

**IMMUNOLOGICAL STUDIES ON OUTER MEMBRANE
PROTEINS OF SALMONELLA TYPHI**

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UNIVERSITY OF BOMBAY**

**FOR THE DEGREE OF
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**SUBMITTED BY
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Vidula Sitaram Alurkar

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Immunological studies on outer membrane proteins
of *Salmonella typhi*

A Thesis submitted to the
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In Microbiology
by
Vidula Sitaram Alurkar

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Signature of the Research Guide

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Introduction

Macromolecules are of interest to Immunologists, because they can interact with cells of immune system in several ways.

- They can act as antigens which are targets of immune response of the clonally selected T and B cells.
- They can have direct effect on non-clonally selected immunocompetent cells such as macrophages and natural killer (NK) cells thus acting as immunomodulatory agents.
- They can act as polyclonal activators of T and/or B cells.

Of all the macromolecules, proteins constitute a diverse class of biologically active molecules to which an immune system can respond. Most proteins function as natural antigens for T and B cells. Proteins such as muramyl dipeptide function as immunomodulators (1). Plant lectins such as poke weed mitogen behave as T and B cell mitogens.

Outer membrane proteins as targets of T cell recognition

Immunological responses directed against surface as well as internal macromolecules of gram negative bacteria are important for the host's defence system (2). But among them, proteins are primary targets for immune recognition by T cells. Bacteria usually have a variety of peptides potentially capable of activating T and B cell clones. However, certain sequences or even individual residues of proteins have been identified as 'immunodominant' sites; i.e. those to which most of the immune response is directed (3). Therefore, identification of T cell target antigens of an organism becomes crucial.

It is now generally accepted that the only effective mechanism in controlling infection by facultative intracellular parasites such as *Salmonella*, is cell mediated immunity (CMI) (4, 5). So, it is important to identify critical antigen/s responsible for induction of CMI. According to Kauffman and White scheme, *Salmonella* are grouped in different serogroups, based on somatic antigens or LPS and proteinic flagellar antigens (6). Obviously, the somatic antigens can not be targets for T cell recognition. Various studies also rule out the possibility of flagellar antigens being involved in CMI (5, 7). Earlier reports from our laboratory (8, 9) have also demonstrated that there is extensive cross reactivity across the serogroup limits when CMI is used as a test parameter. Hence antigenic relationships based

on Kauffman and White scheme may have no relevance as far as target antigens recognised by T cells are concerned.

There is a variety of bacterial surface proteins that play a role in pathogenesis and constitute major antigens against which host immune response is directed (10). Outer membrane proteins (OMP) can be good candidates for T cell target antigens due to their peculiar properties. Antigens recognised by T cells often contain amphipathic peptides (11). Studies have demonstrated the correlation between amphipathicity of peptides and T cell responsiveness (12). Hydrophobic residues of the peptides may act as agretopes interacting with MHC molecules and hydrophilic residues may act as epitopes interacting with T cells (13). Major OMPs of gram negative bacteria are porins, which play a role in cellular permeability. They form transmembrane permeability channels for hydrophilic molecules (14). Porins span the lipid bilayer of the membrane and are exposed on the membrane surface and hence are likely to be amphipathic. Due to their amphipathicity, they have the potential to act as T cell target antigens.

It was therefore decided to study the ability of OMPs of *S.typhi* to act as T cell target antigens and also to study cross-reactivity of the corresponding peptide from different *Salmonella* species.

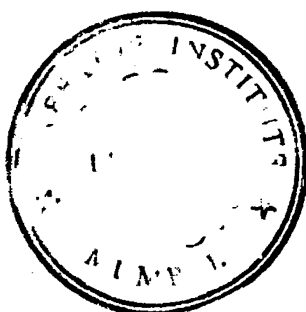
Immunomodulatory properties of OMPs

During the course of our investigation of T cell target antigens of *Salmonella* using delayed type hypersensitivity (DTH) as the test parameter, it was observed that OMPs could induce foot pad swelling in naive non-immune mice. This prompted us to do literature survey. Recently, preparations containing OMPs of *Salmonella* have been shown to induce tissue inflammation in non-immune animals (15). Further experiments have shown that major OMPs (porins) of *Salmonella* induce secretion of TNF- α , IL-1 α and IL-6 by macrophages and IFN- γ and IL-4 by lymphocytes (16). It is therefore logical to expect OMPs of *Salmonella* to have modulatory effects on both afferent and efferent arms of immune response. The OMPs of *Salmonella* could modulate immune response to an unrelated antigen by acting on antigen presenting cells and/or by acting on T/B cells directly. OMPs can also have effects on cell populations involved in non-specific resistance such as macrophages and NK cells. Keeping these issues in mind, immunomodulatory properties of OMPs of *S.typhi* were investigated.

It would be incorrect to view immunomodulators as molecules that cause across the board suppression or stimulation of immune response. Much more interesting are the qualitative changes in the immune response that they may bring about. For example, the ability of an immunomodulator to stimulate selectively one of the two CD4⁺ Th cell subpopulations Th1 or Th2 can be of immense interest.

The biological response modifiers have considerable therapeutic value particularly if they have no direct toxic action. If such immunomodulatory molecules are present in pathogenic organisms, they can play a distinct role in the pathogenesis of the clinical disease.

Among many structural components of micro-organisms that exhibit immunomodulatory activity, LPS that is present in outer membranes of gram negative bacteria has one of the most profound and complex properties (16). Many pathophysiologic mechanisms of gram negative bacterial infections are due to endotoxins acting directly or via mediators such as cytokines (16). Endotoxin can thus interfere at various levels in an immune response. Since *Salmonella* express both, the possibility of OMPs and LPS having synergistic or antagonistic effects in the context of their immunomodulatory properties needs to be considered.



Review of Literature

Immunomodulators are agents that can modify the quality or quantity of an immune response or both. It is a vague term which can be used to describe either enhancement, reduction or even obtaining a particular type of response. Various substances extracted from animal, bacterial, plant, viral or parasitic sources have been shown to be capable of modifying an immune response (17). Many pharmacologic agents also modify immune functions (18). In fact manipulation of immune system has become important in modern medicine with the ultimate objective being immunotherapy. Immunomodulatory agents might be of value in combination with other therapies in treatment of immunodeficiencies, cancer, infection and even autoimmune disorders (19).

