then increased to three doses given at twice-weekly intervals. 12 days after the last oral dose a small peak of IgM AFC was seen in the spleen ($52 \text{ AFC}/10^7$ viable cells), but there were still no intestinal AFC. Therefore oral immunizations alone were ineffective in mice, probably because little of the antigen ever reached the immune system.

FIGURE 5.11

INTRAVENOUS FOLLOWED BY ORAL IMMUNIZATION WITH *V. CHOLERAE* :

SPLEEN IgM PFC/ 10^7 VIABLE LYMPHOID CELLS

A. 111NM SR.

B. 569B IMP SR.

Mice * were given 10^7 vibrios i.v. on day 0, followed by 10^{10} vibrios orally on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

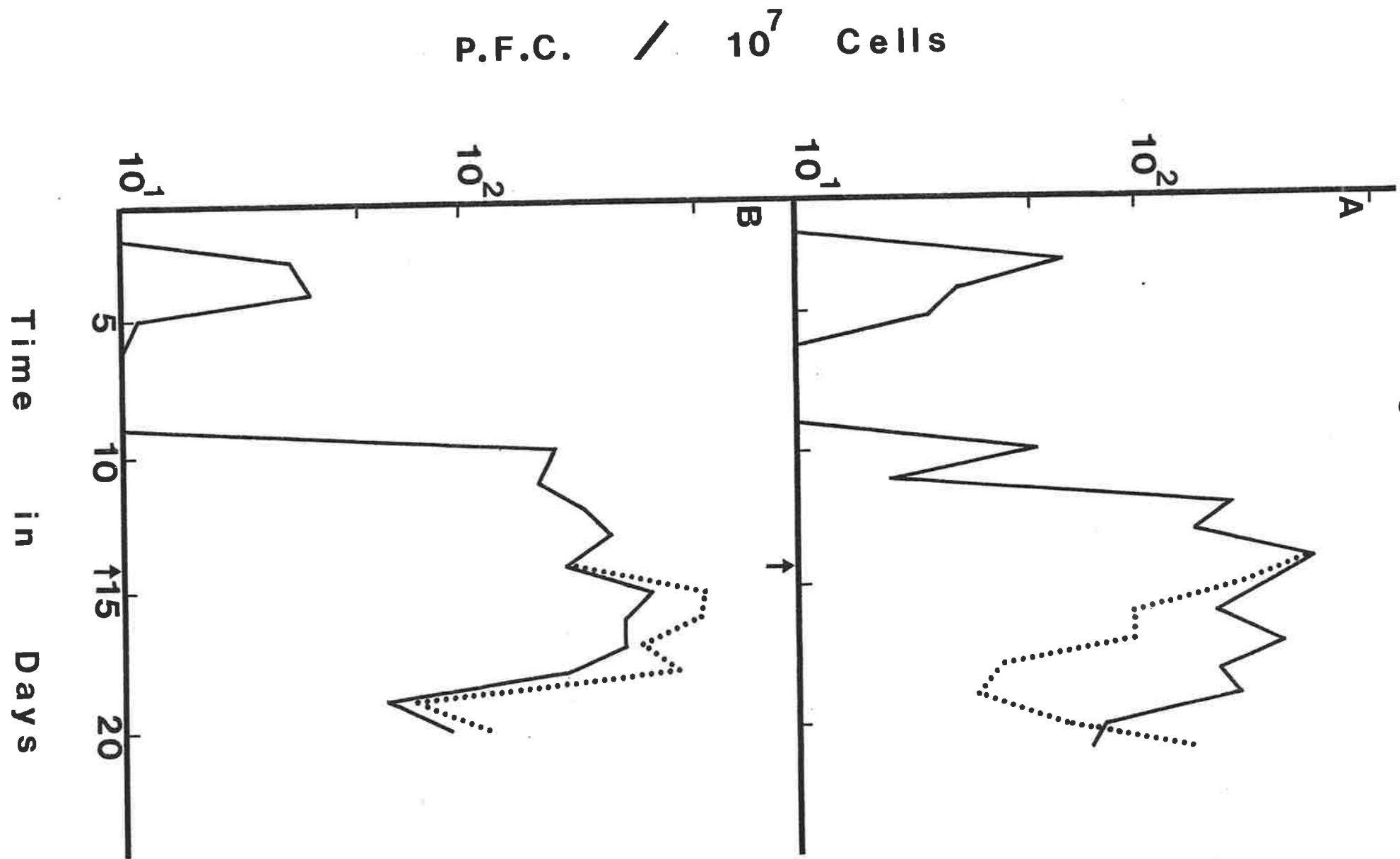
..... Intravenous immunization followed

by a dose of oral vaccine on day 14 (↑)

— Control (Intravenous immunization alone).

*These mice had a conventional bacterial flora which developed while they were kept in the Department animal house for three weeks before they were immunized. In other experiments the mice were used upon delivery from the specific pathogen free unit of the Central Animal House.

Figure 5 . 11



5.7

An Oral Priming with a Parenteral Boosting

This experiment confirmed just what little effect one oral dose of V. cholerae had on mice. Mice were primed orally with 10^{10} V. cholerae and were given an i.v. booster dose of 10^7 organisms 14 days later. The response to the i.v. dose was similar to that shown in Figs. 5.1-3, and was quite unchanged ($p > 0.02$) by the preceding oral immunization.

5.8

A Parenteral Priming and an Oral Boosting

Pierce and Gowans (1975) primed rats i.p. with cholera toxoid in FCA and boosted them with toxoid delivered by an intra-jejunal tube. They were able to demonstrate an immune response in the intestine following this schedule. A similar experiment was done with live V. cholerae. Mice were given 10^7 V. cholerae i.v. on day 0, and 14 days later they were boosted orally with 10^{10} of the organisms. The response of boosted mice was compared with the response of control mice given the i.v. immunization alone.

The study with a live 569B IMP SR vaccine did not duplicate the findings of Pierce and Gowans who used toxoid, as the numbers of antibody forming cells in the spleen after i.v. immunization were not significantly ($p > 0.02$) altered by the subsequent oral dose (Fig. 5.11). There was also no response by the intestine, as measured by the Jerne technique. It will be noted that Fig. 5.11 reveals smaller responses by the spleen to an i.v. immunization than do Figs. 4.1, 2. This was the result of using mice with a conventional bacterial flora in this particular experiment, and such mice generally have a smaller response to immunization than

specific pathogen free animals (personal communication, Dr. D.J. Horsfall; Horsfall and Rowley, 1979).

As mentioned in the introduction to this chapter, the success with cholera toxoid may be related to the particular ability of this antigen to bind to the mucosa of the small intestine (Carpenter, 1972). If the trapping of an antigen by the Peyer's patches is a prerequisite for an immune response in the intestine (Robertson and Cebra, 1976), then cholera toxoid may be at an advantage due to the specifically enhanced trapping. Another possibility is that the i.p. dose of cholera toxoid directly stimulated the Peyer's patches : it has been suggested that the Freunds complete adjuvant may have acted by corroding the serosal surface of the Peyer's patches (Husband, Monie and Gowans, 1977).

It was a different story when the 111 NM SR strain was used. The oral dose induced a marked but temporary suppression of the IgM PFC numbers in the spleen (Fig. 5.11). The suppression was statistically significant ($p < 0.02$), and was observed between one and seven days after the oral dose was given. The phenomenon was presumably due to the absorption of immuno-suppressive quantities of antigen into the circulation. Another example of the suppression of a spleen response by an oral dose of live 111 NM SR will be seen, and discussed further, in Chapter 6.

5.9 Multiple Oral Priming and a Parenteral Boosting

A number of approaches had failed to stimulate an immune response in the small intestine of mice vaccinated

FIGURE 5.12

ORAL IMMUNIZATIONS AT TWICE-WEEKLY INTERVALS AND AN

INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE

569B IMP SR : SPLEEN PFC/10⁷ VIABLE

LYMPHOID CELLS

Mice were given four oral doses of 10^{10} vibrios at twice-weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by †). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.12

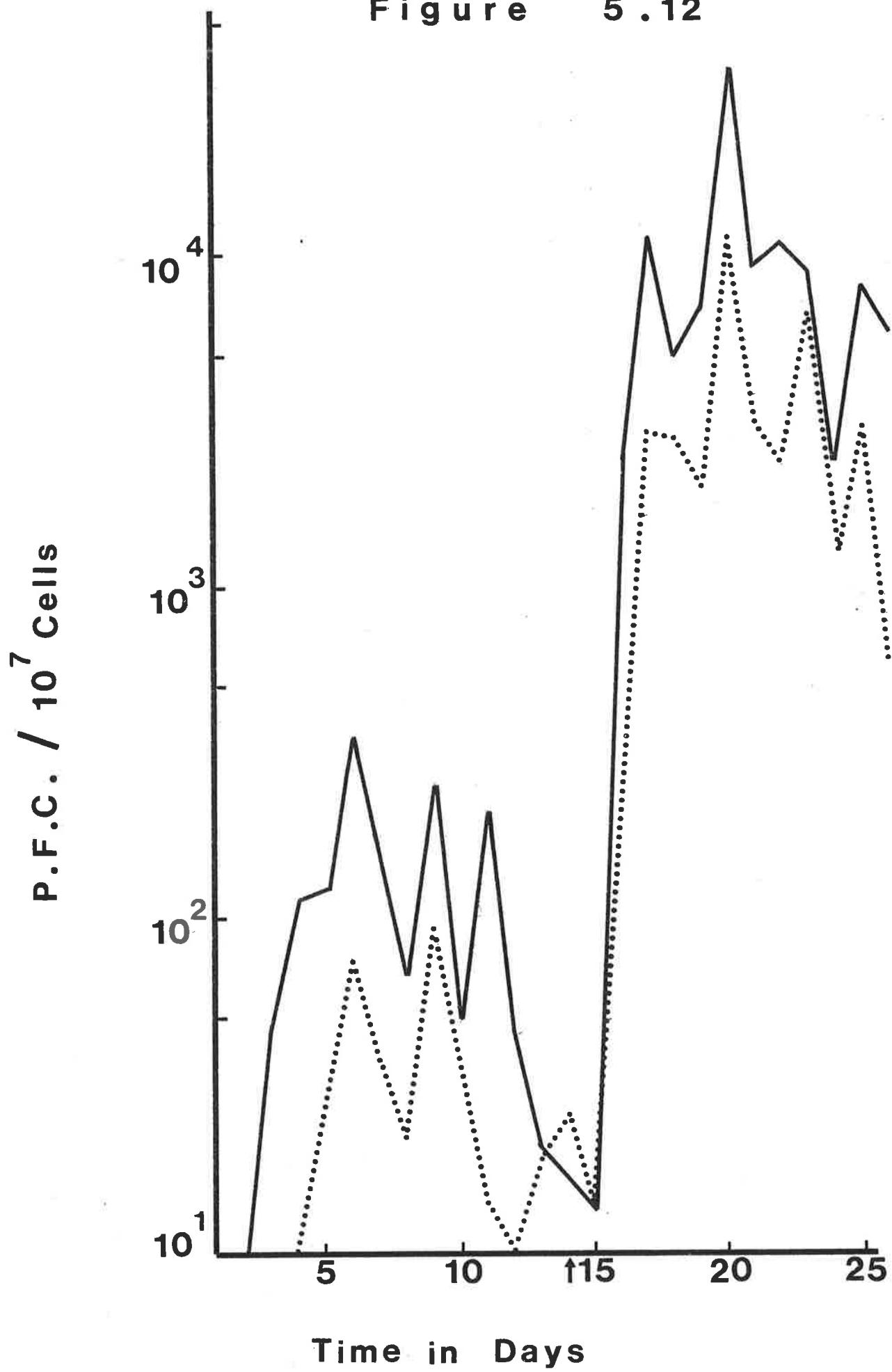


FIGURE 5.13

ORAL IMMUNIZATIONS AT TWICE-WEEKLY INTERVALS AND AN

INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE

111 NM SR : SPLEEN PFC/10⁷ VIABLE

LYMPHOID CELLS

Mice were given four oral doses of 10^{10} vibrios at twice-weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by †). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.13

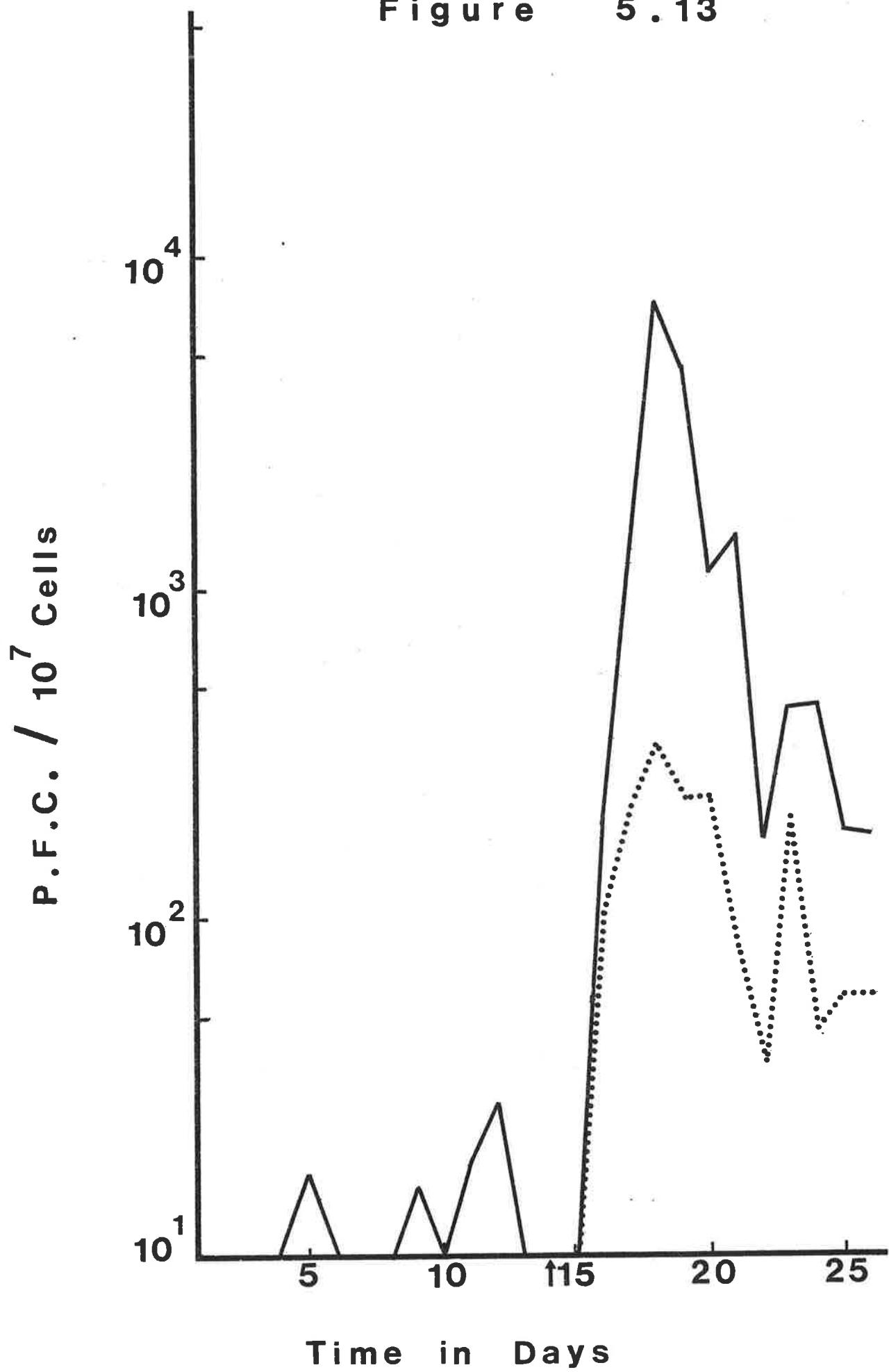


FIGURE 5.14

ORAL IMMUNIZATIONS AT TWICE-WEEKLY INTERVALS AND AN
INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAES : PFC
IN THE MUCOSA OF THE INTESTINE/10⁷ VIABLE
LYMPHOID CELLS

A. 111 NM SR

B. 569B IMP SR

Mice were given four oral doses of 10^{10} vibrios at twice-weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by †). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.14

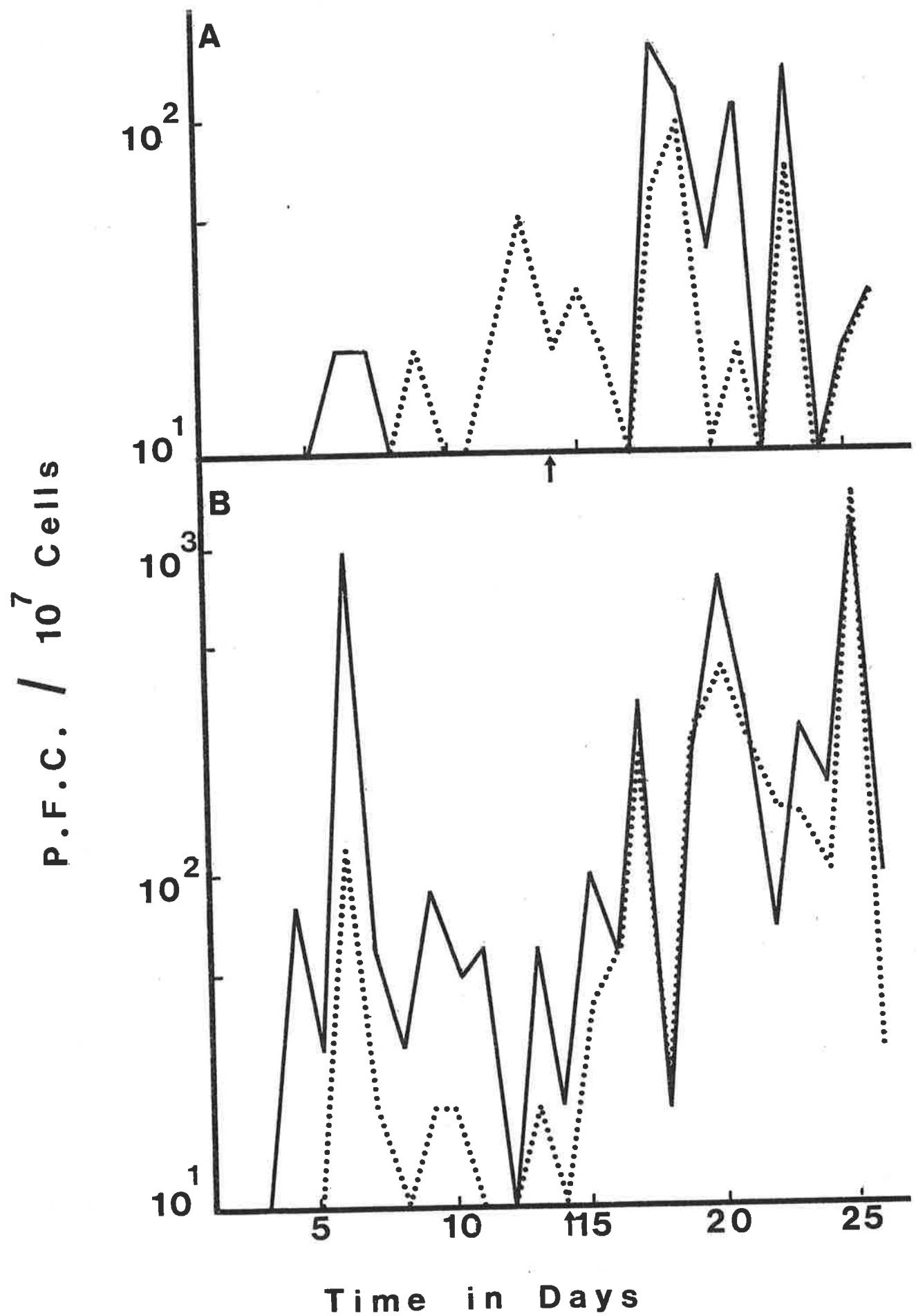


FIGURE 5.15ORAL IMMUNIZATIONS AT TWICE-WEEKLY INTERVALS AND ANINTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE 569BIMP SR : HAEMAGGLUTINATING UNITS OF ANTIBODYPER MOUSE

Mice were given four oral doses of 10^{10} vibrios at twice-weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

— Serum
 Intestinal juice

Coombs enhancement of the direct HA titre, expressed in additional numbers of two-fold dilutions.