Immune response is generated by a complex series of interactions between lymphoreticular cell types like T lymphocytes, B lymphocytes, macrophages and NK cells. Immunomodulators can act on any of the above cells by altering expression of surface adhesion molecules or more importantly by altering secretion of polypeptide mediators or other biologically active molecules that have multiple biological effects (17). These mediators can regulate cellular metabolism of other cells by induction and activation of factors that regulate transcription of DNA sequences in target genes (20).

Microbial components along with induction of a well-known antigen specific immune response, can also exert a wide variety of other type of effects on immune system. Such immunomodulation could be in the form of enhancement of immune response, immunosuppression or even those mechanisms that lead to immunopathology or host tissue damage (21). Bacterial structures provide a major source of immunomodulatory agents (22). Structures like cell wall peptidoglycan, OMPs, LPS from gram negative bacteria are capable of altering immune system without themselves being immunogenic (22). Other bacterial products studied for their immunomodulatory properties include substances like Staphylococcal enterotoxin B (23), MDP (1), microbial enzymes (24) etc.

Extracts from plants like neem (25), *Viscum album* (26) also exhibit immunomodulatory properties. Various other substances like thymic extracts (19), inosine-salt complexes (19), polynucleotides (19), saponines (27), vitamins (28), metals (29) have been investigated for their immunomodulatory potential.

A methodical survey of immunomodulatory agents in the context of their action would be most appropriate in this review.

1. Development of humoral response

An antibody response is the culmination of a series of interactions between B lymphocytes, T lymphocytes and accessory cells, all reacting to the presence of foreign antigen. Immunogenic challenge by foreign antigen prompts activation, proliferation and differentiation of resting B cells, generating a progeny that secretes antibody specific for the immunogen (30).

a. Primary humoral response

First contact of an individual with exogenous antigen generates a primary humoral response, characterised by production of IgM antibody secreting plasma cells and memory B cells.

Antigens that trigger humoral response can be divided in two groups on the basis of additional help they need to provoke B cells into optimal antibody production. T-independent (T_{ind}) antigens, which do not require help from T lymphocytes to trigger B cells, fall in two categories. First category possesses characteristics exhibited by polysaccharide antigens. These include large molecular weight (31), repeating antigenic epitopes which enables them to cross link B cell receptors (32), ability to activate complement cascade (33), poor *in-vivo* degradability (34), and inability to stimulate MHC II dependent T cell help. Many T_{ind} antigens are products of bacteria e.g. endotoxin, dextrans and levans found in bacterial cell walls, polymerised flagellin found in flagellae. Second category unlike polysaccharides shares the characteristic of being mitogenic to B cells, and thus acts via antigen non-specific mechanisms. Many investigators have demonstrated that signalling through T_{ind} antigens alone is not sufficient (35). Humoral response to these antigens may require or at least be modulated by some form of non-T help as well as be regulated by T cells (35).

T-dependent (T_{dep}) antigens require antigen specific T lymphocytes as well as non-antigen specific, non lymphocytic accessory cells to help them induce specific antibody production in B cells. These consist of soluble proteins or peptides. They have the ability to associate with MHC molecules at the surface of an antigen presenting cell (APC), thus allowing the APC to interact with T cell. Activation of B cells by T_{dep} antigen requires a number of sequential steps.

1. Presentation of processed antigen plus class II MHC molecules by an APC to a T helper (T_h) cell specific for that antigen.

2. Activation of Th cell induces expression of CD40 ligand (GP39) on Th cell membrane and directional release of cytokines by Th cell.
3. Generation of signals, by membrane mediated events and localised cytokines that are transduced to B cell, causing changes in gene expression that leads to proliferation and differentiation of B cells.

When an antigen is introduced into the body, it is concentrated in various peripheral lymphoid organs. Blood borne antigen is filtered by spleen which responds to systemic infection, whereas tissue antigen is filtered by regional lymph node. Once the antigen is carried to a regional node by lymph, it is non-specifically trapped, processed and presented together with class II MHC by dendritic cells in the paracortex. Three types of APCs are present in the lymph node, interdigitating dendritic cells in paracortex, macrophages throughout the node or specialised follicular dendritic cells (FDC) in follicles and germinal centres. Interaction between an antigen specific naive Th cell and class II MHC complex on dendritic cells of paracortex leads to activation and proliferation of Th cells. Initial activation of B cells also takes place in paracortex. Once activated, Th and B cells migrate to primary follicles of cortex. Within primary follicle, cellular interactions between FDC, B cells and Th cells take place leading to development of secondary follicle with a central germinal centre. Antigen trapped on membrane of FDC is thought to be very effective in activating B cells (36). Plasma cells leave the germinal centre and migrate to the medulla where they secrete large quantities of antibody.

Marrak and Kappler (37) observed that activated T cells are capable of triggering most if not all resting B lymphocytes to proliferation and antibody secretion without requirement of cross linkage of membrane immunoglobulin. Here antigen is presented to specific Th cell by a B cell that expresses surface membrane immunoglobulin specific for that antigen. Following antigen recognition, B cell internalises the antigen via receptor-mediated endocytosis, processes it into antigenic peptides and then presents it together with MHC class II molecule on the membrane (38).

Kinetics of a primary response, as measured by serum antibody level, varies depending on the nature of antigen, route of antigen administration, presence or absence of adjuvants and species or strain being immunised. During a primary response IgM is secreted initially followed by IgG later in the response.

b. Secondary humoral response

Memory B cells formed during primary response stop dividing and enter G0 phase of cell cycle. These cells have a variable life span. Capacity to develop a secondary response solely depends on existence of these memory B cells and memory T cells. Secondary follicle provides a specialised micro-environment for interaction between B cells, activated Th cells and FDC. FDCs have long processes, along which are present Fc receptors that bind antigen-antibody complexes effectively and retain complexes for months or years on cell membrane. Because FDCs bind antigen that has been complexed to antibody, they are thought to be particularly important for secondary response, when circulating antibody levels are high (36, 39).