	IgM	IgA	IgG2A
Serum	5	4	2
Intestinal juice	2	5	3

F i g u r e 5 . 1 5

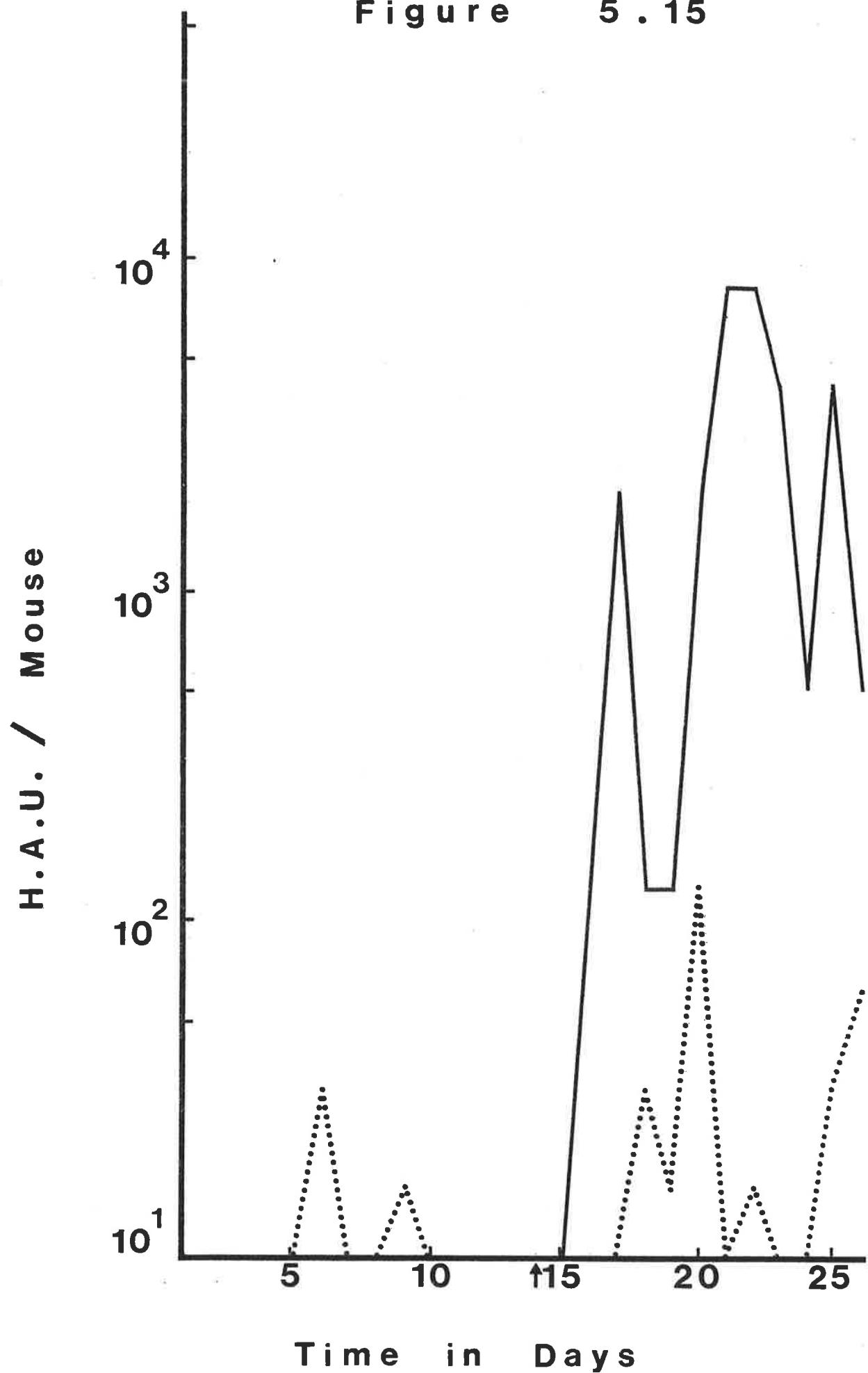


FIGURE 5.16

ORAL IMMUNIZATIONS AT TWICE-WEEKLY INTERVALS AND AN
INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE
111 NM SR. : HAEMAGGLUTINATING UNITS OF
ANTIBODY PER MOUSE

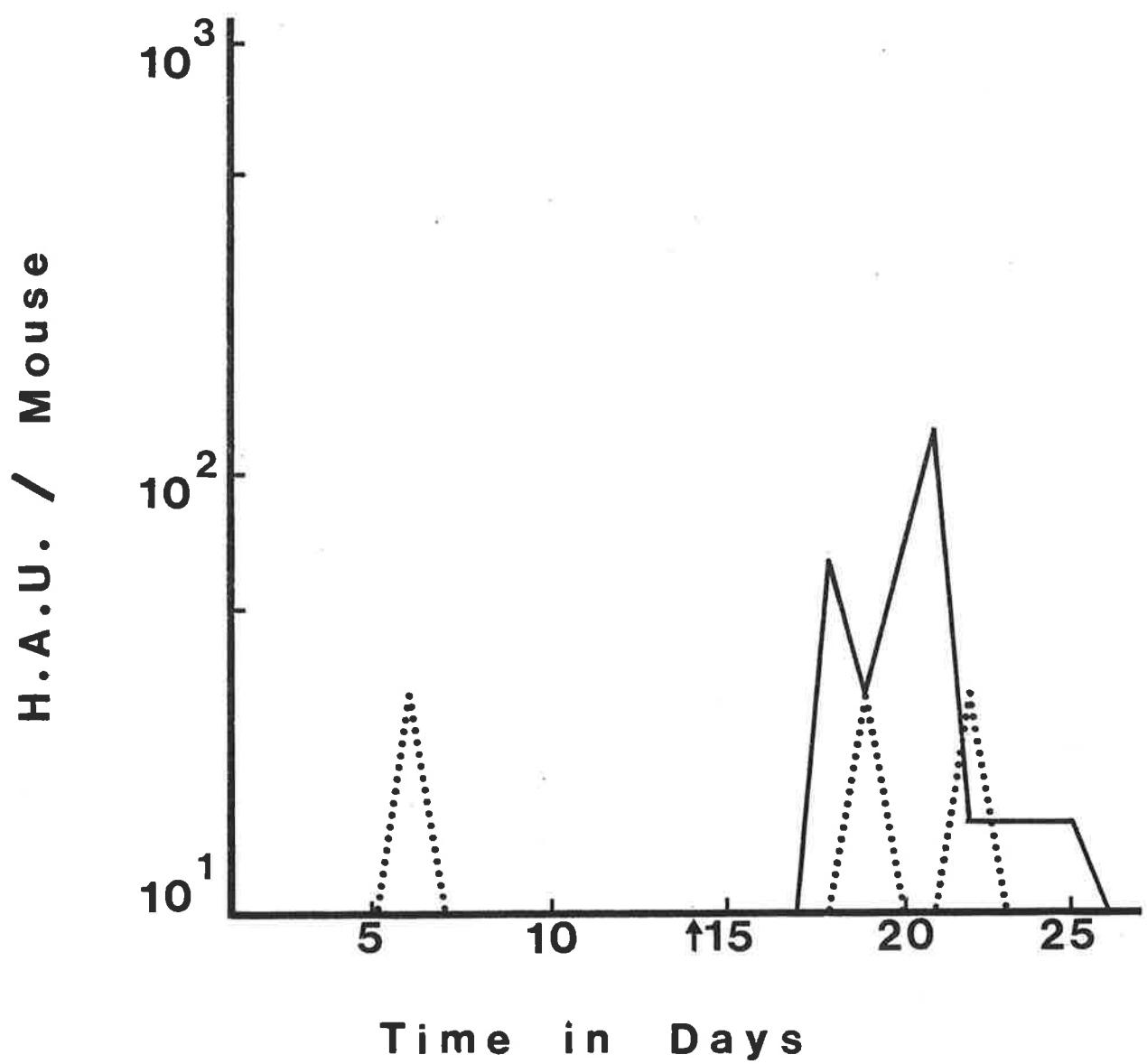
Mice were given four oral doses of 10^{10} vibrios at twice-weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

— Serum
..... Intestinal juice

Coombs enhancement of the direct HA titre, expressed in additional numbers of two-fold dilutions.

	IgM	IgA	IgG2A
Serum	6	2	0
Intestinal juice	0	5	0

Figure 5.16



with V. cholerae. In a final approach an examination was made of the ability of repeated oral immunization to prime the intestine for an immune response to an i.v. dose. As an analogy, people living in cholera areas have immune priming against V. cholerae because of their regular ingestion of the organisms and their secretory immunity can be boosted with a parenteral vaccination (Svennerholm *et al*, 1977).

A course of four oral immunizations was chosen for examination as a course of three could stimulate a predictable, albeit small, IgM PFC response in the spleen (Section 5.6). The four doses of 10^{10} V. cholerae were given orally to mice at twice-weekly intervals. An i.v. boost of 10^7 organisms was given 14 days after the final priming dose.

A difference was evident between the two V. cholerae strains used. Oral immunization with the strain with the greater ability to adhere to the mucosa of the intestine, the 569B IMP SR strain, produced a greater AFC response in the spleen than did the 111 NM SR strain (Figs. 5.12,13). In fact there were no IgA AFC in the primary spleen response to 111 NM SR, and very few IgM AFC. As discussed in Section 5.3, the two strains were equally immunogenic following parenteral vaccination. Therefore, differences in the effectiveness of the oral vaccines must have been due to other characteristics, such as an ability to persist in the small intestine.

After the i.v. boost the strain difference was still obvious (Figs. 5.12,13). Numbers of IgA and IgM antibody forming cells in the spleens of mice were much greater ($p < 0.01$) after 569B IMP SR vaccination than after use of

111 NM SR. The marked difference was also apparent in the levels of HA antibody in the serum (Figs. 5.15,16). A depression in serum antibody levels a few days after an i.v. boost was seen yet again (Fig. 5.15).

In the intestine the oral doses of 569B IMP SR evoked a primary response in which AFC of the IgM class were more numerous ($p < 0.01$) than those of the IgA class. The 111 NM SR strain produced a similarly small IgA response ($p > 0.02$), but a significantly ($p < 0.01$) smaller IgM response (Fig. 5.14). Antibody was also detected in the intestinal juice by the haemagglutination assay (Fig. 5.15,16).

Secondary responses ($p < 0.01$) were produced in the mucosa of the small intestine after intravenous boosting (Fig. 5.14). Immunization with the 569B IMP SR strain resulted in particularly marked secondary responses in both the IgA and the IgM classes; 111 NM SR only stimulated a small IgM secondary response and did not boost IgA AFC numbers. The greater effectiveness of the 569B IMP SR vaccine was also evident from the levels of HA antibody in the intestinal juice (Fig. 5.15,16).

Therefore with this schedule of multiple oral priming doses and an intravenous booster it was possible to stimulate an immune response in the intestine to V. cholerae. The priming course of oral immunizations was essential as multiple parenteral doses did not stimulate the GALT effectively (Section 5.5). The reason for this is obscure, as antigen from an i.v. dose can obviously reach the secretory immune system of the gut if it is able to boost

an immune response there. Also, the requirement for multiple oral doses is puzzling. The need for a continual presentation of antigen to the Peyer's patches may be explained, at least in part, by a lack of antigen trapping reticulum in these lymphoid organs (Bockman and Cooper, 1973). In turn, this would explain the enhanced local immunogenicity of the 569B IMP SR strain, the greater persistence of which would tend to prolong the presentation of antigen to the Peyer's patches.

For a time it was considered that the secretory immune system had no memory (Andre, Bazin and Heremans, 1973). However, the use of sensitive assays to detect antibody forming cells in the intestinal tissues has made it increasingly clear that there is immunological memory in this site (Robertson and Cooper, 1972, 1973; Pierce and Gowans, 1975; Svennerholm and Holmgren, 1977). From the data in Figure 5.14 there can be little doubt that there was a secondary response to V. cholerae in the mucosa of the small intestine.

5.10 Antibody Class of the Intestinal PFC

A feature of the intestinal PFC response to V. cholerae that required some explanation was the high proportion of PFC of the IgM class (Fig. 5.14). A more usual finding is that IgA plasma cells greatly outnumber IgM plasma cells in the mucosa of the intestine (Crabbe, Carbonara and Heremans, 1965). But, as discussed in Section 1.4, a situation nearer IgM and IgA equality may be more frequent than previously believed.

The HA assay on the intestinal juice tended to confirm the Jerne plaque data. For instance, the IgM and IgA secondary responses by the lymphoid cells in the intestine shown in Fig. 5.14 were associated with IgM and IgG2A as well as IgA in the intestinal juice, as determined by Coombs enhancement of the HA assay (Fig. 5.15). There was a predominance of IgA in the intestinal juice, but this could be expected from its greater resistance to proteolysis in comparison with the other Ig classes (Brown, Newcomb and Ishizaka, 1970).

The work of Cebra, Kamat, Gearhart, Robertson and Tseng (1977) added another dimension to this problem. They found that antigens which stimulate Peyer's patch cells naturally and chronically tend to provoke an IgA response. Conversely, the 'rarer' antigens tend to result in an IgM response. By this criterion an IgM response in the intestine to V. cholerae challenge should be expected as the mice do not have a detectable background antibody reactivity to this organism (Section 3.3a).

To test this hypothesis, the mice were immunized with an antigen against which they normally have background AFC in the spleen and the intestine. Such an antigen was found to be the O8 antigen of E. coli. Mice were given four oral doses of 10^{10} organisms at twice weekly intervals and were boosted with 10^7 organisms given i.v. 14 days after the last priming dose. The ratio of the IgA to IgM PFC peaks in the intestine was nearly 10:1 which is more like the ratio often found. As noted in Section 3.7a, the IgA to IgM ratio in the spleen for this

antigen was 1:40. It was concluded that the equality of the IgM and IgA ratios in both the spleen and the gut was a peculiarity of the response of the mice to the antigen used, V. cholerae.

5.11 A Quantitative Comparison of the Systemic and Local Response

When a secondary AFC response was obtained in the lymphoid tissue of the intestine against V. cholerae 569B IMP SR (Fig. 5.14), a question arose as to the significance of the low levels of antibody recorded in the intestinal juice (Fig. 5.15). This quantity of antibody was greatly exceeded by serum antibody.

One approach used to answer this question was to compare AFC numbers in the spleen and the intestine. These numbers were expressed as PFC/ 10^7 viable cells, because although the total number of lymphocytes in the spleen was known (approximately 2×10^8), the total number in the intestine was unknown. However, the lymphoid cell population of the intestine has been estimated to equal that of the spleen (Ferguson, 1972). Therefore PFC numbers/ 10^7 cells would be directly comparable. Fig. 5.17A compares the IgM and IgA response in the spleen to that in the intestine using the Jerne assay. The spleen AFC outnumber those in the intestine by approximately 15 fold.

Next, the quantities of serum and intestinal juice antibodies were compared. Of course the levels of antibodies are reduced by their catabolism and degradation, factors which are difficult to estimate. For the purposes

FIGURE 5.17

COMPARISON OF THE SYSTEMIC AND THE LOCAL IMMUNE RESPONSES
TO V. CHOLERAE 569B IMP SR, USING DATA FROM THE JERNE
ASSAY FOR AFC AND FROM THE HA ASSAY FOR ANTIBODIES

A. PFC (IgM and IgA) per 10^7 viable cells.

— Spleen

..... Mucosa

B. Haemagglutinating units of antibody produced per mouse per 24 hours*. Data of antibody in the intestinal juice is corrected for losses due to peristaltic removal, estimated to reduce the levels of antibody 12 fold per 24 hours.

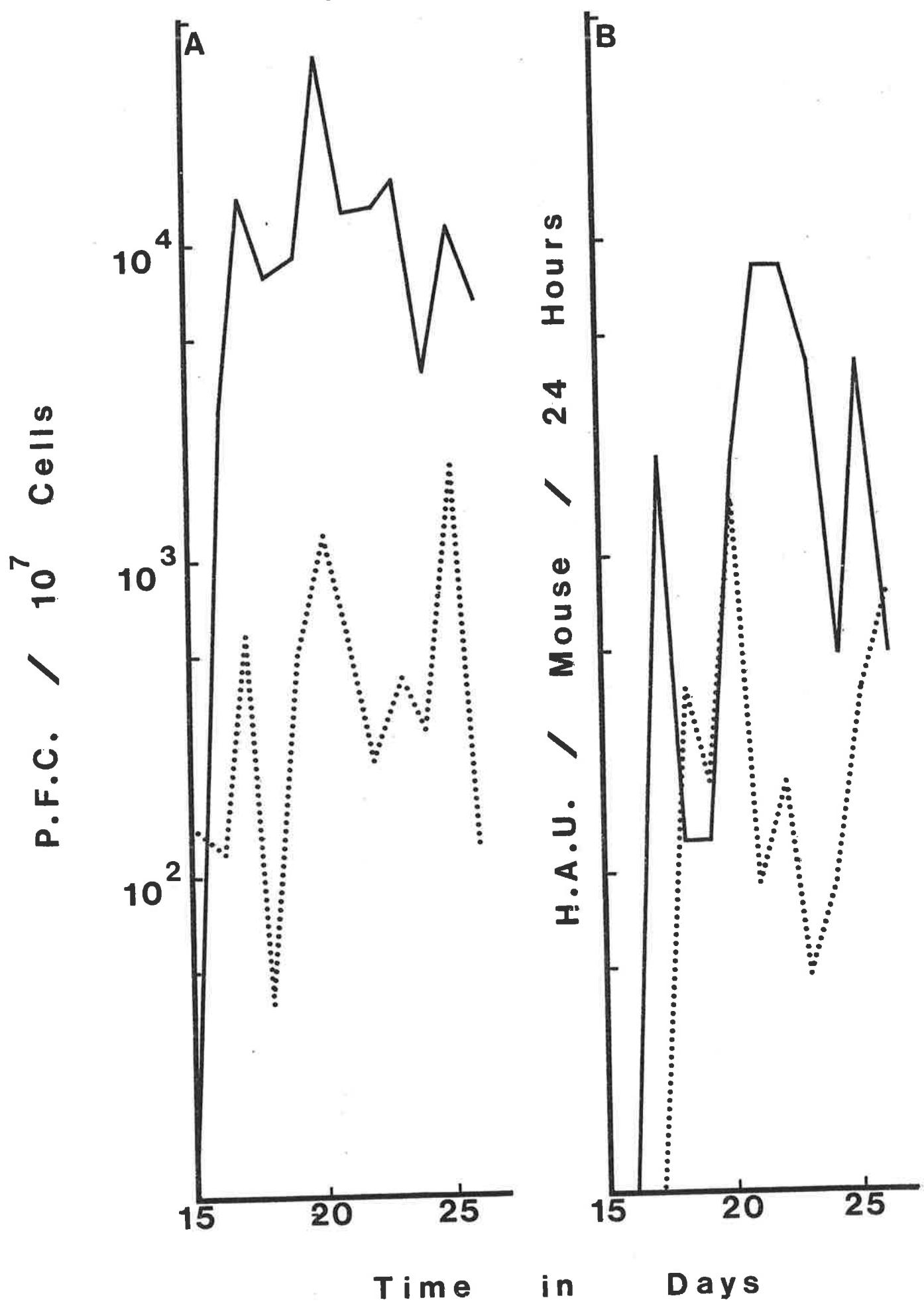
— Serum

..... Intestinal juice

Mice were given four oral doses of 10^{10} vibrios at twice-weekly intervals ending on day 0, and 10^7 vibrios i.v. on day 14. Three mice were sampled at random each day and a geometric mean of the PFC results was obtained. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

* This graph can also be said to represent the reciprocal HA titre per mouse as the volume of serum is nearly 1ml and the volume of intestinal juice is approximately 0.1ml.

Figure 5.17



of this exercise it was assumed that the rates of degradation of antibody were equal in the serum and the intestinal juice and that the half life of the antibodies was approximately 24 hrs (Fahey and Sell, 1965; Bazin and Malet, 1969). Therefore the total amount of antibody per mouse at any one assay time could also represent the total output per day, except that the peristaltic removal of intestinal juice was a major factor resulting in an underestimate of intestinal antibody production. From Table 3.1 it can be seen that vibrios make a complete transit of the small intestine within two hours. Therefore peristaltic action would lead to a 12 fold underestimate of antibody output into the small bowel every 24 hours. Fig. 5.17B compares the 24 hour output of serum and intestinal juice antibody, of which the serum output is approximately 10 fold greater.

Therefore when the flow of antibody out of the intestine is taken into account, its production can be seen to be considerable and also in keeping with the Jerne data. Another point worth considering is that the serum antibody is in a volume of nearly 1 ml, whereas the intestinal juice measures more like 0.1ml. In other words the intestinal juice antibody is in relatively high titre, and Fig. 5.17B could also be labelled HA titre/mouse.

5.12 A Critical Evaluation of the Choice of Antibody

Assays

Mice produced substantial secondary responses to v. cholerae 569B IMP SR, both systemically and in the intestine, when primed orally at twice-weekly intervals

and boosted i.v. 14 days after the final primary dose (Section 5.9). The magnitude of these immune responses provided the opportunity to critically evaluate the choice of antibody assays. In Immunology, as in other fields, the selection of techniques must be appropriate for the study of the particular problem. In this work the goal was qualitative and quantitative information regarding immune responses in mice.

The haemolytic plaque assay was the yardstick against which the other antibody assays were measured. It was certainly the most sensitive; a spleen PFC count of $10^2/10^7$ viable cells was not accompanied by detectable antibody in the serum (Figs. 5.12,15). It also provided accurate information about the antibody class composition of a response, as long as the developing antiserum was of strict class specificity (see Materials and Methods).

Haemolytic PFC are directed against the LPS antigens, whereas vibriolytic PFC are against all of the bacterial antigens. The two assays were compared on duplicate samples, but in general the vibriolytic count was a third lower. It was apparent that the thin layer modification of the Jerne technique aided the identification of haemolytic PFC in contrast to the vibriolytic PFC which had to be distinguished through a base layer.

From the PFC data in Figures 5.12 and 14, the period of the peak response to V. cholerae 569B IMP SR was seen to be from day 17 to day 23. In this period the average PFC numbers per 10^7 cells in the spleen were 10^4 IgM and 3.8×10^3 IgA; for the intestine the numbers per 10^7 cells

were 300 IgM and 260 IgA. Therefore an antibody assay might be expected to show an IgM:IgA ratio of 3:1 in the serum and 1:1 in the intestinal juice.

The data provided by the HA assay compared favourably with the data from the Jerne assay. At the peak of the response the number of H.A.U. was of the same order as AFC/ 10^7 cells (Fig. 5.17). In the serum an average of 8,200 units was found, with a Coombs enhancement of 5 wells for IgM and 4 wells for IgA, an IgM:IgA ratio in line with the AFC data. The intestinal juice averaged 300 units/24 hours at its peak, but with a 5 well enhancement for IgA and a 2 well enhancement for IgM. This result of finding eight fold less IgM than IgA in the intestinal juice was probably due to the proteolysis of IgM (Brown, Newcomb and Ishizaka, 1970).

The radio-immuno-assay (RIA) was found to have a sensitivity similar to that of the HA assay. Peak RIA activity in the serum averaged 5,120 anti LC (light chain) units and 1,280 anti α (α heavy chain) units. The anti LC assay detected all antibody classes, and the anti- α assay only detected IgA. Therefore the ratio of IgA to the other antibody classes in the serum was nearly 1:3, which was also in keeping with PFC data.

The RIA was less useful for assaying antibody levels in the intestinal juice, particularly as no anti-LC activity was ever found in it. This was a puzzle, especially as the anti α assay functioned in the intestinal juice. Peak anti α activity averaged at a titre of 1/64, but with a 1/8 background in normal intestinal juice.

Therefore 672 units were averaged in 24 hours, compared with the estimate of 300 HA units/24 hrs.