Secondary response occurs more rapidly, reaches a greater magnitude and lasts for longer duration. Affinity of antibodies in secondary response is much greater, a process which is referred to as affinity maturation. It is the result of two events; Somatic hypermutation and selection of high affinity clones (36). These processes can be detected late in primary response and increase after second contact with antigen. Secondary response is also characterised by isotype switching which will be discussed later.

c. Memory B cells

Memory B cells are characterised as long lived cells that elaborate recall responses to antigen. In general, memory cells can be identified by the expression of high affinity antigen specific receptors which may include classes of immunoglobulins other than IgM or IgD (36). An immunoglobulin marker that can distinguish naive and memory B cells is a heat stable antigen as recognised by monoclonal antibody J11D (40). Memory B cells express almost no J11D while primary B cells express high levels of the same. Development of memory B cells is a T cell dependent process; antigens that stimulate B cells in a T_{ind} manner do not induce memory (41). Generation of memory B cells has been described in detail by Gray et al (41). Within days of the start of germinal centre proliferation, some cells become resting and the germinal centre becomes polarised to give a basal zone of proliferating cells and an apical zone of non-cycling cells. These resting centrocytes in the absence of rescue signals rapidly die by apoptosis. Cross linking of their surface immunoglobulin by the antigen bound in immune complexes on the surface of FDC can rescue these cells (42). However this signal alone is not enough to confer long term survival. Further secondary signals are required to induce either differentiation to plasma cells (CD23 + IL-1, IL-2) or to memory cells (CD40 ligand) (40, 43). Liu et al have shown that CD40

ligands are necessary for development of memory B cells (44). They injected mice with hamster antibody to CD40 ligand and it resulted in considerable impairment of development of memory B cells. Cytokine signals from T cells ensure survival but also facilitate isotype switching and somatic mutation. Further, at this stage antigen plays an important role in selecting high affinity clones amongst the somatic mutants implying that other cells that are not selected die by apoptosis.

Memory B cells proceed along a different pathway of differentiation from the cells that generate primary antibody response. It could be that two sets of cells have common precursors which are pushed along one or the other course depending on micro-environment in which they find themselves (i.e. the type of T cell or accessory cell with which they interact) (41). This view has been challenged by Klinman et al (45). They propose that memory B cells and B cell precursors of plasma cells of primary antibody response arise from different lineages each of which clonally expands following primary antigenic exposure. Support for this model came from the analysis of primary and secondary antibodies induced by certain antigens. Phosphoryl-choline induces antibodies characterised by a single idiotype in primary response, whereas the antibodies produced in secondary response completely lack this idiotype, which suggests that memory cells generated in primary response arose from a different lineage than did plasma cells (46).

d. Isotype Switching

In addition to generation of higher affinity antibodies in the secondary response, response of memory B cells is also characterised by the expression of isotypes other than IgM and IgD (36). In fact majority of the precursors for IgG secreting cells express IgG on their surface (47), and a number of studies have shown co-expression of IgM with IgG, IgE or IgA on memory B cells (47). Because different heavy chain constant regions can mediate different biological effector functions, isotype switching allows a single B cell clone to alter the effector capability of its secreted antibody while maintaining its original antigenic specificity. Thus not only is the humoral response of higher affinity due to somatic hypermutation and antigen selection, but it is also better adapted functionally due to expression of antibodies with effector capabilities more appropriate for the ongoing immunogenic challenge.

Since early 70's isotype switching and the secondary response have been shown to be highly T dependent (36, 48). Thus while IgM and IgG3 responses are relatively T

independent; IgG1, IgG2a, IgE and IgA responses require T cell help. Lymphokines may direct this switching by increasing the accessibility of a particular switch region to the switch recombination machinery of the cell (36).

The discovery by Isakson and Vitetta that T lymphocyte produced substance could induce LPS activated murine B lymphocytes to secrete IgG1 (49), first suggested the role of lymphokines in selecting the isotype of antibody produced. This was confirmed when Vitetta, Ohara and Paul traced this IgG1 inducing activity to IL-4 (50). Later Coffman showed that IL-4 could induce LPS activated B cells to secrete IgE and IgG1 and that IFN- γ could inhibit this activity (51). He also showed that TGF- β induces LPS-stimulated B cells to switch to IgA expression. IL-5 has also been shown to have IgA inducing activity (48). Snapper et al showed that IFN- γ could induce IgG2a secretion while IL-4 could inhibit the same (52). Based on these findings they suggested that IL-4 and IFN- γ act as non-competitive antagonists with respect to isotype expression and so could regulate relative quantities of IgG1, IgE and IgG2a that are synthesised.

Having looked at the development of humoral response, we would now look at the action of some immunomodulators affecting various stages of the response.

2. Immunomodulatory agents affecting humoral response

Agents that decrease antigen degradation or turn soluble antigen into particulate form enhance antibody response. First use of aluminium compounds as adjuvants was described in 1926 (1). Since that time antigens precipitated with aluminium salts or antigens mixed with or adsorbed on pre-formed aluminium compounds have been used extensively to augment immune response in animals and humans. Aluminium compounds work through following means (1): degradation/excretion of antigen is slowed, thus prolonging the time of interaction between antigen and APC such as macrophage or FDC. Aluminium particles have been demonstrated in regional lymph nodes of rabbits seven days following injection (1). Many studies confirm that antigens administered with aluminium salts lead to activation of Th2 cells and augment humoral response to peptide antigens (22). CMI appears to be only slightly increased as measured by DTH (1). Aluminium hydroxide has also been described as activating complement pathway (1). This may play a role in local inflammatory response as well as antibody production.

Oil emulsions such as Freund's Incomplete adjuvant (FIA) localises the antigen in its initial depot (1) and like aluminium, disseminates to the lymph nodes (1), which has been thought to be more important for its adjuvant function. FIA does not substantially increase CMI.

Liposomes have also been used to enhance humoral immunity as they too function as depots for antigen (1). They can modify uptake of antigen and can also act as vehicles for release of immune mediators (22).

First cellular interaction after antigenic challenge is the interaction between T cells and APC and its outcome largely dictates subsequent events. If sufficient CD4⁺ Th cells are triggered, then activation of B cells almost certainly takes place (53). Activation signals from accessory cells and T cells include both interleukins and factors resulting from cell-cell contact (54). Immunointervention can be at various stages viz. at the level of antigen presentation, cytokine gene transcription, interdiction of cytokine action or at the level of clonal expansion by agents that interfere with DNA synthesis. Intervention can also be at the cell surface antigens involved in critical cell recognition and interaction events (55).

Skea et al developed an adjuvant independent antigen delivery system based on the use of monoclonal antibodies specific for cell surface determinants on APC (56). By coupling protein or peptide antigens to such monoclonal antibodies, they 'delivered' (i.e. immunotargetted) antigen to the surface of APC and generated an adjuvant independent serological response to bound antigen. It has been shown that immunoconjugates formed between certain mouse monoclonal antibodies and either protein or synthetic peptide antigens can, when injected subcutaneously in saline, prime murine immune system for subsequent antigen specific IgG antibody response.

Recently it has been shown that antigenicity of proteins can be modulated by maleylation, to target scavenger receptors on macrophages (57). Maleylation of diphtheria toxoid (DT) induced its targeting to macrophage scavenger receptor. Maleylated DT could generate significant antibody response and also a better T cell proliferative response *in-vitro* than DT.

When Mond et al studied T_{ind} responses, they found that apart from T cells, other cells can possibly replace or synergise with T cells in providing ancillary help for T_{ind} responses (35). Falkoff studied the effect of purified IL-1 on various steps in B cell activation pathway and showed that although IL-1 is not a direct activator of resting B cells, it

synergised well with low concentration of anti- μ in induction of B cell proliferation (58). Macrophages also produce IL-6 and TNF which are B cell activators (35). NK cells also secrete various cytokines including CSF, IFN- γ , IL-1, IL-2, IL-8 all of which play a role in B cell response (35). Certain accessory cells and their products play a role in downregulation of response. Philips et al have shown that one such mechanism is secretion of prostaglandins by macrophages (59). Prostaglandins can induce B cell unresponsiveness and can also induce isotype switching to IgE and IgG1.

Muramyl dipeptide (MDP) is capable of inducing humoral immunity when administered with an antigen in saline and induces an antibody response when given orally, even if the antigen is given by a different route (1). MDP stimulates better processing of antigen by stimulating phagocytosis. Expression of Ia on macrophages has been reported to be unaffected by MDP. MDP induces macrophages to secrete CSF, IL-1, TNF and IL-6 (60). It also enhances macrophage adherence, stimulates chemotaxis and inhibits macrophage migration. All of these contribute to amplification of the response.

Non-ionic block polymer surfactants in oil-in-water emulsions have been studied for their stimulating activity on humoral responses against peptide antigens (22). These surfactants can bind proteins to the surface of oil drops and these complexes act on macrophages to enhance both expression of MHC II molecules on their surface and the molecules' ability to present antigen to T cells. They also shift the isotype distribution from IgG1 towards IgG2a thus shifting the balance of Th1 and Th2 cells induced by the antigen toward Th1 cells.

Endotoxin associated protein (EP) from *Salmonella* species has been shown to induce polyclonal immunoglobulin synthesis. Mangan et al have shown that mononuclear phagocytes are essential for its adjuvant activity (61). EP is a potent inhibitor of apoptosis in human monocytes. This can increase number of macrophages *in-vivo* through enhanced accumulation and subsequent differentiation of viable monocytes. Production of IL-1, which along with upregulation of numerous components of immune system, also prevents monocyte apoptosis, is upregulated by EP. Mangan et al also showed that EP increased number of HLA-DR positive cells and also number of HLA-DR molecules per cell. Expression of IL-2 receptors on monocytes were also upregulated by EP (61).

Dutton and his colleagues first suggested that soluble factors released from Th cells are important mechanism of delivery of T cell help. Since then, many factors, antigen

specific or non-specific have been reported to act on B cells (62). IL-4 is a major T cell derived lymphokine that acts early in B cell activation and proliferation. IL-2 is a proliferation inducer for B and T cells. IL-5 is a B cell activator and IL-6 is a strong signal for B cell differentiation (53). Effects of interferons on B cells are heterogeneous. They usually inhibit cellular growth in B cells but also modulate B cell function (53). A number of interacting pair of molecules have been identified that could send and receive cell contact signals in case of cognate interactions (54). Among them are T cell antigen receptor and class II MHC molecule on B cells, adhesion molecule pairs LFA1 and ICAM1, CD2-LFA3, CD28-B7, CD40-CD40 ligand (63).

Melatonin, which is the main hormone of the pineal gland, showed enhancement of antibody response to T_{dep} antigens. Enhancement of the antibody response was associated with increased induction of Th cell activity and IL-2 production (64).

Wilson et al have demonstrated the effect of keyhole limpet hemocyanine using cholera toxin as an adjuvant (65). The response was characterised by high levels of IL-4, IL-5 and IL-6 with lower levels of IFN- γ , and IL-2 (Th2 type of response).

Cholera toxin and related heat-labile exotoxin from *E.coli*, have been extensively studied as mucosal immunogens. Cholera toxin elevates c-AMP levels in cells. This results in inhibition of IL-2 production and proliferation of Th1 cells but has no effect on IL-4 production and proliferation of Th2 cells (66). It enhances mucosal immunogenicity of many antigens as assessed by humoral responses. The *in-vivo* effects on helper T cell subsets have been shown to favour dominance of IgA humoral response over CMI.

Cyclosporin A can be a suppresser of T_{dep} humoral response (67). As stated earlier, ligand for CD40 is expressed on activated T cells and it delivers contact-dependent activation signals to B cells. Optimal expression of CD40 ligand requires activation of protein kinase C and a rise in intracellular calcium concentration in T cells (Detailed action of Cyclosporin A on T cells will be dealt in the next section). CD40 ligand expression was inhibited by pre-treatment of T cells with Cyclosporin A, thus depriving the B cell of CD40 ligand dependent T cell help (67).

Monophosphoryl lipid A (MLA) is a less toxic derivative of LPS. It promotes IFN- γ production by T cells. It indirectly aids the production of antibodies (22). MLA also

stimulates the release of TNF by macrophages. It preferentially triggers Th1 type of response and favours IgG2a secretion.

Rapamycin is a known T cell suppressive agent (68). It has been shown to inhibit both IL-2 dependent and independent proliferation of B cells (69). Cell cycle analysis indicated that it inhibited the progression of stimulated B cells past the mid G phase of the cell cycle. Thus entry of the B cell into the S phase and subsequent B cell differentiation was hampered. Cepharanthine also causes arrest of the late G1 to S phase transition in the cell cycle causing inhibition of proliferation of B lymphocytes (70).

Among many structural components of micro-organisms that exhibit immunomodulatory activity, LPS has the most profound and complex properties. LPS has both direct action on cells of the immune system and it is also an inducer of cytokines. It has direct action on macrophages and B cells. Adjuvant properties of LPS are due to stimulation of production of monokines like IL-1 and TNF (71). Short exposure times *in-vitro* or *in-vivo* lead to decreased expression of Ia, but longer exposure times increase Ia expression (17). LPS is a T cell independent polyclonal B cell mitogen. LPS stimulates natural antibodies if administered without antigen, and specific antibody if injected with antigen (72). LPS can also inhibit immune responses. Generally inhibition is observed when LPS is administered before antigenic challenge *in-vivo* (17). The demonstration that LPS can enhance immunity under certain conditions and induce suppression when given under other conditions raises the possibility whether the adjuvanticity and suppression inducing properties of LPS are due to qualitatively similar but quantitatively different effects on metabolism of lymphocytes.