Although the RIA was a sensitive assay quite appropriate for the study, it had some distinct disadvantages. In particular, it was very time-consuming to perform, and it involved the use of radio-isotopes of iodine. Also there were unsolved problems in its application for use on intestinal juice. A theoretical disadvantage was the use of whole bacteria as the antigen, but a modification using LPS could have been developed to make it more comparable with the Jerne assay.

The bactericidal assay also employed whole bacteria. Its sensitivity is remarkable, and the serum titre reached $1/4 \times 10^6$ with this technique. It is also notable that this titre was the highest that was obtained by the hyperimmunization of mice with V. cholerae, which emphasizes the effectiveness of the multiple oral priming, i.v. boosting schedule. However, it could not be used to assay intestinal juice because of the anticomplementary activity of this fluid (Freter, 1962).

The average of bactericidal activity in the peak serum response was 2×10^6 units/ml, reduced by 2-ME reduction to 6×10^3 units/ml. However, the 99.7% loss in activity did not truly indicate the proportion of antibody of the IgM class. For instance the pure antibody class preparations used in Section 4.3a were also assayed for vibriocidal antibody. The number of vibriocidal units assayed per PD₅₀ unit of antibody was : < 1 for IgG₁, IgG2B, IgA; 3 for IgG2A; and 160 for IgM. Therefore this assay did

FIGURE 5.18

ORAL IMMUNIZATIONS GIVEN DAILY AND AN INTRAVENOUS

BOOSTER GIVEN WITH *V. CHOLERAE* 569B IMP SR :

SPLEEN PFC/ 10^7 VIABLE LYMPHOID CELLS

Mice were given four oral doses of 10^{10} vibrios daily ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.18

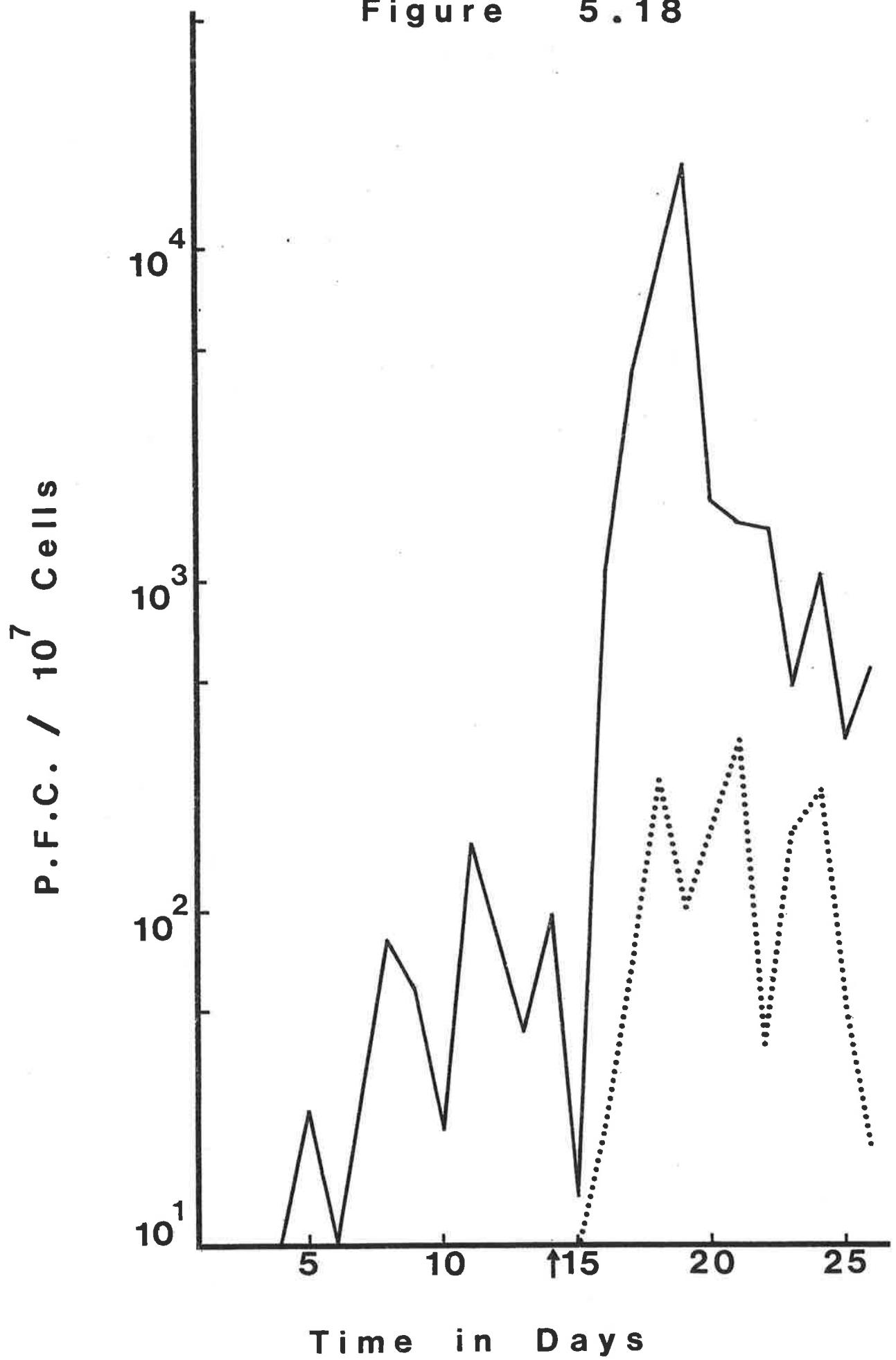


FIGURE 5.19

ORAL IMMUNIZATIONS GIVEN DAILY AND AN INTRAVENOUS

BOOSTER GIVEN WITH V. CHOLERAES 111 NM SR :

SPLEEN PFC/10⁷ VIABLE LYMPHOID CELLS

Mice were given four oral doses of 10^{10} vibrios daily ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.19

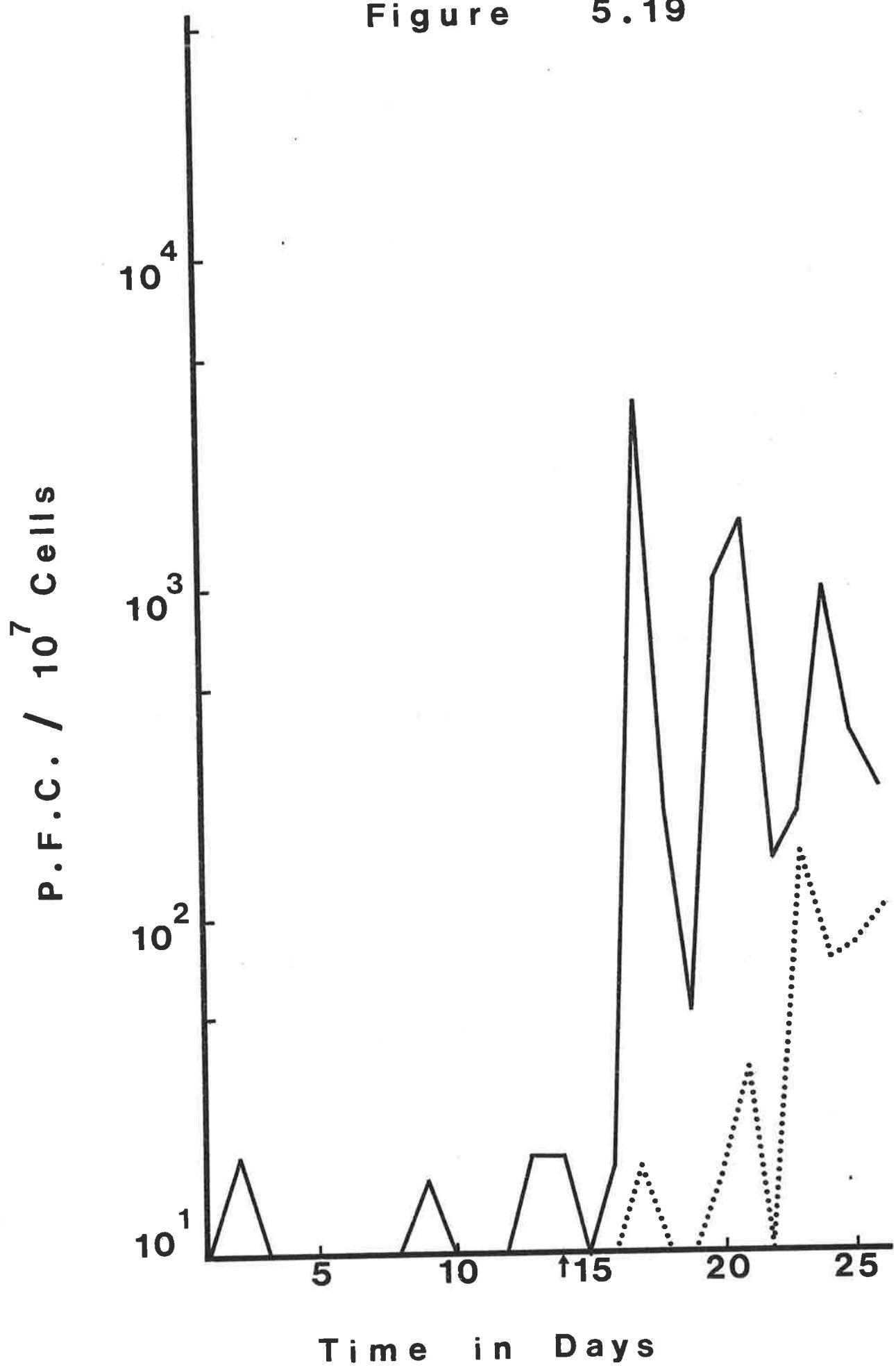


FIGURE 5.20

ORAL IMMUNIZATIONS GIVEN DAILY AND AN INTRAVENOUS
BOOSTER GIVEN WITH V. CHOLERAE : PFC IN THE
MUCOSA OF THE SMALL INTESTINE/10⁷ VIABLE
LYMPHOID CELLS

A. 111 NM SR

B. 569B IMP SR

Mice were given four oral doses of 10^{10} vibrios daily ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.20

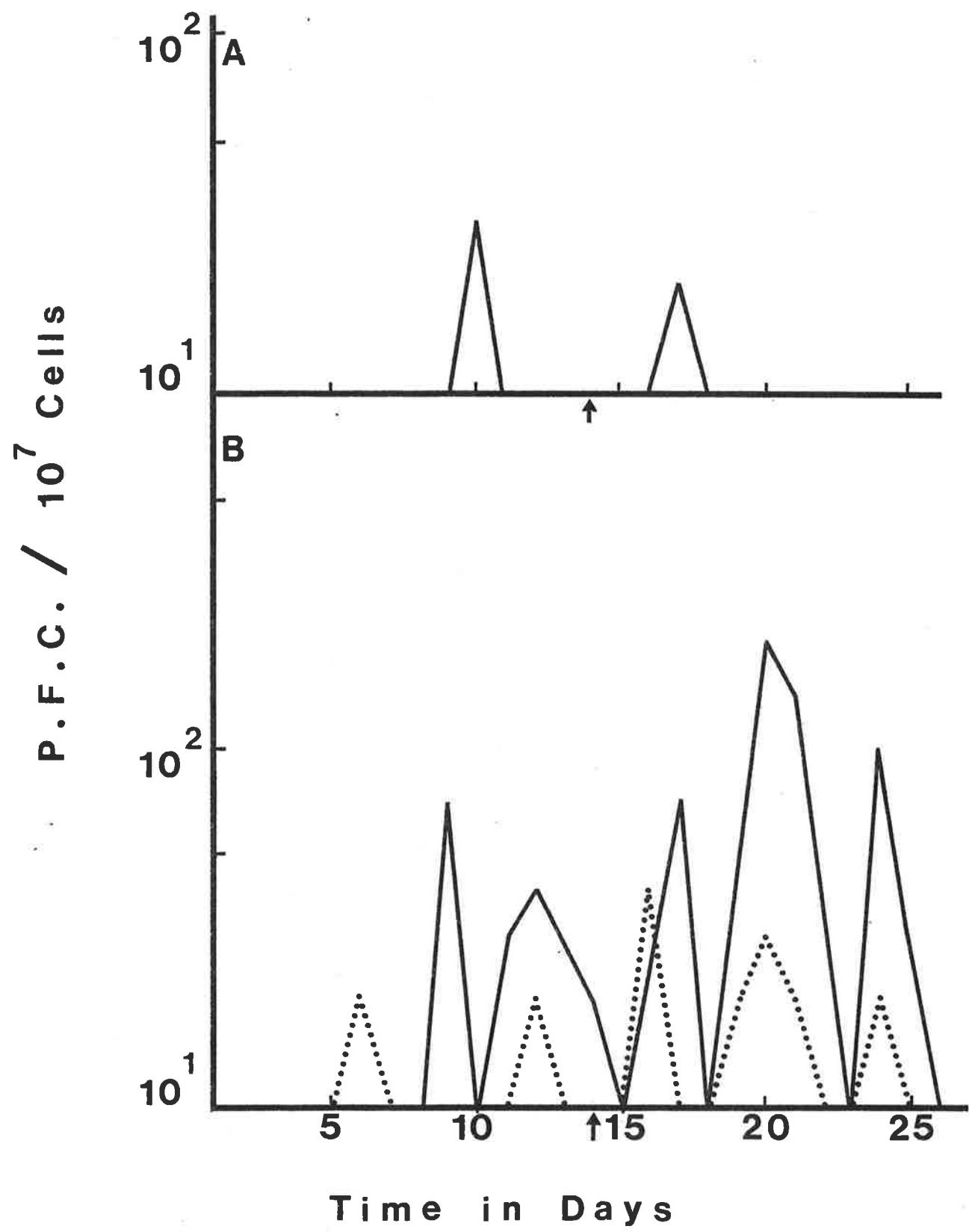


FIGURE 5.21

ORAL IMMUNIZATIONS GIVEN DAILY AND AN INTRAVENOUS BOOSTER
GIVEN WITH V. CHOLERAE : HAEMAGGLUTINATING UNITS OF
ANTIBODY PER MOUSE

A. 111 NM SR.

B. 569B IMP SR.

Mice were given four oral doses of 10^{10} vibrios daily ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

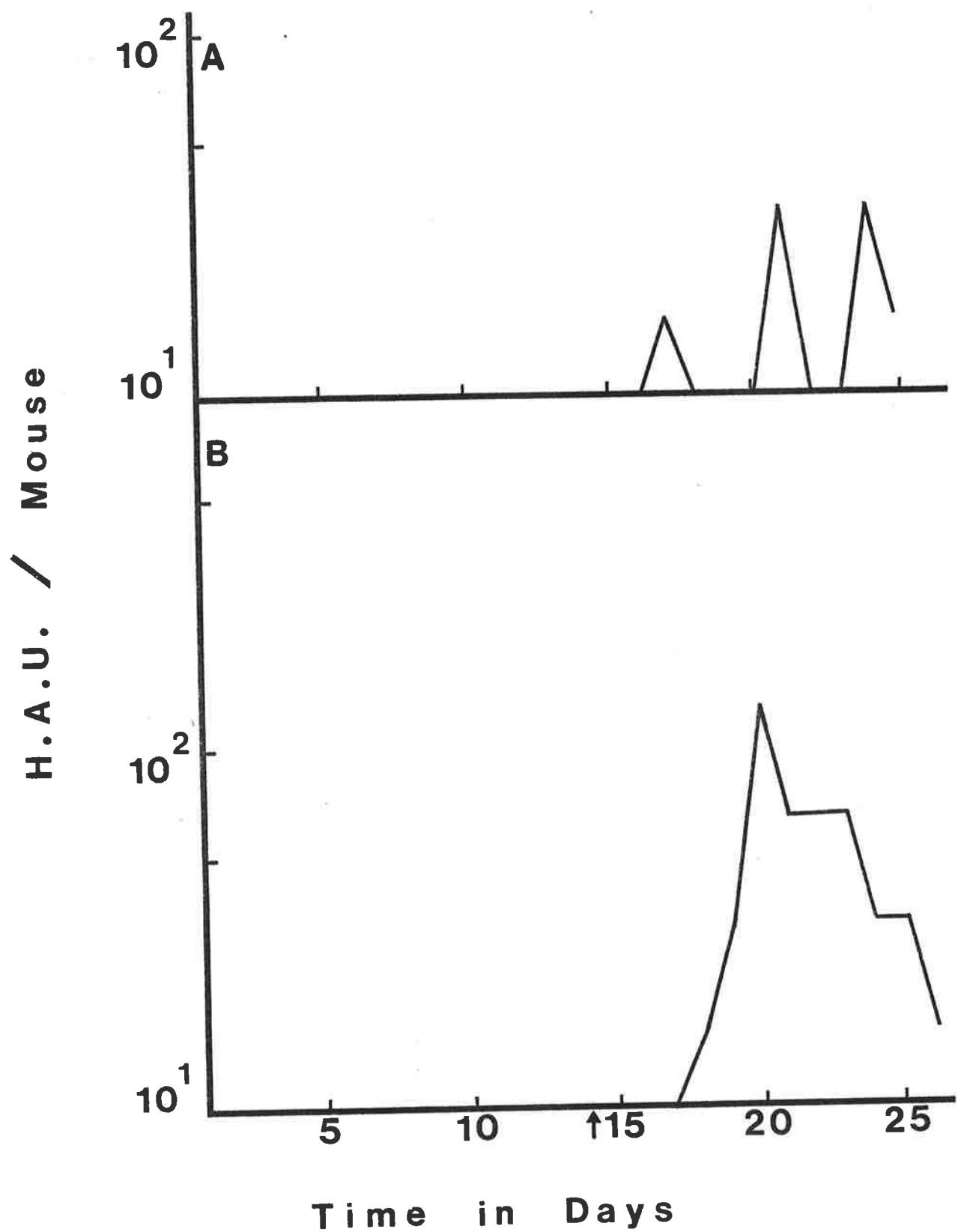
— Serum

..... Intestinal juice (no antibody detected by direct HA in this fluid for this experiment).

Coombs enhancement of the direct HA titre, expressed in additional numbers of two-fold dilutions.

		IgM	IgA	IgG2A
A	Serum	4	0	0
	Intestinal juice	0	2	0
B	Serum	4	0	0
	Intestinal juice	1	2	0

Figure 5.21



not provide the appropriate qualitative information that was required.

5.13 Duration of an Oral Priming Course : A Critical Factor in the Immunization of mice with Vibrio cholerae

5.13a Oral priming compressed over four days : The duration of an oral priming course was the next variable considered in the systematic study of V. cholerae immunization of mice. In the immunization schedule which successfully stimulated intestinal immunity to V. cholerae, the mice were primed with four oral doses of vaccine given at twice-weekly intervals, and were subsequently boosted i.v. (Section 5.9). These results were then compared with the responses of mice given a similar programme of V. cholerae, but with the oral doses delivered in a shorter space of time.

The mice were given an oral dose of 10^{10} V. cholerae on four successive days and were boosted i.v. with 10^7 organisms 14 days after the last priming dose. The crowding of the oral priming course into a four day period reduced its effectiveness. In the spleen the most notable effect was a significant reduction ($p < 0.01$) in IgA PFC numbers, while the IgM response remained unchanged (Figs. 5.18,19 - compare with Figs. 5.12,13). The selective effect on IgA AFC made it clear that the underlying reason for this was the relatively slow prim-

ing for an IgA response. As discussed in Section 5.4, prolonging the interval between immunizations may increase IgA AFC numbers relative to IgM by allowing time for complete priming for both responses.

The profound fluctuations ($p < 0.01$) in the numbers of IgM PFC seen in Fig. 5.19 are reminiscent of those found after a single i.v. immunization with V. cholerae (Figs. 4.1,2). In Chapter 4 evidence was detailed which linked this phenomenon to an immuno-regulatory mechanism.

The compression of the oral priming course from two weeks to four days had an even more striking effect on the intestinal immune responses (Figs. 5.14,20). Whereas the significant effect in the spleen was a reduction in IgA PFC, in the intestine both the IgA and the IgM responses were greatly reduced ($p < 0.01$). As well, the shorter oral immunization course did not prime the gut associated lymphoid tissue for a secondary response to an i.v. booster dose.

These data show a dichotomy between the systemic and the local responses to oral immunization. The effect in the spleen can be adequately explained in terms of the time elapsed from the commencement of oral immunization to the time of the i.v. boosting. In the intestine there was an effect on the IgM response and the formation of immunological memory

which could not be explained in a similar manner. A working hypothesis was constructed which accounted for all the data. Firstly, the period of establishment and multiplication of the 569B IMP SR strain in the small intestine might effectively increase the amount of antigen delivered by its use in oral immunization, and so increase the immune responses in comparison with the 111 NM SR strain. Secondly, the antigen reaching the systemic immune system was effective given over a shorter or a longer period of time because it was retained by reticulo-endothelial tissue (Bockman and Cooper, 1973). The IgA response in the spleen was affected only because of its long priming time. Finally, antigen reaching the local immune system has to be delivered over longer periods of time because the Peyer's patches have a poor capacity for antigen retention (Bockman and Cooper, 1973).

- 5.13b Oral priming expanded over four weeks : A course of four oral doses of V. cholerae was less effective in stimulating an immune response in the intestine when given over four days rather than two weeks. The effect of lengthening the course was then examined. Mice were primed for an immune response with four doses of 10^{10} V. cholerae given orally at weekly intervals. The mice were boosted i.v. with 10^7 organisms 14 days after the last priming dose.

FIGURE 5.22

ORAL IMMUNIZATIONS GIVEN AT WEEKLY INTERVALS AND AN

INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE

569B IMP SR : SPLEEN PFC/ 10^7 VIABLE

LYMPHOID CELLS

Mice were given four oral doses of 10^{10} vibrios at weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.22

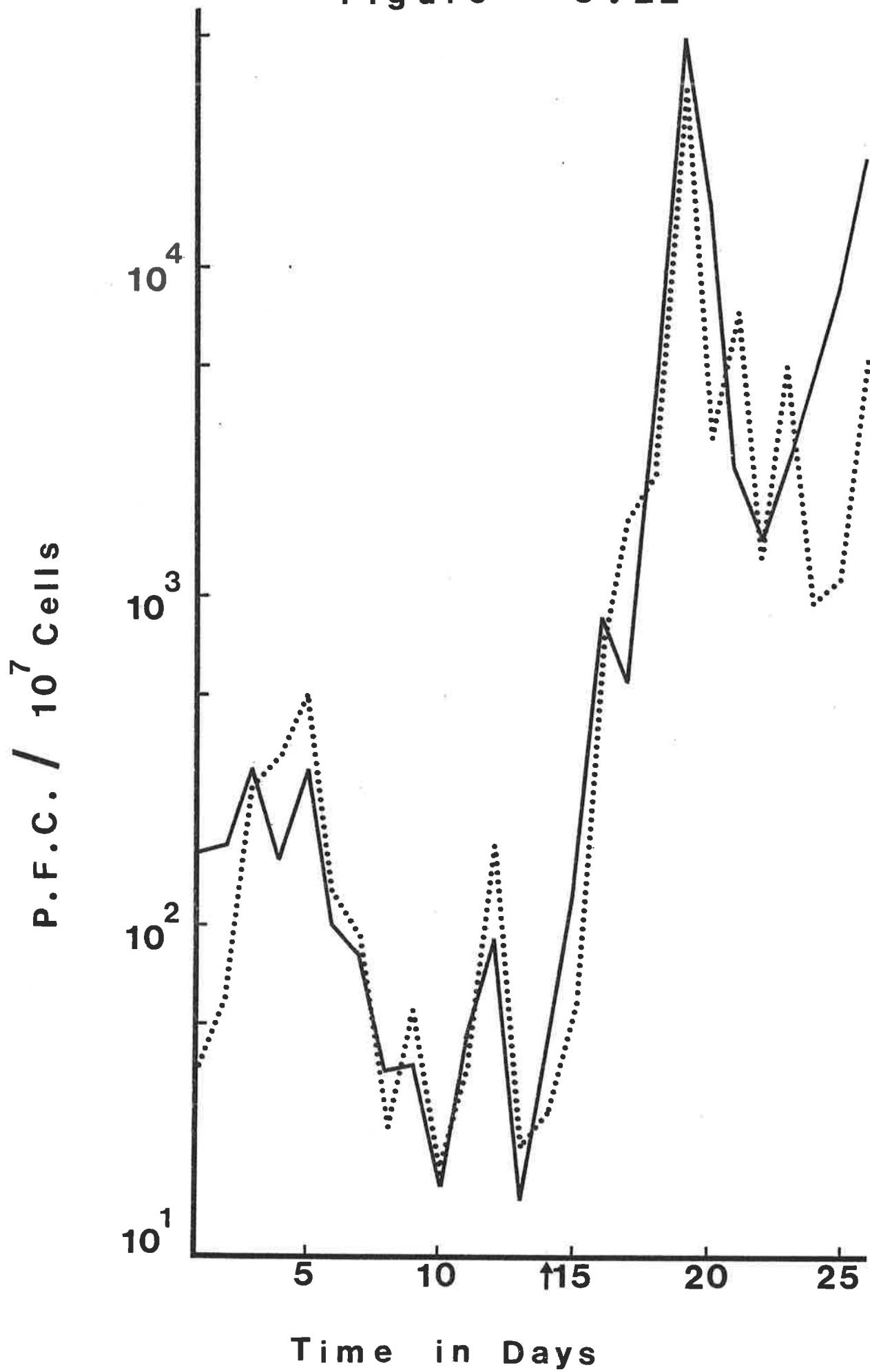


FIGURE 5.23

ORAL IMMUNIZATIONS GIVEN AT WEEKLY INTERVALS AND AN

INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE

111 NM SR : SPLEEN PFC/ 10^7 VIABLE

LYMPHOID CELLS

Mice were given four oral doses of 10^{10} vibrios at weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5 . 23

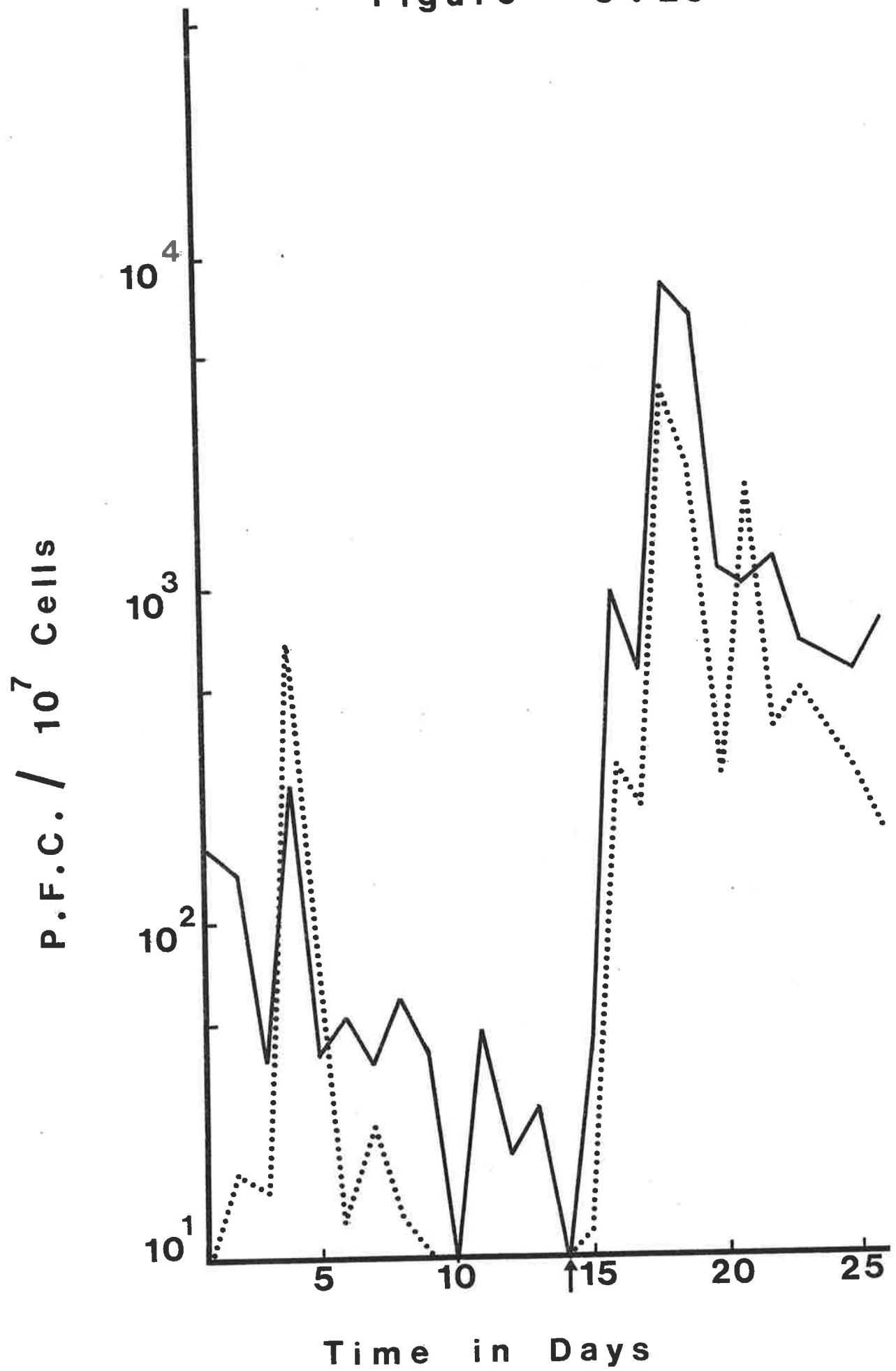


FIGURE 5.24

ORAL IMMUNIZATIONS GIVEN AT WEEKLY INTERVALS AND AN
INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE 569B
IMP SR : PFC IN THE MUCOSA OF THE SMALL
INTESTINE/10⁷ VIABLE LYMPHOID CELLS

Mice were given four oral doses of 10^{10} vibrios at weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by †). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM
..... IgA

Figure 5.24

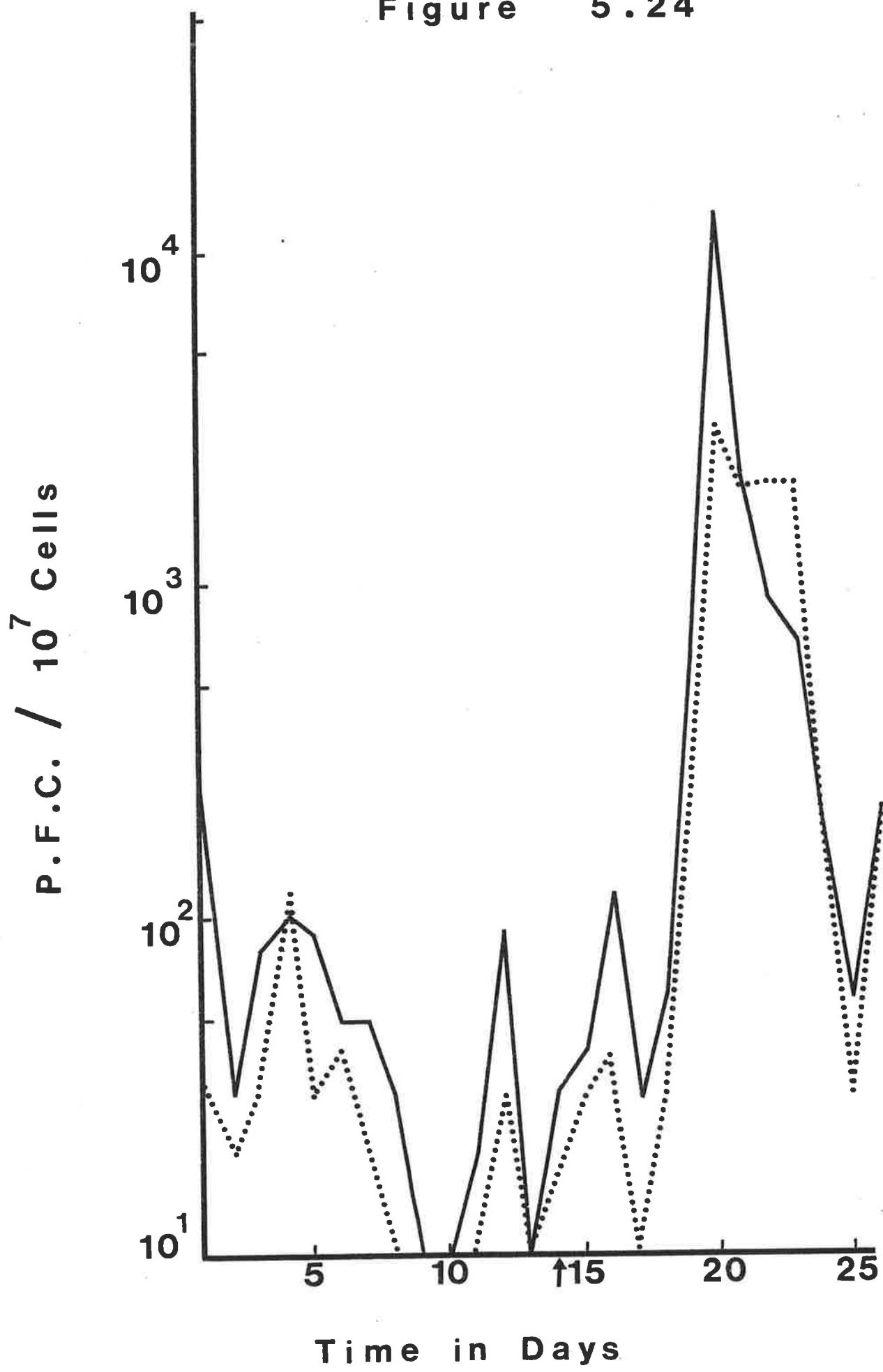


FIGURE 5.25

ORAL IMMUNIZATIONS GIVEN AT WEEKLY INTERVALS AND AN
INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAES 111 NM SR :
PFC IN THE MUCOSA OF THE SMALL INTESTINE/10⁷ VIABLE
LYMPHOID CELLS

Mice were given four oral doses of 10^{10} vibrios at weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random every day and a geometric mean of the results was obtained.

— IgM

..... IgA

Figure 5.25

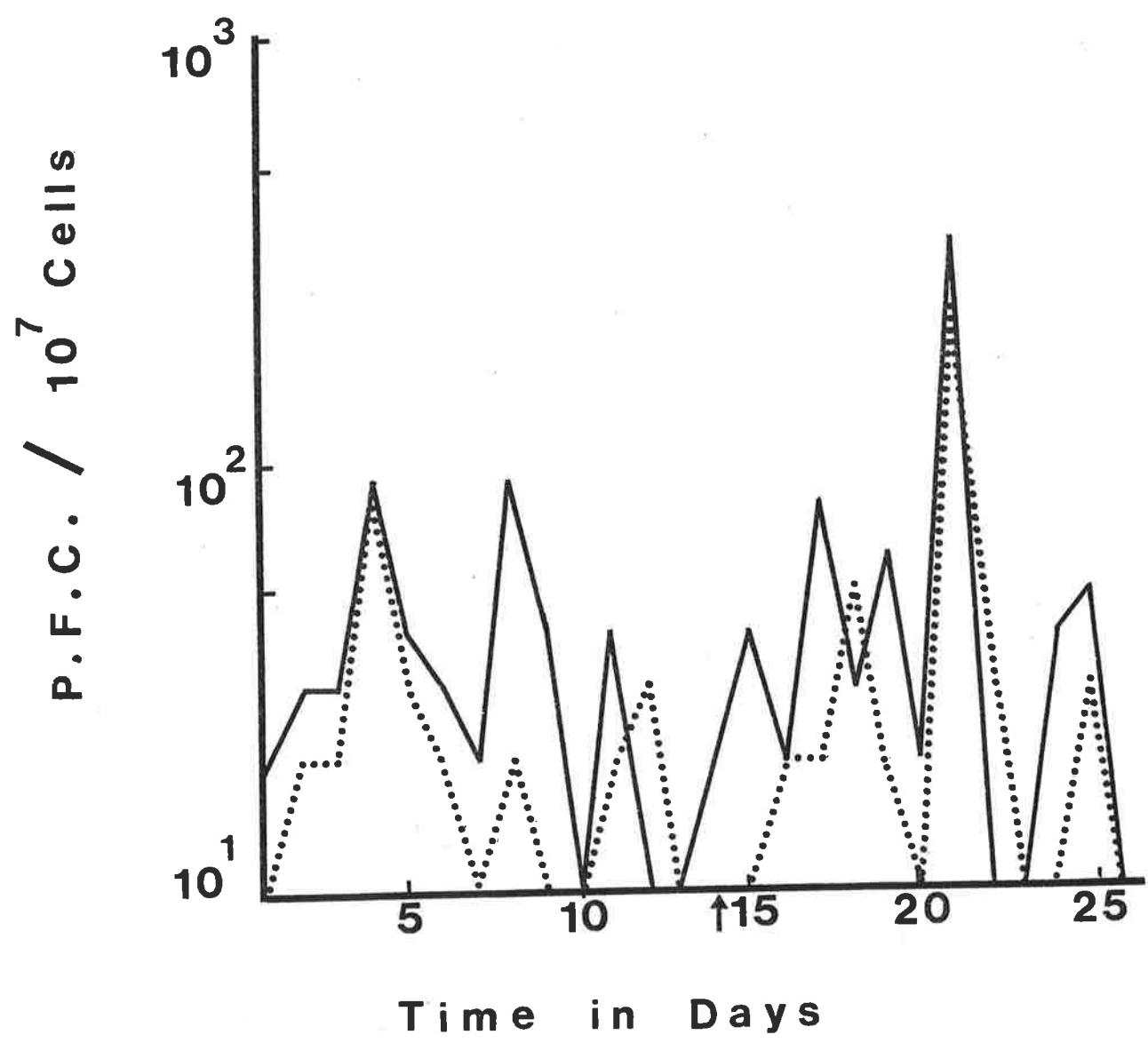


FIGURE 5.26

ORAL IMMUNIZATIONS AT WEEKLY INTERVALS AND AN INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE 569B IMP SR :
HAEMAGGLUTINATING UNITS OF ANTIBODY
PER MOUSE

Mice were given four oral doses of 10^{10} vibrios at weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by ↑). Three mice were sampled at random. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

— Serum
 Intestinal juice

Coombs enhancement of direct HA titre, expressed in additional numbers of two-fold dilutions.

	IgM	IgA	IgG2A
Serum	4	4	2
Intestinal juice	2	4	2

Figure 5.26

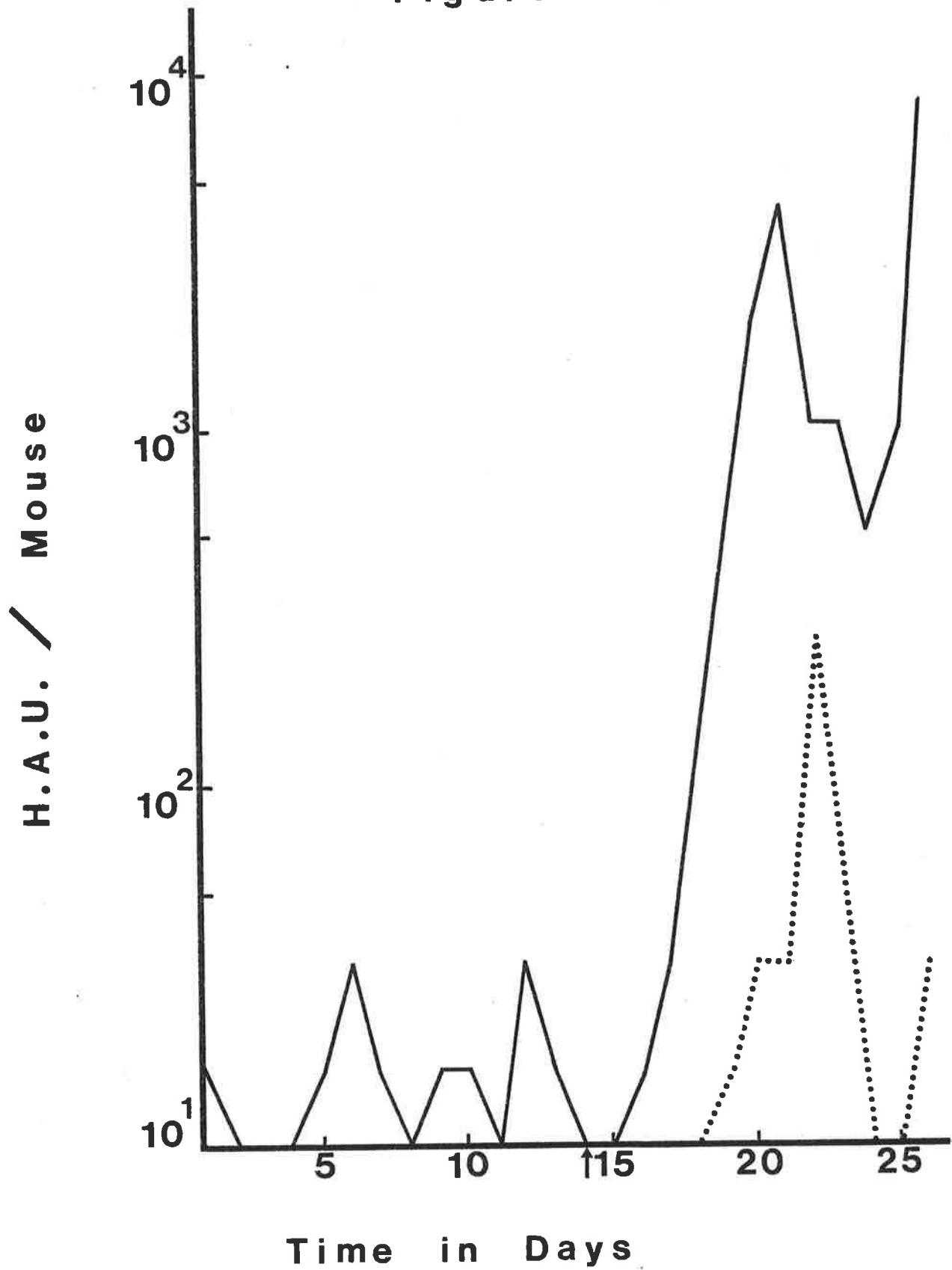


FIGURE 5.27

ORAL IMMUNIZATIONS AT WEEKLY INTERVALS AND AN INTRAVENOUS BOOSTER GIVEN WITH V. CHOLERAE 111 NM SR :
HAEMAGGLUTINATING UNITS OF ANTIBODY
PER MOUSE

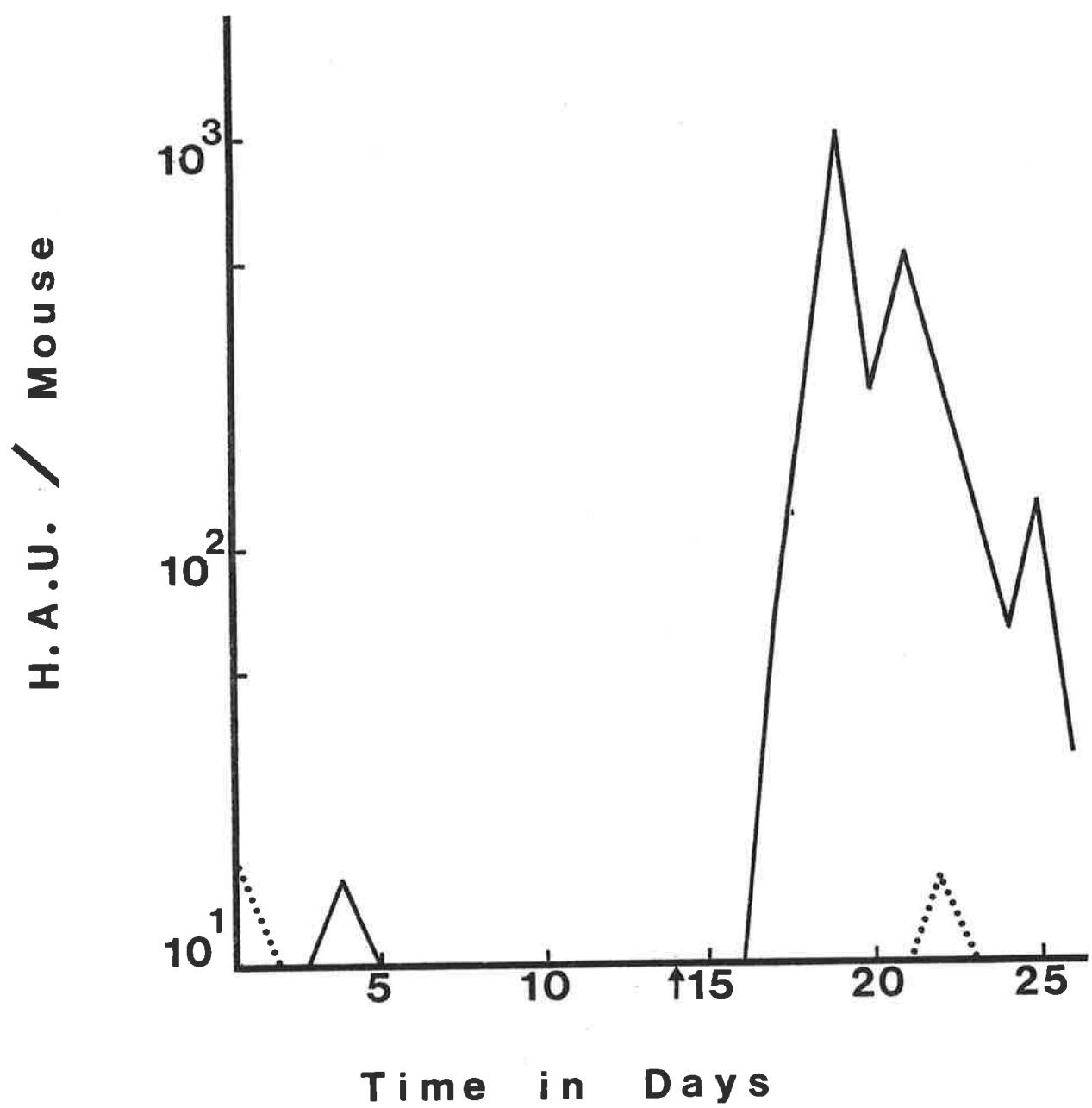
Mice were given four oral doses of 10^{10} vibrios at weekly intervals ending day 0, and 10^7 vibrios i.v. on day 14 (indicated by †). Three mice were sampled at random. Equal volumes of sera were pooled, as were equal volumes of intestinal juice.