Takada et al studied immunobiological activities of porins isolated from *Fusobacterium nucleatum* (73). Porins exhibited immunoadjuvant activity to increase the antibody production against sheep erythrocytes in mice that were immunised with sheep erythrocytes with porin. They also showed that porins show polyclonal B cell activation and also have stimulatory effects on peritoneal macrophages.

3. Development of Cellular Immunity

Cell mediated branch of immune system confers immunity primarily through generation of various effector immune cells. Both antigen specific T cell clones and non-clonally selected cells contribute to CMI. The T cell clones include both CD4⁺ and CD8⁺ cells. Non-clonally selected cells include macrophages, neutrophils, eosinophils and NK cells. Unlike humoral branch of immune system which serves mainly to eliminate

extracellular pathogens and their products, CMI is responsible for clearance of intracellular pathogens, virus infected cells, tumour cells and foreign grafts. The system is evolved to recognise altered self cells and eliminates them from the body.

a. Th cell activation

Central event in generation of both humoral and cellular immune responses is activation and clonal expansion of Th cells. T cells that express CD4 molecules and recognise antigen in association with MHC II molecules and generally provide 'help' for both humoral and cell mediated responses are called as Th cells. In 1986, Mosmann and co-workers proposed a major subdivision of CD4⁺ Th cell clones based on differences in their pattern of cytokine production (74). Th1 cells secrete IL-2 and IFN- γ and Th2 cells make predominantly IL-4, IL-5, IL-6 and IL-10.

Minimal requirement for activation of a T cell consists of an APC and antigen (75). The APC should:

1. express MHC class II and co-stimulatory molecules,
2. possess the capacity to internalise and process antigenic peptides in association with MHC class II molecules, and
3. engage in cluster formation.

Various lymphoid and non-lymphoid cells have been shown to be capable of presenting antigen to T cells. APC function depends on multiple factors including antigen density, level of accessory (adhesion) molecules, net surface charge etc. (76). Potency of APC function is largely a reflection of overall avidity of T-APC interaction. Family of APCs can be divided in two groups (75):

1. Professional APCs such as dendritic cells, Langerhans cells, which constitutively express sufficient levels of MHC II molecules, co-stimulatory B7 molecules, and adhesion molecules.
2. Facultative APCs such as macrophages, endothelial cells and B cells which require to be suitably stimulated to induce MHC II, and B7 expression.

Among these APCs dendritic cells stand out for their typical morphology and unique functional effectiveness in Th lymphocyte activation (75).

After invasion of host, intracellular pathogens are taken up by APCs situated near the site of entry. Paracortex of lymph nodes is populated with naive T cells and also contains dendritic cells which have migrated from tissues to the node. Lymph nodes taken from neonatally thymectomised mice show a severe depletion of cells in paracortical region. The paracortex is therefore sometimes referred to as 'Thymus dependent' area. Dendritic cells are responsible for processing and presenting the antigen together with MHC II molecules to Th cells.

Antigen is internalised by APCs via phagocytosis or pinocytosis. Antigen is then proteolytically degraded in acidic lysosomal compartment and associated with MHC class II molecules. The details of antigen processing and presentation would be discussed in a later section.

Signalling through TCR

Activation signals generated as a result of recognition of antigen MHC complex by TCR are transmitted via CD3, a multimolecular complex expressed in association with TCR on the surface of all mature T cells. It has been shown that activation of T cells can be achieved by cross linking the complex via monoclonal antibody to either CD3 or TCR (77). Activation through TCR-CD3 complex depends directly on number of molecules engaged, the degree and nature of cross linking and the duration of engagement (77). TCR-CD3 complex consists of eight integral membrane proteins; the antigen binding $\alpha\beta$ (or $\gamma\delta$) TCR heterodimers and invariant CD3- γ , δ , ϵ and ζ and η chains (78). This complex arrangement with multiple transmembrane and cytoplasmic domains can be compared with that of the G protein coupled receptor family (79). Protein tyrosine kinases (PTK) are considered to be essential components of the proximal signalling cascade operated by TCR-CD3 complex. However none of the CD3 sub-units possess a cytoplasmic domain with intrinsic protein kinase activity (78). These observations suggest that TCR-CD3 is non-covalently coupled to PTK. Two PTKs are associated with CD3 ($p59^{fyn}$ and ZAP-70). Cytoplasmic domains of CD4 and CD8 that are associated with PTK $p56^{lck}$ are also essential for T cell activation. Dephosphorylation of the carboxyl terminal tyrosine is thought to activate these kinases. This is mediated by CD45 (78). CD45 has two protein tyrosine phosphatases which dephosphorylate tyrosine residue of $p56^{lck}$ and $p59^{fyn}$. This activates the two PTKs. But neither $p59^{fyn}$ or $p56^{lck}$ are considered to be the proximal PTKs involved in signal transduction by TCR. These PTKs phosphorylate phospholipase C and ζ chain of CD3.

Phosphorylation of ζ chain does not appear to be essential for TCR induced activation (78). Activation of phospholipase C results in second messengers. That is, it hydrolyses phosphatidyl inositol biphosphate to inositol triphosphate and diacyl glycerol (DAG). Inositol triphosphate triggers an increase in intracellular calcium which leads to activation of Ca^{++} /calmodulin dependent phosphatase calcineurin (80). DAG activates protein kinase C which mediates serine-phosphorylation of various substrates as well as mediates release of nuclear factors which participate in specific pattern of gene expression (78).

TCR-CD3 complex is not the only set of T cell surface molecules engaged during interaction with APCs nor the sole pathway generating signals capable of activating T cells. T cells receive a multiplicity of signals during interaction with APC that summate or synergise thereby facilitating T cell activation.

In past few years it has become apparent that CD28 is the major co-stimulatory signal receptor for Th cells and B7, a molecule induced or constitutively expressed on APC, is its natural ligand (81). Results have demonstrated that some of the requirement for APC function can be replaced by an antibody to CD28 that delivers an activation signal independent of CD3 (77). Both murine and human T cell clones are anergised in absence of CD28 co-stimulation. Results from experiments performed also show that immune responses can be either inhibited or augmented by manipulation of CD28-B7 interaction (82). Recently an additional co-stimulatory ligand for CD28 was found. Proof of this came from analysis of B7 deficient mice (83). Two CD28 counter-receptors are B7-1 and B7-2. They both equivalently co-stimulate IL-2 and IFN- γ production and IL-2R expression. B7-2 has been shown to induce significantly more IL-4 production than B7-1. Stimulation with B7-1 results in high levels of IL-2 and low levels of IL-4 (84). Therefore it appears that B7-1 and B7-2 co-stimulation mediate distinct outcomes (84). B7-2 probably provides an initial signal to induce naive T cells to become IL-4 producers, thereby directing the immune response more towards Th0/Th2, whereas B7-1 is likely to be a more neutral differentiating signal (84).

Other molecules capable of providing co-stimulatory signal to T cells include CD2, LFA1, monomorphic regions on MHC class II, ICAM-1 on APC. Recent work has suggested that even molecules originally considered to have only a role in cellular adhesion are also capable of conveying activation signals (77).

In addition to signals mediated by TCR and its associated accessory molecules, activation of Th cell requires additional costimulatory signal in the form of cytokines from

APC. Data from several laboratories suggests that IL-1 and IL-6 may be particularly important in this regard. IL-1 which was originally identified as Lymphocyte activating factor, is one of the most potent cytokine signal (85). IL-1 potentiates Th cell activity through several pathways:

1. It increases release of IL-2.
2. It also increases IL-2 receptor expression.
3. It raises body temperature thus enhancing response of T cells.

TNF- α has also been shown to potentiate Th cell activity in presence of supplementary IL-2 (77).

Signal transduction through cytokines, adhesion molecules (also in B cells through membrane IgD or IgM (86)) occurs by a series of protein phosphorylation events catalysed by protein kinases and dephosphorylation events catalysed by protein phosphatases (87).

Interaction of T cells and antigen-APC initiates a cascade of biochemical events that induces resting Th cell to enter cell cycle (G0 to G1 transition). It leads to activation of genes encoding IL-2 and IL-2 receptor (IL-2R). Although both low and high affinity receptors are induced following T cell activation, the physiological response mediated by IL-2 has been correlated with occupancy of high affinity sites (88). Secreted IL-2 then binds to newly expressed receptor and autostimulates Th cell proliferation. IL-2 can promote proliferation of any IL-2R positive cell regardless of its antigen specificity. Specificity of the response is maintained at the level of induction of both IL-2 and IL-2R. As a Th cell is activated it becomes effector cell secreting various cytokines. It participates in DTH response. Cytokines also play an important role in activating B cells, T cytotoxic (Tc) cells. They also regulate proliferation and differentiation of a number of non-specific effector cells like macrophages and NK cells that play various roles in CMI. These non-specific cells do not possess immunologic specificity and memory, so regulation of their activity depends on activity of antigen specific Th cells.

Th cell recognition of an antigenic peptide-MHC complex on APC results either in activation or a state of non-responsiveness called clonal anergy (89). Anergy is a state of inactivation marked by inability of T cells to proliferate in response to an antigen-MHC complex. One of the major factors which decides whether clonal expansion or clonal anergy ensues is the presence or absence of a co-stimulatory signal, such as that provided by

interaction of CD28-B7. If resting T cell receives TCR mediated signal in absence of a suitable co-stimulating signal, it will become anergic (89).

b. Activation of Tc cells

Tc cells predominantly express CD8 molecules and generally recognise antigen in association with MHC class I. They generally secrete TNF and IFN- γ but mainly exhibit cytotoxic properties.

Generation of Tc cells from precursors requires several signals. One signal is provided when TCR and CD8 on Tc cell precursor interact with antigenic peptide-class I MHC on membrane of target cell. Second signal is provided by cytokines from activated CD4 Th cells. IL-2 is principle cytokine required for proliferation and differentiation of Tc cell precursors into effector Tc cells. IL-2 produced by proliferating Th cells binds to IL-2R on Tc cell precursors inducing it to proliferate and differentiate into effector Tc cells. The fact that IL-2R is not expressed until after a Tc cells precursor has been activated by antigen plus MHC I molecule, ensures that only antigen specific Tc cell precursors are clonally expanded by IL-2 and acquire cytotoxicity. Several other cytokines e.g. IL-4, IL-6 and IFN- γ also play a role in activating Tc cells (76).

c. Effector arm of CMI

Cytotoxic T cell mediated destruction

Effector phase of Tc cells mediated response involves a sequence of events culminating in target cell lysis.

Primary events in Tc cell mediated cytotoxicity are conjugate formation, membrane attack, Tc cell dissociation and target cell destruction. First step is antigen specific recognition of a target cell by Tc cell. Electron microscopy of Tc cell reveals several electron dense granules. These contain various cytokines (e.g. TNF- β) and a pore forming protein called perforin (90). After formation of Tc cell - target cell conjugate, perforin monomers are released by exocytosis into junctional space. Monomers after contact with target cell get inserted into the cell membrane, polymerise in presence of calcium to form a cylindrical pore. This pore facilitates entry of various lytic substances that destroy the target cell.

Some investigators have shown that target cell killing by Tc cell sometime is achieved in absence of calcium (91). They have shown morphologic hallmarks of apoptosis in target cell. Tc cell either secretes a factor that induces DNA fragmentation, or else Tc cell

- target cell interaction induces a transmembrane signal that results in apoptosis. One of the Tc cell derived factors that has been implicated in apoptosis is TNF- β .

DTH response

Encounter of an antigen with a subpopulation of effector Th cells induces a DTH response. Activated T cells secrete a variety of cytokines that are responsible for the recruitment and activation of macrophages and other non-specific inflammatory cells. The delayed onset of the response reflects the time required for the cytokines to induce localised influxes of macrophages and activation of these cells. Once a DTH response begins, a complex interplay of non-specific cells and mediators is set in motion that can result in amplification of response. Cells harbouring intracellular pathogens are rapidly destroyed by activated macrophages which exhibit increased levels of phagocytosis and increased ability to kill micro-organisms. They also express high levels of MHC II and cell adhesion molecules, therefore function as more effective APCs.

Role of macrophages

Role of macrophages in eliminating intracellular pathogens is crucial. However, most of its microbicidal activities are only induced by appropriate activation which could be optimally achieved by IFN- γ although other interleukins like IL-2, TNF and IL-6 may also participate (92). It has also been shown that IL-4, IL-10 and IL-13 which are Th2 cytokines downregulate activation of macrophages (93). A number of anti-microbial and cytotoxic substances produced by activated macrophages are responsible for intracellular destruction of phagocytosed pathogen. Toxic effect of these substances involve both oxygen-dependent and oxygen-independent mechanisms.