— Serum
..... Intestinal juice

Coombs enhancement of direct HA titre expressed in additional numbers of two-fold dilutions.

	IgM	IgA	IgG2A
Serum	3	3	2
Intestinal juice	0	2	0

Figure 5.27



The expansion of the course of oral vaccinations from a period of two weeks to four weeks did not have a great deal of effect on the responses obtained. In the spleen the IgM PFC numbers were not significantly changed, apart from an increase ($p < 0.01$) in the primary response to 111 NM SR (Figs. 5.22,23 - compare with Figs. 5.12,13). The shape of the secondary IgM PFC peak in mice immunized with 569B IMP SR showed the beginning of another peak (Fig. 5.22). The formation of two peaks was also seen with HA antibody in the serum (Fig. 5.26). There was no immediate explanation for this, and as there was a correspondence between the Jerne and the HA data, this was not a phenomenon like that described in Section 5.3.

Once again IgA AFC numbers in the spleen were influenced by the length of the immunization programme. They increased significantly in the primary response ($p < 0.01$) to both strains, and in the secondary response to 111 NM SR. IgA AFC reached a record peak of $3.5 \times 10^4 / 10^7$ viable spleen cells in the response to 569B IMP SR, but like the IgM AFC, the high peak was not sustained and the overall magnitude of the response was no different ($p > 0.02$) to that obtained with twice weekly oral vaccinations (Fig. 5.12,22).

The picture in the intestine was essentially unchanged by the spacing of the course of oral doses

over four weeks. The only significant difference recorded ($p < 0.01$) was an increase in IgM AFC numbers in the primary response to 111 NM SR (Figs. 5.24,25 - compare with Fig. 5.14). However this experiment re-emphasized the basic difference between the two V. cholerae strains. Immunization with 569B IMP SR stimulated a large, unmistakable secondary response in the intestine (Fig. 5.24). As in the spleen, this was a sharper PFC peak than found with oral immunization twice weekly, but it was of similar overall magnitude. In great contrast, oral doses of 111 NM SR did not prime the lymphoid tissue of the intestine for a secondary response to an i.v. booster (Fig. 5.25). The strain difference is also clearly seen in the antibody data (Figs. 5.26,27).

5.14

Summary

The systemic and intestinal immune response of mice to Vibrio cholerae were examined and some very basic differences emerged from the work. There was a marked difference between the ease with which the spleen was stimulated, and the difficulty in obtaining a secretory immune response in the intestine. Only one immunization schedule effectively stimulated the lymphoid tissue of the intestine and that was a course of oral immunizations followed by an i.v. booster dose. The reason for this was not readily apparent because the antigen from an i.v. dose could reach the local lymphoid tissue to

stimulate a secondary response there. It then becomes hard to understand why multiple i.v. immunizations could not prime the intestine for a secondary response to an i.v. booster.

The time over which the oral immunizations were given influenced the responses. Longer courses increased the numbers of IgA PFC in the spleen and the intestine, probably because the time required for IgA priming was a limiting factor. IgM PFC in the spleen were uninfluenced by the length of the immunization schedule, which was understandable because IgM development was shown to be more rapid than that for IgA. However, the IgM response of the intestine could be greatly reduced by the delivery of the oral doses over too short a period of time. This was interpreted as showing the gut lymphoid tissue to have a lower antigen retaining capacity in comparison with systemic tissue.

The work confirmed that the capacity of a V. cholerae strain to establish and multiply in the small intestine increased its effectiveness as an oral vaccine. 569B IMP SR and 111 NM SR were shown to be equally immunogenic given in parenteral vaccination schedules, and yet wide strain differences were seen with oral vaccinations. Apart from the greater systemic and local responses to oral immunization with 569B IMP SR, only this strain primed the intestine for a large secondary response to i.v. boosting. The superiority of 569B IMP SR was seen to be related to its ability to adhere to the mucosa of

the small intestine, whereas ¹¹¹ NM SR was rapidly and completely eliminated.

An evaluation of the techniques used confirmed that they were the most appropriate for this study. The data from the Jerne and the HA assays complemented each other and gave reliable information as to the extent and antibody composition of an immune response.

CHAPTER 6

CHAPTER 6RECALL OF THE IMMUNE RESPONSE OF MICE
TO VIBRIO CHOLERAE6.1 Introduction

There is a problem in maintaining immunity to cholera, as it lasts for a year or two at the most following vaccination (review in Section 1.20). Freter (1962) has suggested that regular oral doses of heat inactivated V. cholerae would sustain the protection afforded by an immunization program. However, it is now well established that oral vaccines prepared from killed bacteria are of less immunogenic effectiveness than similar vaccines prepared from live organisms. The organisms with which this has been found include polioviruses (Ogra, Karzon, Righthand and Mac Gillivray, 1968; Ogra and Karzon, 1969), salmonellae (Hornick et al, 1970), shigellae (W.H.O. Techn. Rep. 500, 1972), BCG (Muller-Schoop and Good, 1975) and Vibrio cholerae (Cash et al, 1974a; Freter, 1962; Freter and Gangarosa, 1963).

It must be considered whether the preparation of oral vaccines from killed organisms results in the loss of antigens which are important for eliciting a protective immune response. The majority of antibodies in a serum raised against V. cholerae are specific for the heat stable lipopolysaccharide antigenic complex (Neoh and Rowley, 1970), although some seem to be specific for heat labile H-antigens (Steel, 1975). The latter may include antibodies

against an antigen associated with flagella which seem particularly protective in the infant mouse cholera model (Eubanks, Guentzel and Berry, 1977).

The inactivation of bacteria also results in the loss of properties such as motility, and this too may influence the immune response to an oral vaccine. It was seen in Chapter 5 how the ability of V. cholerae to populate the small intestine was related to its immunogenicity as an oral vaccine. This ability is enhanced by the degree to which the bacteria adhere to the mucosa of the small intestine (Chapter 3; Nelson, Clements and Finkelstein, 1976), and by the motility of the organisms (Guentzel, Field, Eubanks and Berry, 1977). In fact the adhesiveness of V. cholerae is closely related to its motility (Jones and Freter, 1976; Freter and Jones, 1976; personal communication, S.R. Attridge). The inactivation of V. cholerae probably reduces its adhesiveness as it is mediated by a haemagglutinin (Finkelstein et al, 1977) which is extremely labile (Zinnaka, Shinodori and Takeya, 1964). Therefore, the loss of motility and adhesiveness may be important factors in the relatively poor immune response to killed vibrios given orally.

It has been questioned whether the recall of a secretory immune response is best achieved by a local application of antigen, alive or dead, on the grounds that secretory antibodies may prevent contact between the antigen and the submucosal lymphoid tissue (Svennerholm et al, 1977). Svennerholm suggests that this could explain the commonly experienced difficulty in boosting an immune

response in the mucosa by local administration of antigen, and she recommends a parenteral approach. The work in this Chapter examines these issues by comparing the recall of the immune response of mice to V. cholerae using different methods of immunization.

6.2 Experimental Method

The experiments detailed in this chapter examine the systemic and local immune responses of mice given a single dose of V. cholerae following the end of an intensive vaccination program. This program consisted of four doses of 10^{10} V. cholerae given orally at twice weekly intervals and ending on day 0, followed by 10^7 V. cholerae given intravenously on day 14. The regime was the same as the one described in Section 5.9, which elicited strong systemic and local immune responses to the organism.

The objective of these experiments was to determine the optimal way in which to recall the immune response of mice to V. cholerae. Three alternatives were studied : a parenteral dose of 10^7 organisms, or an oral dose of 10^{11} organisms alive or disrupted mechanically. These were given 12 days after the i.v. immunization, that is, on day 26 of the vaccination program.

6.3 Vaccines of Live V. cholerae 569B IMP SR

Mice were intensively immunized with V. cholerae 569B IMP SR as described in the last section. They were challenged with live organisms of the same strain on day

FIGURE 6.1

RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OF
LIVE V. CHOLERAE 569B IMP SR : SPLEEN PFC PER 10^7
VIABLE LYMPHOID CELLS

A. Intravenous challenge

B. Oral challenge

Mice were given four oral doses of 10^{10} organisms at twice-weekly intervals ending day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with either A. 10^7 i.v. or B. 10^{11} orally.

Each day three mice were selected at random from both groups, and the results were expressed as a geometric mean.

— IgM PFC

..... IgA PFC

Figure 6.1

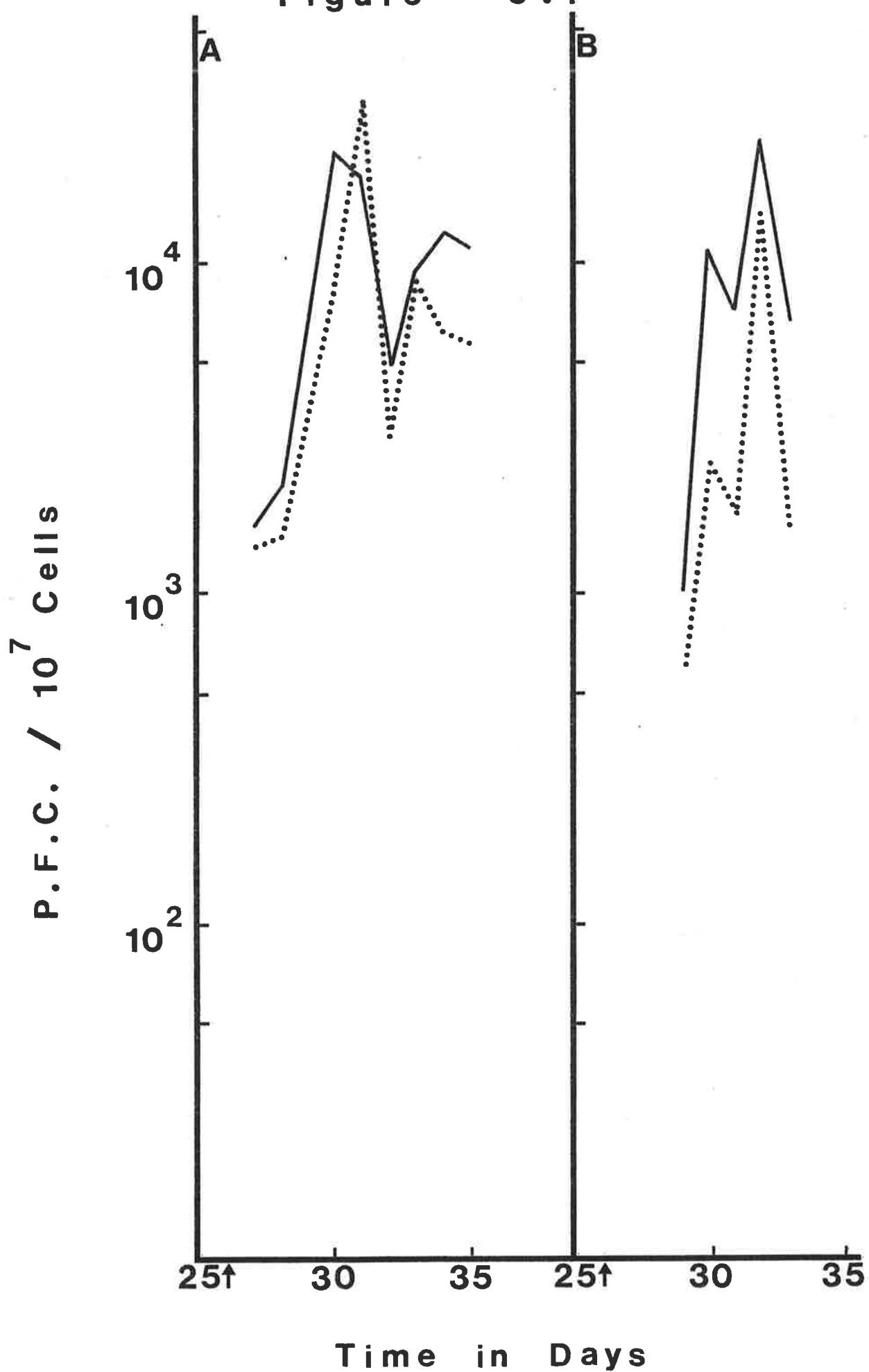


FIGURE 6.2

RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OF LIVE

V. CHOLERAE 569B IMP SR : PFC IN THE MUCOSA OF THE

SMALL INTESTINE PER 10^7 VIABLE LYMPHOID CELLS

A. Intravenous challenge

B. Oral challenge

Mice were given four oral doses of 10^{10} organisms at twice-weekly intervals, ending day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with either A. 10^7 i.v. or B. 10^{11} orally.

Each day three mice were selected at random from both groups, and the results were expressed as a geometric mean.

— IgM PFC
..... IgA PFC

Figure 6.2

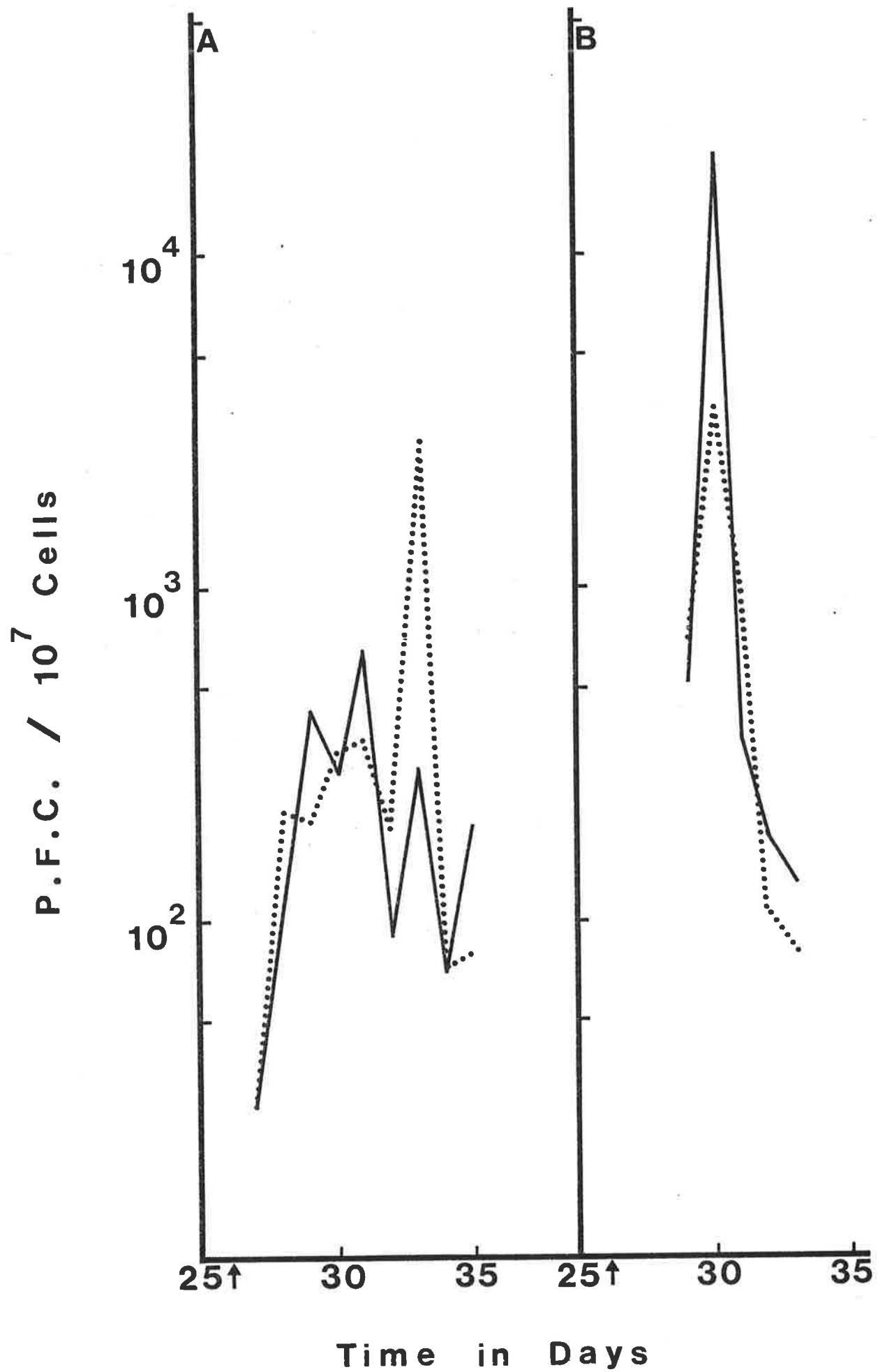


FIGURE 6.3

RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OF LIVE
V. CHOLERAE 569B IMP SR : UNITS OF HAEMAGGLUTINATING
ANTIBODY PER MOUSE

A. Intravenous challenge.

B. Oral challenge.

Mice were given four oral doses of 10^{10} organisms at twice-weekly intervals ending on day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with either A. 10^7 i.v. or B. 10^{11} orally.

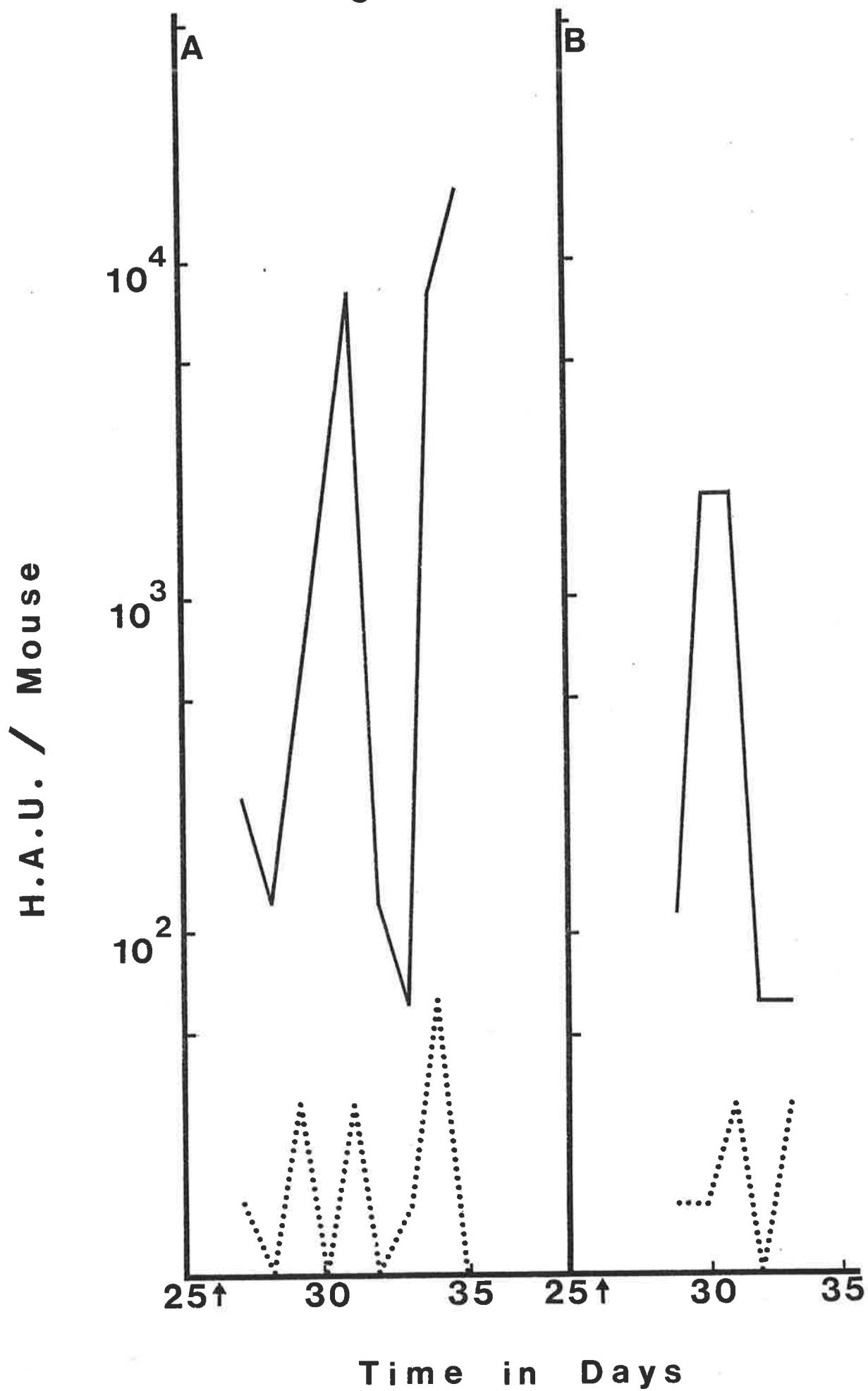
Each day three mice were selected at random from both groups. Equal volumes of serum were pooled, as were equal volumes of intestinal juice.