Oxygen-dependent mechanisms: Activated macrophages produce a number of reactive oxygen and reactive nitrogen intermediates. During phagocytosis, a metabolic process known as respiratory burst occurs which results in activation of membrane bound oxidase that catalyses the reduction of oxygen to superoxide anion, which is toxic to pathogen (94). Activated macrophages also express high levels of nitric oxide synthase, which oxidises L-arginine to yield citrulline and nitric oxide (NO) (95). Biochemical basis of cytotoxicity induced by NO depends on the combination of NO with iron-containing moieties in key enzymes of the respiratory cycle and of synthesis of DNA in target cells. Much of the anti-microbial activity of macrophages against bacterial, fungal, helminthic and protozoal pathogens is due to NO and NO-derived substances.

Oxygen-independent mechanisms: During phagocytosis, the endosomal pH is increased to basic levels for a brief period that provides optimum condition for action of defensins (96). Defensins are a group of anti-microbial cytotoxic peptides with an anti-microbial spectrum of gram positive, gram negative bacteria, mycobacteria, fungi and some enveloped viruses. Defensins get inserted into cell membranes forming voltage regulated iron-permeable channels. They also act as opsonins. When pH decreases to acidic levels, it supports optimum activity of acidic lysosomal enzymes.

Role of NK cells

NK cell is another non-specific effector cell whose activity is enhanced by IL-2 and IFN- γ produced by activated Th cell. NK cells are large granular lymphocytes whose lineage remains unclear as they express some membrane markers of T lymphocytes and some markers of monocytes and granulocytes. NK cells kill tumour cells and virus infected cells by a process similar to Tc cell mediated killing (perforin mediated killing and induction of apoptosis by secretion of agents like TNF- α). However NK cells differ from Tc cells in that there is no immunological memory.

A number of cells that have cytotoxic potential express membrane receptors for Fc region of antibody. When antibody is specifically bound to a target cell, these receptor bearing cells can bind to Fc region and thus to target cells and subsequently cause lysis of target cells. Thus, although cytotoxic cells involved are non-specific, specificity of antibodies directs them to specific target cells.

d. T cell memory

After a primary encounter with antigen, naive T cells proliferate extensively and an expanded pool of memory T cells develops and persists when the transient effectors have come and gone. Memory and effector T cells are believed to be generated by differential outcomes after activation; however possibility of T effectors being converted to memory is still being debated (97).

Earlier memory T cells were thought to be long lived cells (98). However, later evidences have shown that individual memory cells may be short lived and turn over more rapidly than naive cells (99). Studies showing decay of Tc and Th cell memory in absence of antigen suggests that memory cell survival can be driven by specific antigen which persists *in-vivo* and provides continuous low level stimulation (40). One such source of antigen could

be FDC which store antigen-antibody complexes on their surface for months. However their role in maintaining T cell memory is not yet fully understood. Physical properties of antigen, *in-vivo* persistence, route of exposure, dose and frequency of re-exposure, all can influence the length of memory. The life span of memory cells is also regulated by expression of molecules like bcl-2 that influence the choice between life and death (41).

Several surface markers have been used to distinguish memory cells. Markers that are lost on activation are high molecular weight isoforms of CD45R, lymph node homing receptor L-selectin (mel14), LAM-1 which mediates binding of lymph node high endothelial venules (41). Several adhesion molecules are up-regulated by activation like CD44, CD2, CD29, VLA4, VLA5, VLA6 and integrins (100). The latter appear to participate in selective migration of memory T cells to peripheral tissues.

Memory T cells are found to have less stringent requirements for activation than naive T cells (97). Naive T cells respond to antigen only when it is presented on APC such as dendritic cells or activated B cells. Their response if any is poor to unstimulated macrophages or resting B cells. In contrast, resting memory cells can be restimulated efficiently by resting B cells or macrophages, although response is still less than with dendritic cells or activated B cells (97). Thus, memory T cells have low and naive cells have high dependence on costimuli for activation.

Unlike naive T cells, memory T cells secrete multiple cytokines, namely IL-2, IL-3, IL-4, IL-6 and IFN- γ like Th0 cells (40). Swain et al have challenged this view. They suggest that memory CD4⁺ T cells can be committed to a particular pattern of cytokine secretion (Th1 or Th2) during initial priming, although they may require further differentiation for optimum functioning (97).

Memory T cells recirculate in blood and lymph. But they tend to return to the tissue in which they were originally stimulated. They are the predominant cell type within an inflammatory lesion.

Having looked at how cellular and humoral immune responses develop, it is necessary to know how immunity to infectious diseases is generated and which arm of immune response affords protection against a particular pathogen.

4. Immunity to Bacteria

Infection by extracellular bacteria induces production of humoral antibodies which are secreted by plasma cells in regional lymph nodes and submucosa of respiratory and GI tract. Antibody plays a role in dealing with bacterial toxins. It can also interfere with motility by binding to flagellae, and can also act as an opsonin. Antibody mediated activation of complement system can induce localised production of immune effector molecules that help to develop an inflammatory response.

Infections caused by bacteria that are capable of intracellular growth within phagocytic cells tend to induce CMI responses. At an early stage NK cells activate antibacterial defence mechanisms. Role of T cell subsets and cytokines generated in regulation of bacterial infection has been reviewed by Philip Scott and Stephen Kauffman (101). In many bacterial infections like *L.monocytogenes*, Th1 kind of response rather than Th2 has been associated with protection (102). CD4⁺ Th1 cells activate phagocytes and Tc cells converting them to effector cells. Evidence for involvement of class I restricted CD8⁺ T cells in antibacterial immunity was first obtained in murine listeriosis and later confirmed in a variety of other intracellular bacteria including *M.tuberculosis*/*M.bovis* and *S.typhimurium* (102). Tc cells exhibit cytotoxicity and also secrete IFN- γ . Cytokines like IFN- γ , TNF, IL-1 activate NK cells which also exhibit cytotoxic activity and activate macrophages through secretion of IFN- γ .

IFN- γ produced by CD4⁺ Th1 cells, CD8⁺ T cells, $\gamma\delta$ T cells and NK cells plays a central role in resistance to infection. This has been shown in case of *L.monocytogenes* and *M.leprae* infections (102). Other interleukins like TNF, IL-1, IFN- α , IL-6 and IL-8 also participate in immune response to intracellular bacteria (102).

5. Immunity to Viruses

A number of specific immune effector mechanisms together with non-specific defence mechanisms are called into play to eliminate an infecting virus. Antibodies specific for viral surface antigens are often crucial in containing the spread of virus. Antibodies may block viral penetration by binding to epitopes that are necessary to mediate fusion of viral envelope with plasma membrane. Most viruses like influenza virus, rhino virus, EB virus express surface receptor molecules that enable them to initiate infection by binding

specifically to host cell membrane molecules (103). IgG and IgM are limited in their action to plasma and tissue fluids whereas secretory IgA may protect epithelial surfaces.