— Serum H.A. units
 Intestinal juice H.A. units

Coombs enhancement of the H.A. titres, expressed as additional numbers of two fold dilutions

		IgM	IgA	IgG2A
Serum	A	3	3	2
	B	3	3	2
Intestinal juice	A	0	3	0
	B	0	4	0

Figure 6.3



26 in order to recall the immune response : in this experiment the efficacy of challenge via the parenteral route was compared with that of the oral route.

It can be seen from Figures 5.12,14 that on the day of challenge, day 26, there were still substantial numbers of antibody forming cells in the spleen and in the mucosa of the small intestine. There were also 64 haemagglutinating units of antibody per mouse intestine, a relatively high figure which can also be expressed as a titre of 1/640 or more (Fig. 5.15). Therefore it might be expected that antigen delivered orally would be excluded from the local immune system and would not stimulate a response as well as an i.v. dose (Svennerholm et al, 1977). It must be emphasized that the i.v. dose was 10^7 bacteria and that the oral dose was 10^{11} , which means that comparisons must be viewed with caution.

Both the i.v. and the oral immunization recalled the responses in the spleen and in the lymphoid tissue of the small intestine (Fig. 6.1,2), but did not increase them significantly ($p > 0.5$) overall in comparison with the previous data (Figs. 5.12,14). However, the peak numbers of antibody forming cells in the intestine after the oral challenge reached the very high figures of 2×10^4 IgM AFC/ 10^7 viable cells and 4×10^3 IgA AFC/ 10^7 viable cells. It could be concluded that despite the intestinal antibody, oral challenge with enormous numbers of live 569B IMP SR was as effective immunogenically as a parenteral challenge.

The data bring out an interesting point in regard

to the sequence of the spleen and the gut immune responses. The oral challenge was followed by a peak of AFC numbers in the intestine on day 30 (Fig. 6.2B), and in the spleen on day 32 (Fig. 6.1B). In contrast, the i.v. dose evoked an AFC peak in the spleen on days 31, 32 (Figs. 6.1A), with an IgM AFC maximum in the intestine on day 31 and an IgA AFC maximum on day 33 (Fig. 6.2A). Although it could be argued that the asynchrony of the responses revealed an AFC migration between the two lymphoid organs (Robertson and Cooper, 1972, 1973), this does not accord with our knowledge of the separate nature of the systemic and the secretory lymphocyte pools (Section 1.3). More probably, the data show that antigen given i.v. stimulates the spleen first, and that the oral dose encounters the lymphoid tissue of the intestine first.

Once again there was a profound depression in the level of haemagglutinating antibodies in the serum between six and seven days after immunization (Fig. 6.3) which did not have a counterpart in the spleen using the Jerne assay (Fig. 6.1). In this instance there was a slight depression in the spleen PFC count on day 32, after i.v. immunization. It was not large enough to account for the apparent loss of serum antibody, but it might have reflected the same process. The response to the oral challenge was only measured to day 33, but it is likely that the serum antibody levels would have increased within a day or two (Fig. 6.3B).

The antibody activity in the intestinal juice constantly fluctuated (Fig. 6.3). The main reason for this

FIGURE 6.4

RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OF LIVE
V. CHOLERAE 111 NM SR : SPLEEN PFC PER 10^7 VIABLE
LYMPHOID CELLS

A. Intravenous challenge

B. Oral challenge

Mice were given four oral doses of 10^{10} organisms at twice-weekly intervals, ending day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with either A. 10^7 or B. 10^{11} orally.

Each day three mice were selected at random from both groups, and the results were expressed as a geometric mean.

— IgM PFC
..... IgA PFC

Figure 6.4

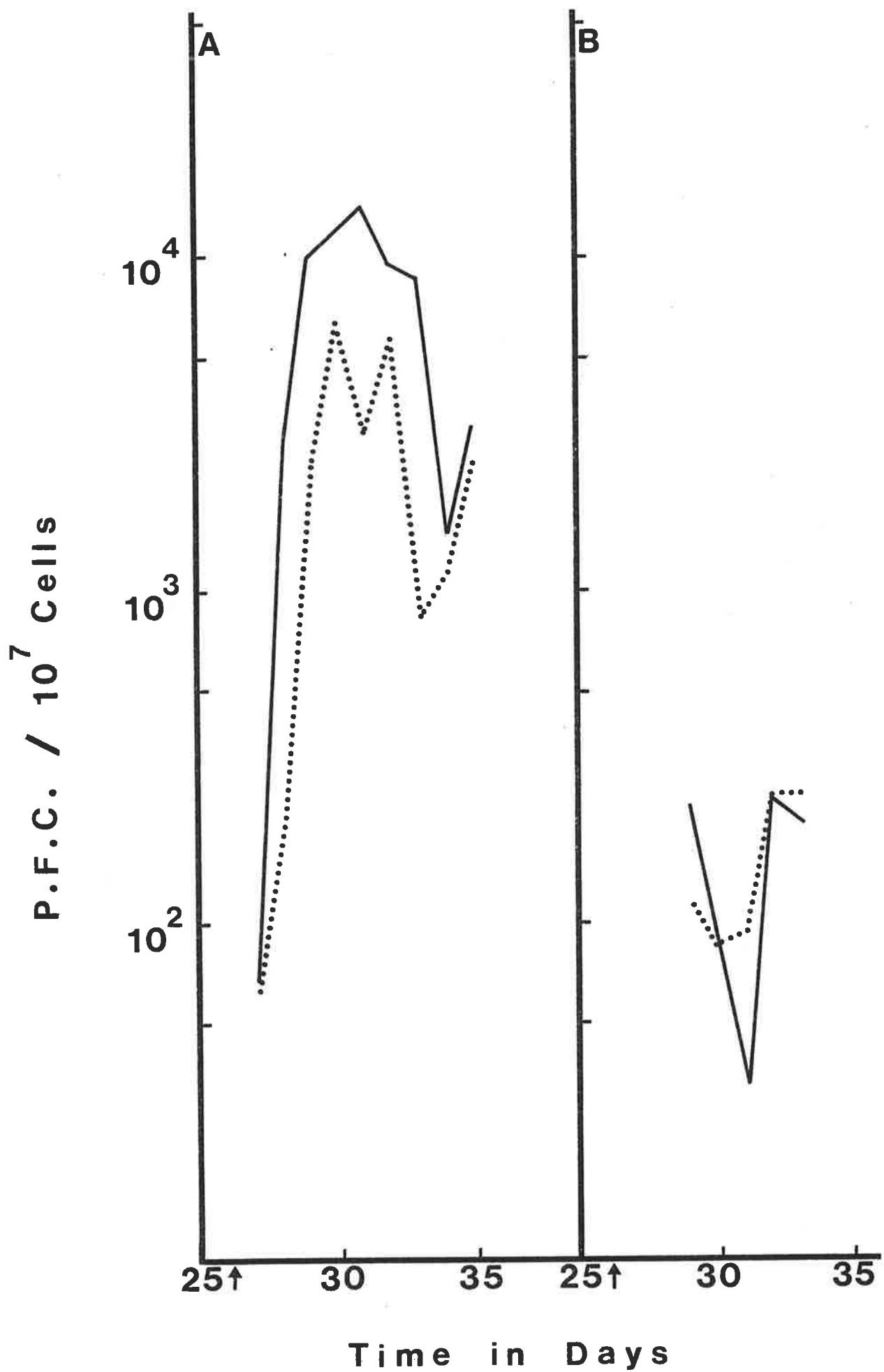


FIGURE 6.5

RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OF LIVE
V. CHOLERAE ·111 NM SR : PFC IN THE MUCOSA OF THE SMALL
INTESTINE PER 10^7 VIABLE LYMPHOID CELLS

A. Intravenous challenge

B. Oral challenge

Mice were given four oral doses of 10^{10} organisms at twice-weekly intervals, ending day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with either A. 10^7 or B. 10^{11} orally.

Each day three mice were selected at random from both groups, and the results were expressed as a geometric mean.

— IgM PFC

..... IgA PFC

Figure 6 . 5

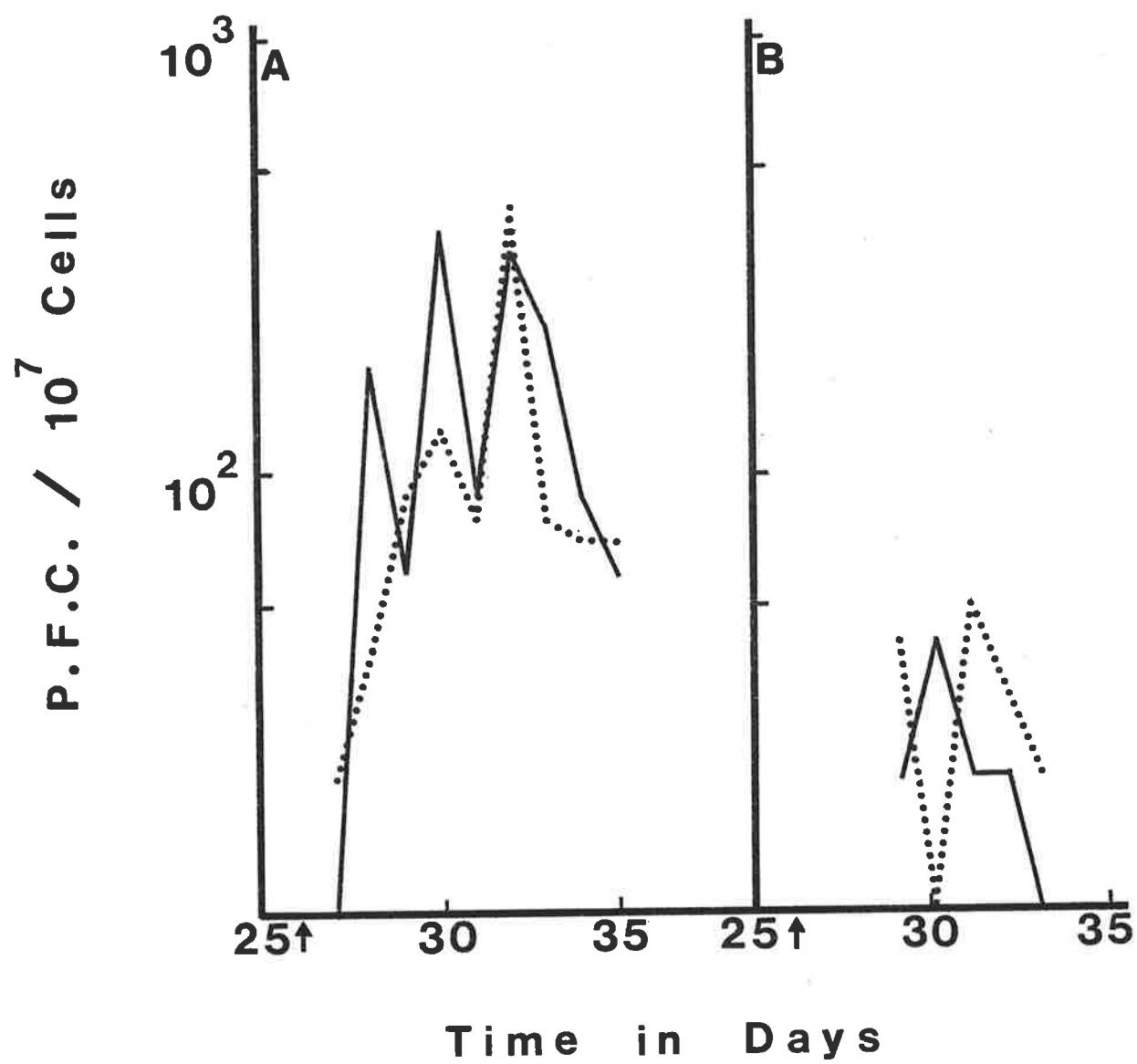


FIGURE 6.6

RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OF LIVE
V. CHOLERAE 111 NM SR : UNITS OF HAEMAGGLUTINATING
ANTIBODY PER MOUSE

A. Intravenous challenge

B. Oral challenge

Mice were given four oral doses of 10^{10} organisms at twice-weekly intervals ending day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with either A. 10^7 or B. 10^{11} orally.

Each day three mice were selected at random from both groups. Equal volumes of serum were pooled, as were equal volumes of intestinal juice.

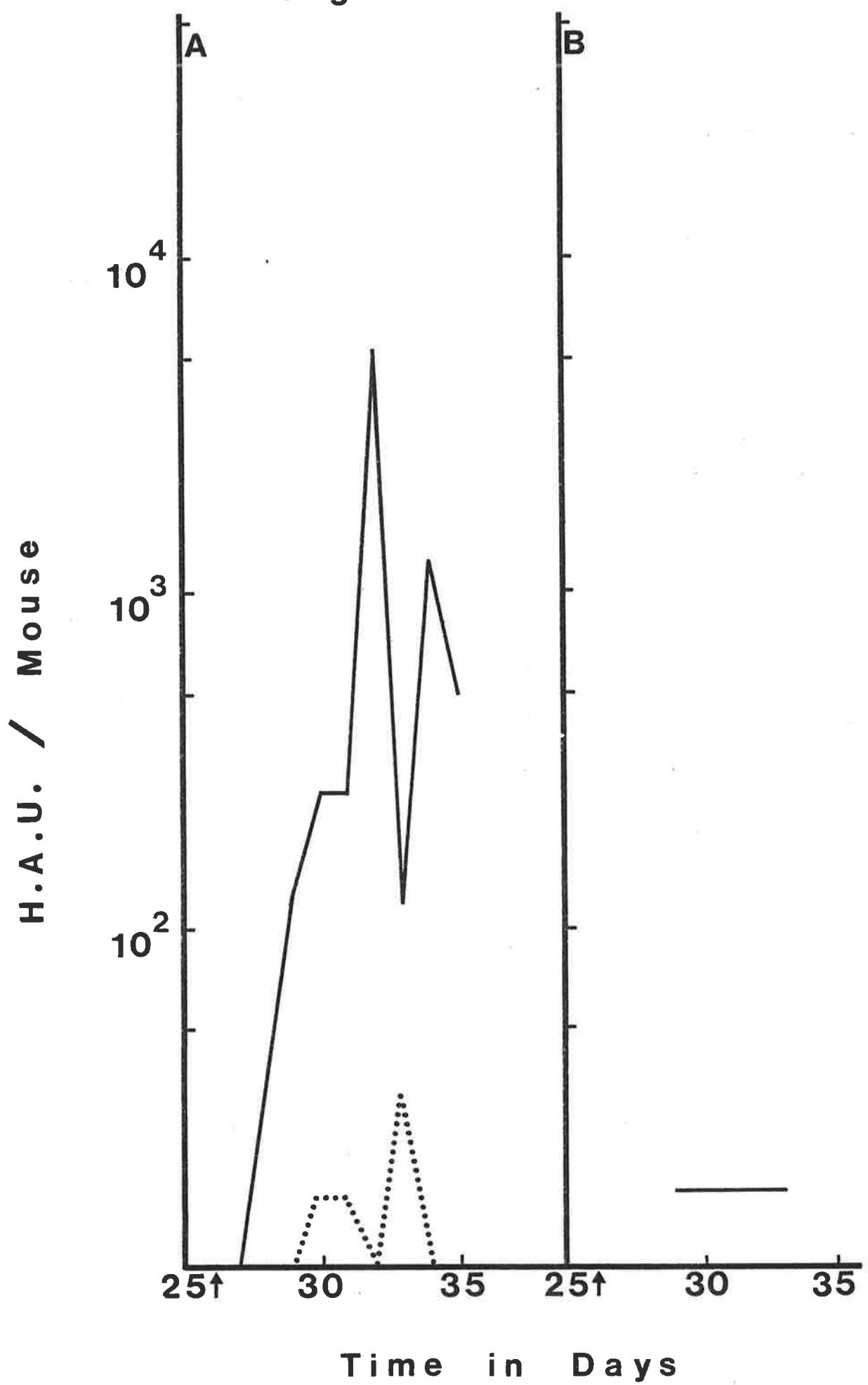
— Serum H.A. units

..... Intestinal juice H.A. units

Coombs enhancement of the H.A. Titres, expressed as additional numbers of two fold dilutions.

		IgM	IgA	IgG2A
Serum	A	5	4	2
	B	3	3	0
Intestinal juice	A	0	4	0
	B	0	4	0

Figure 6.6



would have been changes in the flow of intestinal juice causing changes in the concentration of secretory antibodies. Even though the IgM response in the mucosa of the intestine reached a very high peak after oral immunization (Fig. 6.2B), no IgM was detected in the intestinal juice by Coombs enhancement (Fig. 6.3B). The IgM was probably degraded, but its fragments could still have been active and it may have been possible to assay them in the baby mouse protection test (Steele, Chaicumpa and Rowley, 1975).

6.4 Vaccines of Live *V. cholerae* 111 NM SR

The adherent 569B IMP SR strain could stimulate the systemic and the secretory immune systems of previously immunized mice when given by the oral or the intravenous route (Section 6.3). A parallel experiment was performed with the non-adherent 111 NM SR strain. It was expected that the intravenous immunization with this strain would recall the immune response but that an oral dose would not, going on the results detailed in Chapter 5.

10^7 live organisms given i.v. on day 26 provoked a statistically significant increase in the IgM ($p = 0.02$) and the IgA ($p < 0.02$) PFC responses in spleen and the gut (Figs. 6.4A, 5A) compared with those shown previously (Figs. 5.13,14). The splenic response was similar to that seen after two i.v. immunizations with 111 NM SR (Fig. 5.2) but the numbers of AFC in the mucosa of the small intestine were the highest that were attained with this strain. It was apparent that unlike 569B IMP SR,

four oral doses of 111 NM SR could not maximally prime mice for an immune response to a parenteral immunization.

An oral dose of 10^{11} of these organisms failed to recall the immune response in either the spleen (Fig. 6.4B) or the intestine (Fig. 6.5B) of previously immunized mice. This startling contrast between oral and parenteral immunization provided further evidence that the inability of 111 NM SR to proliferate in the gastro-intestinal tract of mice is directly related to its relative failure as an oral immunogen.

If anything, oral immunization with live 111 NM SR temporarily depressed the IgM AFC count in the spleen (Fig. 6.4B). This effect of an oral vaccine of live 111 NM SR was seen in Fig. 5.11 where it was shown to be statistically significant. It is possible that the rapid elimination of the organisms from the intestine only permitted sub-immunogenic amounts of specific antigen to enter the circulation, but in concentrations also low enough to induce a temporary suppression of the immune response (Thomas and Parrott, 1974).

The data obtained with the haemagglutination assay (Fig. 6.6) confirmed the results from the Jerne assay, with serum antibody reaching a high level after i.v. immunization, but not after the oral dose. It must be concluded that oral vaccines will only maintain immunity to cholera if they are prepared from an appropriate strain of V. cholerae, whereas parenteral vaccines do not have this restriction.

FIGURE 6.7

RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OF
INACTIVATED *V. CHOLERAES* : SPLEEN PFC PER 10^7 VIABLE
LYMPHOID CELLS

A. *V. cholerae* 569B IMP SR

B. *V. cholerae* 111 NM SR

Mice were given four oral doses of 10^{10} organisms at twice-weekly intervals, ending day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with 10^{11} inactivated* *V. cholerae* of the same strain given orally.

Each day three mice were selected at random from both groups, and the results were expressed as a geometric mean.

— IgM PFC
..... IgA PFC

*The bacteria were inactivated by two passages through a French press. 0.1% of the organisms remained viable.

Figure 6.7

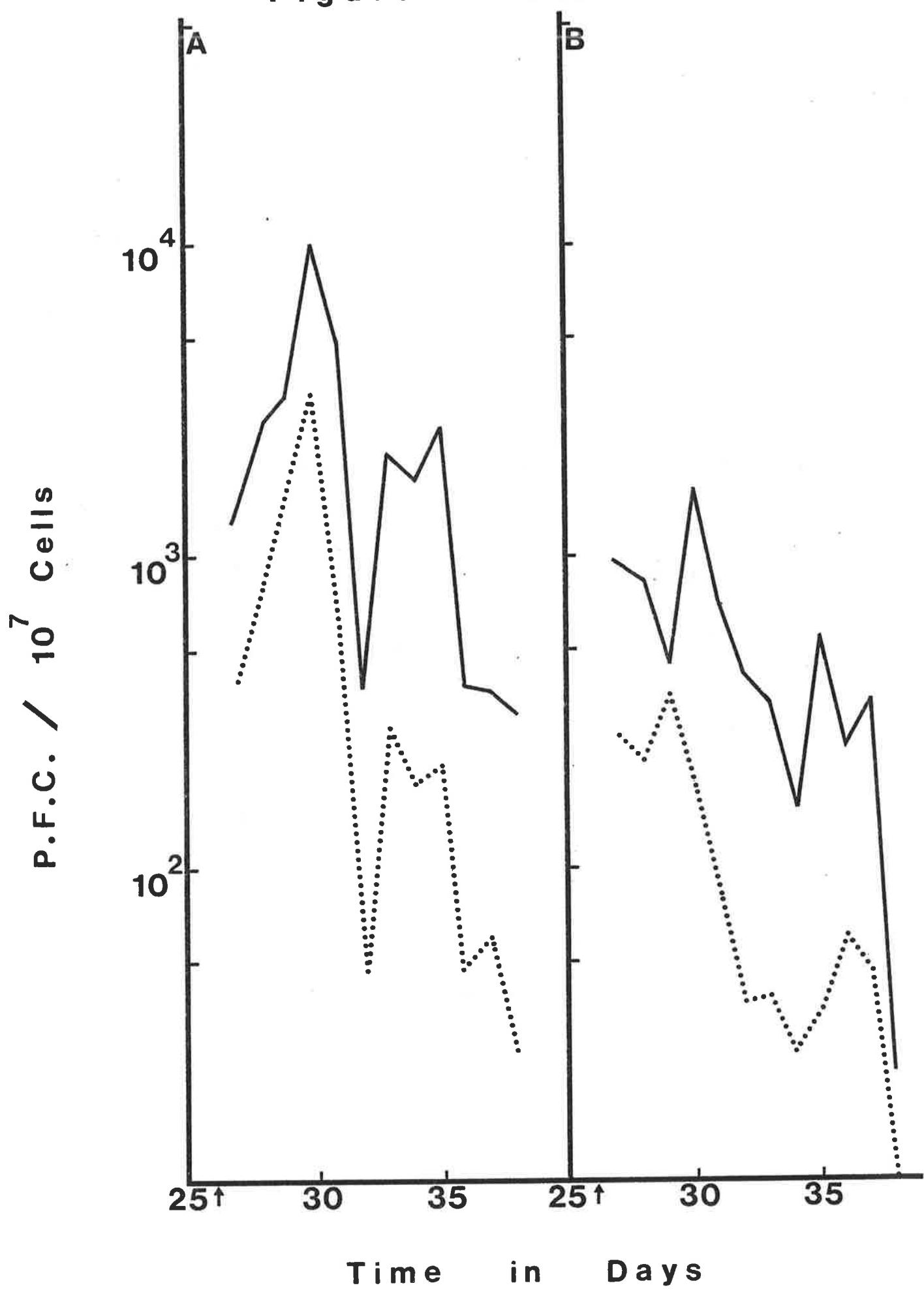


FIGURE 6.8RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OFV. CHOLERAE : PFC IN THE MUCOSA OF THE SMALL
INTESTINE PER 10^7 VIABLE LYMPHOID CELLSA. V. cholerae 569B IMP SRB. V. cholerae 111 NM SR

Mice were given four oral doses of 10^{10} organisms at twice-weekly intervals, ending day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with 10^{10} inactivated* V. cholerae of the same strain given orally.

Each day three mice were selected at random from both groups, and the results were expressed as a geometric mean.

— IgM PFC

.... IgA PFC

*The bacteria were inactivated by two passages through a French press. 0.1% of the organisms remained viable.

Figure 6.8

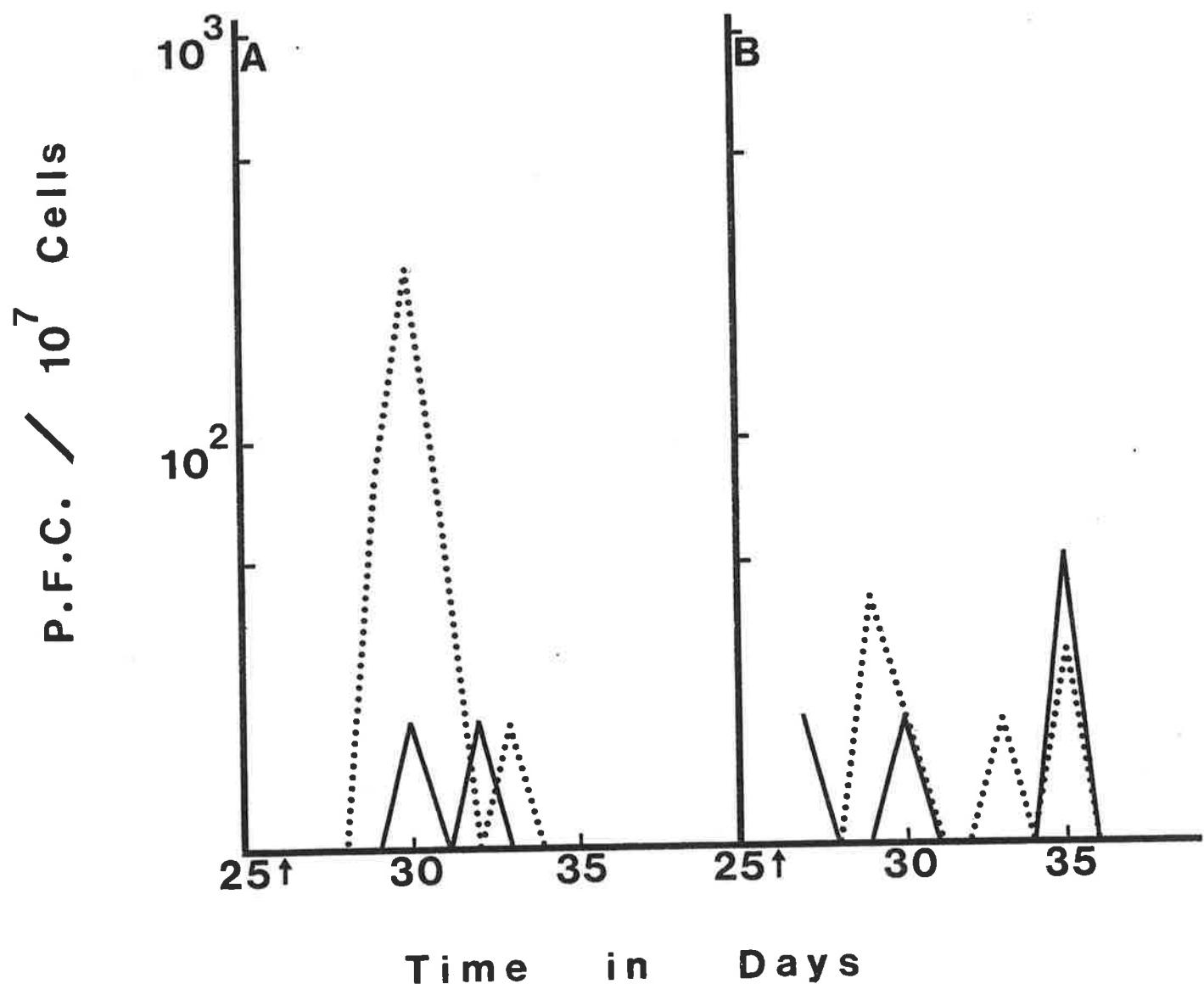


FIGURE 6.9

RECALL OF THE IMMUNE RESPONSE OF MICE WITH VACCINES OF
INACTIVATED *V. CHOLERAЕ* : UNITS OF HAEMAGGLUTINATING
ANTIBODY PER MOUSE

A. *V. cholerae* 569B IMP SR.

B. *V. cholerae* 111 NM SR.

Mice were given four oral doses of 10^{10} organisms at twice weekly intervals, ending day 0, followed by 10^7 i.v. on day 14. They were challenged on day 26 with 10^{11} inactivated* *v. cholerae* of the same strain given orally.

Each day three mice were selected at random from both groups. Equal volumes of serum were pooled, as were equal volumes of intestinal juice.

— Serum H.A. units

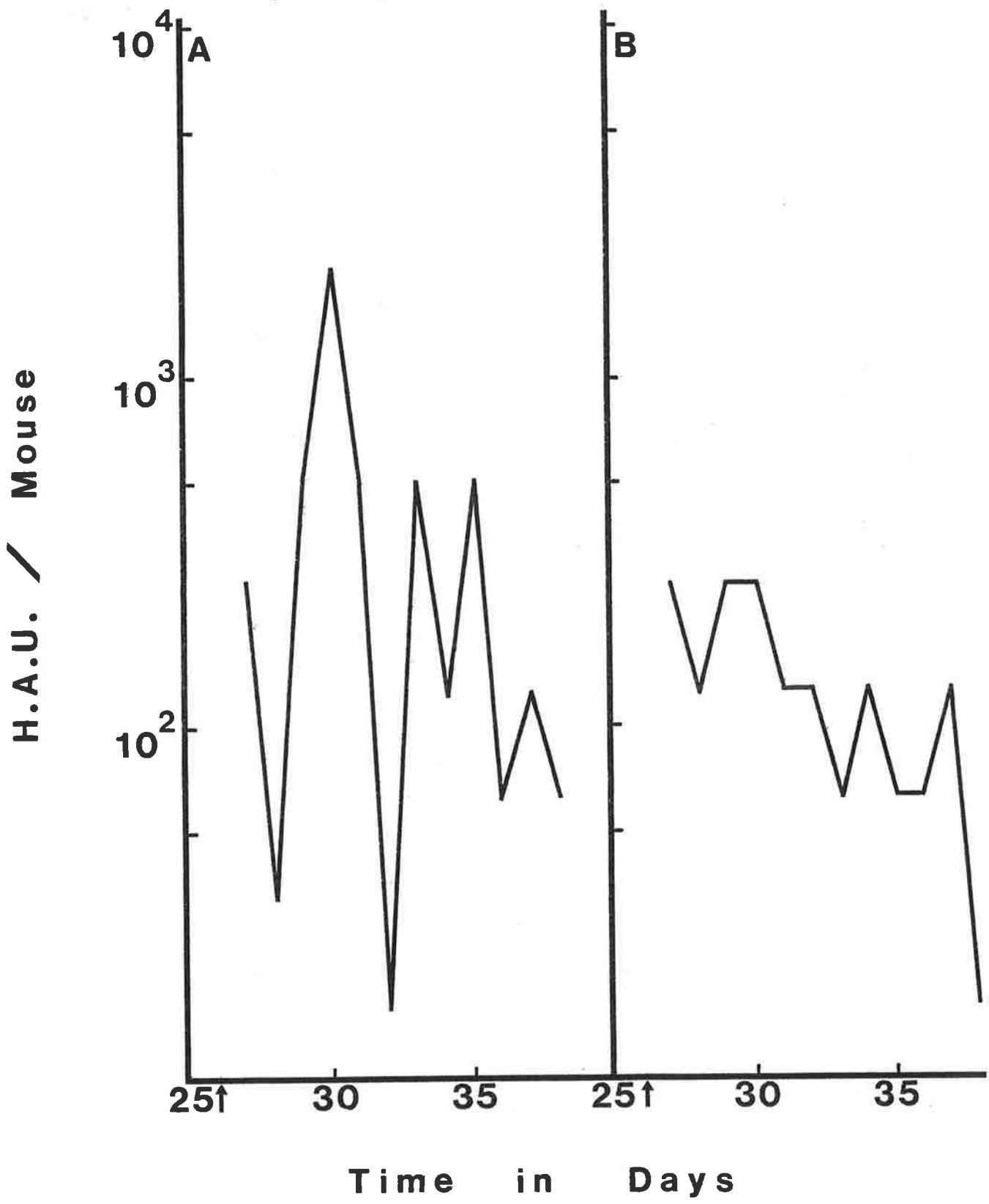
..... Intestinal juice H.A. units

*The bacteria were inactivated by two passages through a French press. 0.1% of the organisms remained viable.

Coombs enhancement of the H.A. titres, expressed as numbers of two fold dilutions.

		IgM	IgA	IgG2A
Serum	A	4	3	1
	B	4	3	2
Intestinal juice	A	0	4	0
	B	0	4	0

Figure 6.9



6.5 Oral Vaccines of Mechanically Disrupted *Vibrio cholerae*

In general, oral vaccines prepared from killed bacteria do not stimulate immune responses as well as those prepared from live bacteria (Section 6.1). However, it is still of great interest to examine the potential of inactivated oral vaccines as they would have advantages in terms of storage, distribution, ease of administration and safety.

An attempt was made to avoid as far as possible the loss of labile antigens when the *V. cholerae* were inactivated. For this reason the methods of heat killing, formalin treatment, glutaraldehyde fixation, and ultraviolet irradiation were considered to be unsuitable. The mechanical method of French pressing was chosen because its action is to extrude bacteria through a fine aperture at high pressure, with the result that they rupture and lose their cytoplasm. The released cytoplasmic enzymes may attack the surface antigens of the bacterial envelope, and to minimize this possibility the vaccines were fed to mice immediately after preparation. This method does not kill all of the treated bacteria : after two passes through the French press the viability was reduced to 0.1%.

The mice were given the preliminary program of four oral and one intravenous immunization (Section 6.2), and were challenged orally on day 26 with a preparation of the same *V. cholerae* strain. Each oral challenge dose contained 10^{11} disrupted bacteria and 10^8 viable organisms.

Once again an oral vaccine of V. cholerae 111 NM SR failed to recall the immune response in either the spleen (Fig. 6.7B) or the mucosa of the small intestine (Fig. 6.8B), which was also shown by the levels of antibody in the serum and the intestinal juice (Fig. 6.9B). The inactivated 569B IMP SR organisms stimulated significantly ($p < 0.01$) lower responses in the spleen (Fig. 6.7A) and the intestine (Fig. 6.8A) than an oral vaccine of live organisms (Fig. 6.1B, 2B). The spleen PFC appeared in two phases (Fig. 6.7A), as did the serum haemagglutinating antibody (Fig. 6.9A). This too was probably another example of a temporary immune suppression on the sixth day after immunization as noted previously.

The results clearly demonstrate that inactivation of V. cholerae reduces its effectiveness as an oral immunogen despite the precautions taken against the loss of labile antigens. It is probable that there was a loss of factors which aid the delivery of bacterial antigen to the gut associated lymphoid tissue, and these may have included replication, motility and the adhesive haemagglutinin. The data also show that 10^8 live V. cholerae given orally are not sufficient to recall the response of previously immunized mice.

6.6 Summary

Mice were intensively immunized with Vibrio cholerae and attempts were made to recall the immune response with different vaccination methods. It was concluded that large doses of live bacteria given orally could recall the

response in both the lymphoid tissue of the spleen and the mucosa of the small intestine, but only if the strain was able to populate the gut for at least a short time, like 569B IMP SR. Even the effectiveness of an oral vaccine prepared from the relatively persistent 569B IMP SR strain was lost if the bacteria were inactivated. This occurred despite the use of a mechanical process which minimized damage to the bacterial antigens, and which even left 0.1% of the bacteria living. It is likely that the disrupted bacteria lost their ability to adhere to the mucosa of the small intestine, so reducing the delivery of antigen to the immune system.

It was apparent that parenteral immunization was the most reliable method for maintaining the immune response of mice to V. cholerae. By this route different bacterial strains were effective, and fewer organisms were required than via the oral route.

CHAPTER 7

CHAPTER 7DISCUSSION7.1 Introduction

The Discussion will centre upon the stimulation of local immunity in the intestine, in particular to Vibrio cholerae, in light of the experimental findings. Firstly, the factors which mediate the virulence of V. cholerae, and their inhibition by the action of antibody, will be considered. Then the current approaches to cholera immunization, and future possibilities which may be used to improve the efficacy of vaccination, will be assessed. Finally, the secretory immune system of the intestine will be discussed in relation to its special attributes leading to the induction of local immune responses, memory and tolerance.

7.2 Interactions between Vibrio cholerae and the Intestine

The data in Chapter 3 suggest that the adherence of vibrios to the mucosa of the intestine is essential for the initiation of an infection, a conclusion reached in numerous other reports (Freter, 1969, 1970, 1972; Guentzel and Berry, 1975; Lankford, 1960; La Brec, Sprinz, Schneider and Formal, 1965). The adhesive interaction appears to be mediated by a cell bound haemagglutinin (Jones, Abrams and Freter, 1976; Finkelstein, Arita, Clements and Nelson, 1977). Other reports indicate that motility is also an important virulence factor in V. cholerae.

(Eubanks, Guentzel and Berry, 1976; Guentzel and Berry, 1975). The reason for this is uncertain, but it seems that motility is correlated with the presence of the adhesive haemagglutinin, even though this is not present on the flagellum (Jones and Freter, 1976; Freter and Jones, 1976). Other workers have concluded that motility per se is a direct virulence factor as it distributes the vibrios between the villi and deep within the crypts of Lieberkuhn (Schrink and Verwey, 1976; Guentzel, Field, Eubanks and Berry, 1977).

Antibody might act by neutralizing the virulence factors of V. cholerae, which could include adherence, motility and its enterotoxin. It has also been proposed that antibody may directly mediate the killing of the organisms in the intestine (Freter, 1970, 1971; Chaicumpa and Rowley, 1972; Knop and Rowley, 1975b,c). However, the evidence in Chapter 3 suggests that the initial protective action of antibody is to block the adherence of the vibrios to the mucosa. Other workers have shown that the adherence may be blocked directly (Freter, 1969, 1970, 1972), or indirectly by the agglutination of the bacteria (Bellamy, Knop, Steele, Chaicumpa and Rowley, 1975; Steele, Chaicumpa and Rowley, 1975; Schrank and Verwey, 1976). Antibody may also act by immobilizing the flagellum (Guentzel et al, 1977). These observations are also in keeping with the ability of IgA, F(ab)₂ and Fab to mediate protection of baby mice against an oral challenge with V. cholerae, as the sole property remaining in the

fractions would seem to be the capacity to bind antigen (Steele, Chaicumpa and Rowley, 1974, 1975).

7.3 Immunization against Cholera

7.3a Current status : The best immunity to cholera which has been induced in volunteers followed an actual cholera infection (Cash et al, 1974a). Oral vaccines can be prepared from attenuated organisms which do not establish and grow in the intestine, however, they do not stimulate effective immunity (Cash et al, 1974b). Oral doses of heat killed vibrios given many times over a long period of time can confer some protection from the disease, but this would be an impractical approach in the areas of greatest need (Freter, 1962; Freter and Gangarosa, 1963).

Parenteral immunization provides a limited degree of protection in field trial populations, but more so for adults than for children (Benenson et al, 1968). The value of such immunizations seems to lie in a boost to secretory immunity, and there is no clear correlation between titres of antibody in the serum and the degree of protection obtained (Mosley et al, 1972). Other workers have also found that the levels of serum antibody bear little relation to cholera attack rates (Cash et al, 1974a) or to the clinical presentations (Sack et al, 1966a).

In summary, there is no immediate solution to

the problem of poor efficacy of immunization against cholera. The parenteral immunization programs being currently employed against cholera in Asia are only providing incomplete or short-lived protection, and safe, effective oral vaccines have not been developed.

7.3b Experimental data : Animal experiments are a requirement before one can ethically apply vaccines to man, but experience has shown that the results from such experiments may not be directly applicable to the situation in humans. However, certain encouraging parallels emerged from the present work.

Systemic immune responses to Vibrio cholerae were readily obtained in mice, for instance by two well spaced parenteral vaccinations. No purely parenteral schedule stimulated more than a minimal immune response in the intestine, as measured by the Jerne assay for antibody forming cells, and no antibody was detected in the intestinal juice, as found by the haemagglutination assay. It is possible that serum antibodies of the IgG class were transported into the intestine (Pierce and Reynolds, 1974; Heddle and Rowley, 1978), and were then degraded into fragments which would not haemagglutinate but which would have been protective in the baby mouse cholera model (Steele, Chaicumpa and Rowley, 1975). This was probably not the case as similar parenteral immunization of mice resulted in high levels of protective activity in the serum but none in the intestinal

juice (Horsfall, 1977). It was concluded that emphasis should be placed on stimulating secretory immunity.

It was difficult to obtain an immune response in the lymphoid tissue of the intestine, and it was only substantial after a course of oral vaccinations followed by a parenteral booster dose. There was a clear parallel between this result and the boost to naturally acquired secretory immunity to cholera that can be evoked with a parenteral vaccination (Svennerholm *et al*, 1977; Benenson *et al*, 1968). The special requirements of the oral vaccine, that it be prepared from live organisms which are able to grow to a certain extent in the intestine, were also in accordance with the studies in humans (Freter, 1962; Cash *et al*, 1974a,b). Therefore few improvements in cholera immunization can be expected using the current parenteral approach, if the mouse model has any relevance to humans.

7.3c Future options with whole cell vaccines : Oral vaccines cannot be made with virulent V. cholerae, but maybe with a mutant which shows a similar growth pattern in the intestine but without the production of enterotoxin. However, toxin production could be plasmid mediated (Robins-Browne, Still, Isaacson, Koornhof, Appelbaum and Scragg, 1977), and the ability of V. cholerae to accept prophages into its genetic complement gives it the potential for numerous genetic

and phenotypic variations (Ogg, Shrestha and Poudayl, 1968). In fact serotype conversions in cholera patients have been reported (Gangarosa, Santi, Saghari and Feeley, 1967). For this reason during any oral cholera vaccination program the strain would need to be constantly re-isolated from recipients and checked for toxin production.

Alternatively, oral vaccines could be prepared from suitably killed organisms which retain their ability to adhere to the mucosa of the intestine. This may be difficult because even mechanical disruption rendered an oral vaccine ineffective in mice. The mediator of adherence is apparently a haemagglutinin (Finkelstein *et al*, 1977) which is extremely labile (Zinnaka, Shinodori and Takeya, 1964). Recent work with gamma irradiation of bacteria may offer a solution to this problem. It has been found that there is a level of irradiation which prevents multiplication of *V. cholerae* but which still permits the adherence of the organism to intestinal strips *in vitro* (personal communication, Prof. D. Rowley).

There may be other bacterial, or even non-bacterial factors which enhance the uptake of antigen into the local immune system of the intestine, and these should be sought. As well, there is some promise of adjuvants for oral immunization. Local or parenteral treatment with Vitamin A can increase the local immune response of mice to BSA fed to them and

can prevent the development of unresponsiveness to large doses of the antigen (Falchuk, Walker, Perrotto and Isselbacher, 1977).

The potential of serum antibodies to protect human's against cholera attack probably has not been fully explored. It is known that serum IgG can enter the intestine in dogs in sufficient amounts to prevent cholera (Pierce and Reynolds, 1974; Heddle and Rowley, 1978). Also, it has been discovered that large quantities of immunoglobulin enter the rodent gut from the bile (personal communication, Dr. G.D.F. Jackson). Therefore it might be possible to provide humans with adequate protection with the use of parenteral vaccines, despite the disappointing results from field trials (Levine *et al*, 1977a).

7.3d Somatic antigens : Some bacteria have somatic pili, which are colonization and virulence factors (Nagy, Moon, Isaacson, To and Brinton, 1978). The adhesion factors of enteropathogenic E. coli are pili which are species specific in their action, for instance K88 in swine (Jones and Rutter, 1972), K99 in calves (Myers and Guinee, 1976) and CFA in humans (Evans, Evans, Tjoa and Du Pont, 1978). Three somatic pilus vaccines have been found to be safe and effective in hosts experimentally infected with Neisseria gonorrhoeae, Pseudomonas aeruginosa and Escherichia coli (Nagy *et al*, 1978). These apparently function

by stimulating the production of antibodies which prevent the bacteria from adhering to their target tissue.

The colonization and virulence factors of V. cholerae seem to be the cell bound haemagglutinin (Finkelstein *et al*, 1977) and motility (Guentzel, Field, Eubanks and Berry, 1977). Clearly vaccination against cholera should result in the blocking of the organism's adherence and motility in the intestine. Unfortunately there are no clearly defined surface structures of V. cholerae which mediate adhesion, and which can be extracted for use in a vaccine. The adhesive haemagglutinin is part of the surface coat of the organism (Lankford and Legsomburana, 1965; Nelson, Clements and Finkelstein, 1976), even though some studies with the electron microscope have shown an apparently fimbriate structure (Zinnaka, Shinodori and Takeya, 1964; Chulasamaya and Lankford, 1970).

There are labile antigens associated with motile vibrios which can stimulate immunity in animals (Steele, Chaicumpa and Rowley, 1975; Eubanks *et al*, 1977), and one reported immune action was the inhibition of vibrio motility (Guentzel *et al*, 1977). However, it should be noted that the major portion of antibodies in an antiserum raised against V. cholerae are specific for LPS determinants (Neoh and Rowley, 1970; Holmgren and Svennerholm, 1977). Antibodies against LPS can effectively inhibit the motility of V. cholerae.

(Benenson, Islam and Greenough, 1964) because the flagellum of this organism is sheathed (Follett and Gordon, 1963). These antibodies can also prevent the adhesive interactions of vibrios (Freter and Jones, 1976). Therefore further development of LPS vaccines should not be overlooked.

7.3e V. cholerae enterotoxin : Immunization with cholera toxoid results in significant levels of antitoxin in the serum of volunteers, whether it is given by the parenteral (Levine et al, 1977a), or the oral (Levine et al, 1978) route. However, this does not protect the volunteers against a cholera challenge (Levine et al, 1977a). A field trial has also shown that the toxoid given parenterally confers minimal protection from the disease (Curlin, Levine, Aziz, Rahman and Verwey, 1976). Despite the disappointing results with cholera toxoid in the human studies there is reason to believe that it may yet perform an important role in immunization against cholera. One encouraging observation has been that a combination of V. cholerae toxin and LPS antigens induced a more than 100-fold higher degree of immunity in the rabbit against challenge with live vibrios than did immunization with either of the two antigens alone (Svennerholm and Holmgren, 1976).

Data from animal models may shed some light on the failure of a parenteral vaccine prepared from cholera toxoid to stimulate local immunity to the disease. In general, parenteral vaccinations fail to

evoke immune responses in the intestine, and some local exposure to antigen is required (Pierce and Gowans, 1975). For instance, a parenteral dose of cholera toxoid stimulates little response in the intestine of rats (Pierce and Gowans, 1975) or dogs (Pierce, Cray and Sircar, 1978), but primes for a response to locally applied antigen. There is also evidence that, unlike the toxin, the toxoid given orally is a poor antigen with which to prime for a local response (Pierce, 1978; Pierce, Cray and Sircar, 1978). It may be less effective than cholera toxin because of two factors : only the toxin binds to the GM₁ ganglioside receptors of cells (Cuatrecasas, 1973), including lymphocytes (Holmgren, Lindholm and Lonnroth, 1974), which may enhance its trapping in the immune system; and only the toxin activates adenyl cyclase (Flores, Witkum and Sharp, 1976), which may potentiate antibody production (Ishizuka, Braun and Matsumoto, 1971).

It is not possible to feed people active cholera toxin, but it may be possible to detoxify it in such a way as to retain some of the adjuvant activity of membrane binding and adenyl cyclase activation. Alternatively, a schedule of parenteral followed by oral immunization could be adopted, using cholera toxoid (Pierce, Cray and Sircar, 1978). Such a regime was initially suggested for immunization with viruses (Ogra *et al*, 1974) and although it may not be of general use for bacterial antigens (Chapter 5) it may be

particularly applicable to bacterial toxins (Pierce and Gowans, 1975; Pierce and Reynolds, 1975). Another avenue might be to couple toxoid and LPS fragments, and this could conceivably combine the immunogenic properties of both in a synergistic fashion (Holmgren and Svennerholm, 1977).

7.3f Vibrios which penetrate the mucosa : There is some very circumstantial evidence that organisms which have the ability to penetrate the mucosa of the intestine may have an advantage as oral immunogens, for instance the successful Sabin oral polio vaccine. The local immune response to dinitrophenol-keyhole limpet hemocyanin (DNP-KLH) placed in a loop of rabbit ileum isolated in situ depends upon the presence of a Peyer's patch in the test segment (Robertson and Cebra, 1976). However, it is interesting that a vigorous response can be elicited to Shigella in segments of intestine that lack a Peyer's patch, maybe because the organism penetrates the mucosa (Keren, Holt, Collins, Genski and Formal, 1978). Hybrid bacteria which proliferate in the intestine of volunteers, but which do not penetrate the mucosa, have been constructed from E. coli exhibiting the surface antigens of Shigella (Levine et al., 1977b). An oral vaccine prepared from this potentially ideal hybrid was ineffective in promoting immunity to shigellosis, which may be another indication of the role of mucosal penetrance.

The role of penetrance in the local immuno-

genicity of bacteria should be assessed in controlled experiments, as has been done for the property of mucosal adhesiveness. There have been reports of non-agglutinable (NAG) vibrios with invasive properties (Robins-Browne *et al*, 1977). Such strains might be compared with non-invasive vibrios of the same serotype.

7.3g Assessment of new vaccines for humans : Ultimately, new cholera vaccines will have to be tested in humans, and preferably in field trials as the conditions are different in healthy volunteers (Music *et al*, 1971). Assessment of their immunogenic effectiveness should include the measurement of the immune response in the intestine, as the systemic response does not correlate as well with protection from cholera. Also, enteric immunity may persist beyond the period during which specific antibodies can be measured in the intestinal juice, and this protection may be demonstrable only by challenge (Pierce, Sack and Sircar, 1977).

7.4 Stimulation of the Secretory Immune System

The secretory immune system of the intestine proved to be far more refractory to stimulation of an immune response than the systemic immune system, using a vaccine of live V. cholerae in mice. The intestine has been recognized to be weakly responsive to immunization in many species (Robertson and Cebra, 1976), and this may indeed be a major function of the gut associated lymphoid tissue (Ferguson, 1977). Ferguson has suggested that the intestine has a very limited

immune capacity in three areas: its major secretory immunoglobulin is IgA, an antibody which is capable of combining with antigens and then doing little else; its major reticulo-endothelial function is in the Kupffer cell, a cell which reduces rather than increases the immunogenicity of ingested antigen; and it has an abundance of inter-epithelial lymphocytes which may be de-differentiated T-cells capable of binding with antigen but with few or no other properties. These properties may prevent harmful immune reactions with ingested antigens but they constitute a barrier to oral immunization against gut pathogens.

The intestine requires at least one local exposure to antigen in order to mount an immune response, although parenteral immunization can prime for a local response (Pierce and Gowans, 1975) and it can evoke a local secondary response (Chapter 5). There is evidence that only antigens with specific tissue binding properties are able to prime for a local immune response when given by the parenteral route (Pierce, 1978). Other antigens given parenterally may also stimulate the local lymphoid tissue, but only after it has been initially sensitized by oral immunization (Pierce, 1978). This suggests that the gut associated lymphoid tissue (GALT) is normally isolated from the circulation unless there is a specific tissue or immune attraction to parenterally given antigen. Such a view is consistent with the observation that Peyer's patches lack afferent lymphatics: they receive antigen either directly or from the circulation (Bockman and Cooper, 1973).

Oral immunizations with inactive antigens usually have

to be given a number of times in order to be effective, and with vaccines prepared from live organisms the duration of colonization is crucial (Robertson and Cebra, 1976; Chapter 5). Once again explanations have been sought in terms of the unusual structure and function of the Peyer's patches. The patches lack an organised antigen trapping reticulum (Bockman and Cooper, 1973), and so a single presentation of antigen may not endure long enough to stimulate the lymphocytes. Antigens with tissue binding and other adjuvant properties may be an exception yet again (Pierce, 1978).

A number of approaches have been tried to reduce the virulence of V. cholerae so that it can be given orally in a viable form. These have included the derivation of mutants which are streptomycin-dependent or temperature-sensitive, or form colonies which are rough or dwarf (W.H.O. techn. rep. 500, 1972). The attenuated organisms are usually well tolerated by volunteers, but they stimulate little immunity locally (Cash *et al.*, 1974b; Sanyal and Mukerjee, 1969). All the mutants proved to have a low potential for growth in the human intestine and this was linked to the poor response they evoked. It is possible that mutants which do not have the growth characteristics of pathogens are treated with indifference by the immune system of the intestine. The lack of bacterial growth in the bowel and association with the mucosa may also mean that little antigen is delivered to the immune system.

Stronger evidence that the growth of bacteria in the bowel is correlated with local immunogenicity comes from

studies with enteropathogenic E. coli (Evans, Satterwhite, Evans and Du Pont, 1978). These workers isolated a virulent strain from diarrheal stool (Evans and Evans, 1973) and found an avirulent mutant which only differed by the lack of a plasmid-controlled fimbriate antigen which mediates adhesion to the epithelium (Evans, Silver, Evans, Chase and Gorbach, 1975). Volunteers were given oral challenges of the bacteria, but only the fimbriate strain showed an extended excretion pattern, and also resulted in a systemic immune response. This strain also caused overt disease in higher doses, which may have been a factor in stimulating the immune response.

The question of bacterial adhesiveness and local immunogenicity was examined more closely in the experiments detailed in this thesis. The two strains of V. cholerae which were selected were equally effective as immunogens when given parenterally. They were found to differ in their ability to adhere to the bowel in vivo and hence to persist there, and this apparently accounted for a marked difference in the immune responses they elicited when given by the oral route. The organisms were not pathogenic for the adult mouse, and so toxin production could not account for the observations.

7.5 Memory in the Immune Response to Vibrio cholerae

7.5a Secondary IgM responses : The first immune response to antigens such as sheep red cells leads to the production of antibodies of the IgM class. A booster

vaccination does not increase the IgM response, but cells forming antibody of other classes appear more rapidly and in greater numbers (Sell, Park and Nordin, 1970). Even though Gram negative bacteria typically stimulate IgM production, a booster dose of organisms increases the number of IgM forming cells only marginally or not at all (Kateley, Patel and Friedman, 1974). It is observations such as these which have suggested that there are no secondary responses in the IgM class.

In Figures 5.1 and 5.2 it can be seen that a second intravenous dose of V. cholerae increased the numbers of IgM AFC in the spleen by at least ten fold, and this was termed a secondary response. There are, however, other explanations.

(i) The number of IgM forming cells was related to the total dose of antigen. As the increase in the response was not additive, this seems to be unlikely. Also, a dose of 10^8 rather than 10^7 V. cholerae stimulates an identical response (personal observations).

(ii) The increase was a mitogenic effect. Bacterial LPS and exotoxins can have immunopotentiating activity (Chedid and Audibert, 1977), and so this possibility had to be considered. The Jerne assay enumerated cells producing antibody to LPS coated SRBC, and from this count the number of PFC against SRBC was subtracted. The background activity to SRBC was always negligible (about 2AFC/spleen), even at the height of a response

to V. cholerae. Therefore no general mitogenic effect of any magnitude was seen in the study.

(iii) There was merely an expansion of the ongoing primary response, and no true IgM memory was established. There were still low numbers of IgM and IgA AFC in the spleen 49 days after an intravenous immunization of mice with V. cholerae (Figs. 4.1,2), presumably due to some active LPS remaining in the body (Britton, Wepsic and Moller, 1968). A second immunization boosted the numbers of antibody forming cells in the spleen, whether it was given after 14 days (Figs. 5.1,2) or 49 days (Fig. 5.6A,B), but there were significant ($p < 0.01$) differences between the two results. Widening the interval between vaccinations increased the IgA responses, but decreased the IgM response to the 111 NM SR strain. As the primary response had diminished markedly in this interval the data are consistent with the suggestion that the IgM boost was only an expansion of the ongoing response. If the boost had been delayed for a few more weeks, a rapid diminution in the IgM response to both 569B IMP SR and 111 NM SR might have been seen. By contrast, there may have been true memory in the IgA class. However, IgM cells appeared more rapidly and in greater numbers after the second immunization compared to the first, and so it could be termed a secondary response according to one of the current definitions.

7.5b Secondary responses in the secretory immune system :

Many workers have not been able to elicit secretory antibodies in higher titres than those obtained following primary vaccination, and so it has been doubted that the secretory IgA system has memory (Andre, Bazin and Heremans, 1973). Different conclusions have been reached with the measurement of antibody forming cells in the tissues of the intestine (Robertson and Cooper, 1972, 1973; Pierce and Gowans, 1975; Svennerholm and Holmgren, 1977).

The most striking example of a secondary response in the secretory immune system in this thesis is that shown in Figure 5.24. It was obtained in mice which had been given four oral doses of 569B IMP SR, followed by an i.v. booster dose of the same organism. The IgA and IgM peaks occurred in the intestine (Fig. 5.24) one day after they occurred in the spleen (Fig. 5.22), a sequence which was seen again in Figs. 6.1A and 6.2A. It could be concluded that AFC had migrated from the spleen to the intestine. However, the peak immune response to an oral boost was noted to occur in the intestine before it occurred in the spleen (Figs. 6.1B, 6.2B). Therefore the asynchrony in the response of the two organs seems to be related to the mode of antigen presentation, rather than to a migration of immunocytes.

It must be asked whether the i.v. booster merely caused a proliferation of immunoblasts after they had migrated to the lamina propria, which would be no

indication of immune memory. There is insufficient evidence to answer this question as definitely as Pierce and Gowans (1975) have done. They immunized rats parenterally with V. cholerae toxoid, and followed this with an oral booster dose of the antigen. The boosted AFC response in the lamina propria was abolished by drainage of the thoracic duct instituted at the time of boosting. Therefore the increase in the numbers of AFC was predominantly by migration.

There is some evidence from the present data that there was local immune memory. Four oral vaccinations of mice with V. cholerae stimulated the appearance of PFC in the mucosa of the small intestine in numbers which were similar for either bacterial strain (Figs. 5.24,25). However, a parenteral vaccination only boosted the response to the 569B IMP SR strain (Figs. 5.24,25). There are at least three interpretations of this data:

(i) 569B had a mitogenic effect. As in the spleen, the background response to SRBC did not increase even when the specific response was at a maximum, and so it is improbable that there was a general mitogenic effect. Also, the two V. cholerae strains stimulate similar responses when they are given to mice as one or two parenteral doses (Figs. 4.1,2; 5.1,2), and so there is no reason to believe that 569B IMP SR was any more 'mitogenic' than 111 NM SR.

(ii) 569B caused a specific in situ proliferation of

immunoblasts in the lamina propria of the intestine. Once again it is difficult to see why 569B IMP SR given i.v. could cause such a proliferation whereas 111 NM SR could not. A parenteral dose of the latter strain was able to boost the intestinal response of well primed mice (Fig. 6.5A).

(iii) 569B given orally resulted in the local formation of immune memory. This alternative is favoured by exclusion, and explains why a primary immune response in the intestine could not necessarily be boosted. It should be noted that oral immunizations with both strains could prime the spleen for a response to an i.v. booster dose (Figs. 5.22,23). Therefore special conditions pertain to the induction of immune memory in the intestine. The ability of the 569B IMP SR strain to adhere to the mucosa of the intestine meets these conditions, as does cholera enterotoxin (Pierce, 1978). It is a matter of speculation as to how certain antigens can stimulate local memory whereas others cannot, but it will be of vital importance for the future of oral immunization to select antigens with this property. This distinction between antigens could well explain the earlier conclusion that the secretory IgA system has no memory (Andre, Bazin and Heremans, 1973).

7.5c Recall of the secretory immune response by an oral challenge : Volunteers convalescent from cholera are immune to rechallenge with the organism for more than

a year (Cash *et al*, 1974a). The mechanism of the protection is unknown, and it may involve: a prolonged primary response which is sufficient to cope with the rechallenge; a residual response which is amplified by the re-encounter with antigen; or a secondary response to the oral challenge. The debate as to whether a boosted response is truly secondary or just an amplified primary becomes academic if the end result is prolonged immunity. However, the longer the duration of this immunity the more acceptable it is to call it immune memory.

There is experimental evidence that there can be protection from challenge with V. cholerae because it stimulates the recall of the local immune response (Pierce, Cray and Sircar, 1978). They immunized dogs with toxoid and observed protection against oral challenge with living V. cholerae when significant responses in the serum or jejunal lamina propria were essentially undetectable. Although the number of AFC in the lamina propria of the jejunum had only started to rise during the incubation period of the disease, it seems that the infection was terminated early by the booster response. The incubation period is a short 12 hours in dogs (Sack and Carpenter, 1969), and so the exposure to V. cholerae could lead to a more effective secondary response in humans where the incubation period is at least two days (Phillips, 1968).

The local immune response to somatic antigens of V. cholerae can also be recalled with an oral challenge with the organisms (Chapter 6), and this may be more important in humans than the antitoxin response (Levine *et al*, 1977a). However, the recall was achieved with the adherent 569B IMP SR strain (Fig. 6.2B) but not with the non-adherent 111 NM SR strain (Fig. 6.5B), which implies that immunity will be less effective against a V. cholerae strain of higher toxigenicity but with a lesser ability to persist in the intestine. For instance, the el tor biotype is the more 'persistent' and the classical biotype is the more toxigenic (Finkelstein *et al*, 1977), and infection to case ratios are lower for the classical biotype (Bart, Huq, Khan and Mosley, 1970). Part of the reason may be that the classical organism can challenge its host with less liability to stimulate an immune response, in comparison with el tor.

7.6

Immunological Tolerance to *Vibrio cholerae*

Oral vaccination can be followed by a hypo-responsive state like the one reported in mice fed sheep erythrocytes (Andre, Heremans, Vaerman and Cambiaso, 1975). This is by no means a general finding, and similar oral vaccination of mice with V. cholerae does not induce tolerance but in fact primes the animals for a secondary response to an i.v. immunization (Chapters 5; Horsfall and Rowley, 1979).

Recent studies with the immunization of rats with forms of cholera toxin emphasize that vaccination may inadvertently

lead to immune suppression (Pierce and Koster, 1978). They found that an intraduodenal dose of crude toxoid/toxin induced local memory which was undiminished for at least 16 weeks, but purified toxin induced a long lasting immune suppression after two weeks. When rats were given toxoid by a parenteral route, a transient local priming was seen with certain combinations of immunization route and adjuvant, but long lasting suppression of the mucosal antitoxin response was consistently induced within a few weeks. Similar suppressive effects of parenteral toxoid have been observed in rabbits (Yardley, Keren, Hamilton and Brown, 1978) but not in dogs (Pierce, Cray and Sircar, 1978). The suppression was antigen specific, and it could be transferred with spleen cells from animals given toxoid subcutaneously four weeks previously; hyperimmune serum was also suppressive. They concluded that the balance between local priming and suppression after immunization was determined in part by antigen form, immunization route and the use of adjuvants.

The findings of Pierce and Koster are in some ways similar to an immune suppression in mice which followed a parenteral immunization with V. cholerae (Chapter 4). In both systems a parenteral immunization was followed some weeks later by an immune suppression which was associated with an antigen specific factor in the serum. The suppressive factor in mice was not specific antibody, and was less than 50,000 Daltons in size. It could be a product of suppressor T-cells (Thomas, Roberts and Talmage, 1975), and it acted so rapidly that its effect may have been to directly depress the

activity of plasma cells (Warren and Davie, 1977). Further studies are required in order to characterize the serum factor and to decide if it is a T-cell product.

In view of the results in rats it would be interesting to see if the local priming in the intestine induced by the oral vaccination of mice with V. cholerae 569B IMP SR could be abolished by an i.v. immunization performed four weeks previously. An evaluation of vaccination should in the future not only consider its effectiveness or ineffectiveness, but also whether it is positively detrimental to the state of immunity. Because of this potential hazard, an understanding of the properties of antigens and adjuvants which determine the balance between immunity and suppression is an urgent need. There may also be a special hazard associated with age, as it has been shown that the inhibitory effects of suppressor T-cells are predominant over the activity of amplifier T-cells in young mice (Baker, Morse, Cross, Stashak and Prescott, 1977). These workers question the advisability of administering certain bacterial antigens as vaccines to children, as such vaccines could interfere with the development of protective immunity in later years.

7.7 Future Work

The results of the work recorded in this thesis gave rise to many questions which should be addressed in future work.

1. Antibody mediated antibacterial processes in the mucus layer of the intestine were not examined. They may be of

minor magnitude in comparison to the processes at work in the lumen of the intestine, but it is possible that they are of critical importance in resistance to infection.

2. The relative protective ability of antibodies against LPS, adhesin, flagellin and exotoxin should be defined for the baby mouse cholera model. The question is important in that vaccine development should be aimed at the most relevant area.

3. Means of inactivating V. cholerae without the loss of adhesive properties should be sought. The isolation of the adhesin could also be of importance in the further development of an oral vaccine.

4. A number of results were interpreted in terms of antigen retention in the gut wall. The theories could be tested by the oral or parenteral administration of radiolabeled antigens.

5. The role of antigen penetration into the Peyer's patches in the stimulation of a local immune response remains unanswered. Invasive and non-invasive vibrios could be compared in an attempt to answer this interesting possibility.

6. There were strong suggestions of immune memory in the systemic and secretory response to V. cholerae. More evidence might be obtained by examining longer intervals between immunizations, and by thoracic duct drainage. The startling difference between the ability of the 569B and 111 strains to stimulate local secondary responses could be explored in further detail.

7. The suppressive factor in the serum is of great interest,

both in terms of the potential hazard for immunization, and for application to the problem of atopy. Its characterization requires considerably more work.

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