Substantial number of macrophages, neutrophils and NK cells can be lavaged from the influenza virus infected lung prior to detection of virus specific T cells (103). Later entry of activated T cells may simply be a continuation of this non-specific process. Activation of Th1 cells leads to several effector functions causing cytotoxicity and so progress of an immune response toward Th1 pathway would be appropriate for dealing with viruses (104). Immunity is conferred by specifically sensitised CD8⁺ T cells which localise to the site of pathology and eliminate the cells supporting virus growth. Role of Tc cells in defence against virus was demonstrated by ability of influenza specific Tc clones to confer immunity to a lethal dose of influenza virus on syngenic adoptive transfer recipients (103).

Various cytokines like IL-2, IFN- γ and TNF serve to defend against virus. IFN- γ acts directly by inducing antiviral state in cells. Antiviral effects of IFN- γ include:

1. Increased expression of class I and class II MHC glycoproteins facilitating recognition of viral antigens by immune system.
2. Activation of cells with the ability to destroy virus infected targets, like NK cells and macrophages.
3. Direct inhibition of viral replication.

6. Immunity to Parasitic Infections

Protozoans are unicellular eukaryotic organisms. They are responsible for several diseases like malaria, leishmaniasis, toxoplasmosis. The type of immune response that develops depends on location of parasite within the host. Many protozoans have stages when they are free in blood stream. In this stage antibody is most effective. Many of these pathogens are capable of intracellular growth and during this stage cell-mediated reactions are effective. Substantial evidence exists in case of Leishmania infection that resistance and susceptibility represent the development of strong Th1 and Th2 responses respectively. Healing disease is accompanied by strong DTH reactivity but poor antibody response, whereas non-healing animals show very high IgE levels but no DTH reactivity (105).

Because of their size, helminths are extracellular parasites. Studies have been done on protective immunity to schistosomiasis. Cytokines produced by Th2-like subset are

important for protection. IL-4 induces B cell to switch immunoglobulin class to IgE production. IL-5 induces bone marrow precursors to differentiate into eosinophils. IL-3 stimulates mast cell growth. Degranulation of mast cells releases mediators that increase infiltration of macrophages and eosinophils. They bind to parasites through their Fc receptors. Once bound, they participate in ADCC, releasing mediators that damage the parasite. However, there is also evidence that CD4⁺ T cells are important for affording protection (106). Results show that *in-vivo* depletion of CD4⁺ T cells results into abrogation of immunity in mice that were vaccinated to *Schistosoma*.

7. Role of Th cell subsets

As seen earlier CD4⁺ helper T lymphocytes appear to perform most functions via the action of secreted lymphokines. These factors modify the activity of T cells that produce them in an autocrine fashion, as well as modulate responses of other cells in paracrine fashion (107). In past few years evidence has accumulated suggesting that differences in cytokine-secretion patterns among Th cell subsets play a major role in regulating the choice of immune functional modality. Therefore, factors that differentially regulate activation of these subsets are vital in determining whether cellular or humoral response is generated.

Mosmann and co-workers proposed a major subdivision of mouse CD4⁺ Th cell clones based on differences in their pattern of cytokine production (74). Th1 cells make predominantly IL-2, TNF- β and IFN- γ and Th2 cells make predominantly IL-4, IL-5, IL-6 and IL-10. Generation of Th1 response results in activation of macrophages, in DTH and in IgG2a and not IgE antibody response (108). Generation of Th2 response results in IgG1 and IgE antibody response and eosinophilia. A phenotype which expresses cytokines made by both Th1 and Th2 clones is known as Th0 (109, 110). Recent evidence now favours the view that Th1 and Th2 cells develop from Th0 cell which could be the common post-thymic precursor for both phenotypes (111).

Many suggestions have been made about what factors may influence activated primary Th cells to give rise to daughter cells with the functions and cytokine profiles of Th1 and Th2 cells. They could be 1) Cytokines 2) APCs 3) Density of presented antigen.

Scott has reviewed the data showing that cytokine environment that is present as the Th cells differentiate influences the subset that develops (112). This cytokine environment is largely established early in a response by cells participating in innate immune reactions such

as APC, mast cells and NK cells. NK cells appear to be a major source of IFN- γ which drives the differentiation to Th1 kind by inhibiting proliferation but not lymphokine generation by Th2 (113). IL-4 can drive the differentiation to Th2 pathway (114). Cells producing IL-4 during initial stages of infection have not been identified. Possible sources could be mast cells or basophils or T cells themselves (115). Another potential source of cytokines during early stages of immunity are $\gamma\delta$ T cells. In listeria infections these cells have been shown to produce IFN- γ (112). Macrophages and B cells produce IL-12 which induces IFN- γ production by both NK cells and T cells and drives the differentiation to Th1 pathway (116). IL-10 from B cells and activated macrophages has been shown to inhibit lymphokine production by Th1 clones but not Th2 clones (113). IL-10 exerts its effects indirectly by acting on APCs to reduce their ability to stimulate lymphokine production by Th1 cell clones (117). IL-10 downregulates production of IL-12 by APC as well as inhibits the expression of co-stimulatory molecule B7 (112). TGF- β has also been shown to augment Th2 cell development (118). IL-13 is a recently reported cytokine thought to regulate selective differentiation of CD4⁺ Th cells. IL-13 probably acts like IL-4, suppressing the development of Th1 cells. Through downregulation of IL-12 production by monocytes, it favours Th2 developmental pathway (119).

Also, cytokines produced by Th1 and Th2 subsets exhibit cross regulation i.e. cytokine secreted by one subset can block production or activity of cytokines secreted by other subset.

Co-stimulatory signals from APCs control expression of cytokines by Th cells. Different types of APCs like macrophages, B cells or dendritic cells may induce expression of different cytokines (113). Gajewsky et al have shown that purified splenic B cells stimulate optimal proliferation of Th2 cells while adherent spleen cells stimulate optimal proliferation of Th1 cells (107, 120). It is likely that cytokines or cell surface molecules important for cellular interaction or cell signalling either in Th1 or Th2 clones are differentially expanded depending on source and prior treatment of APC population (112).

High concentration of antigen has been shown to inhibit proliferation of Th1 clones but not Th2 clones. This may account for the observation that DTH reaction is favoured by low doses of antigen while antibody responses are favoured by high doses of antigen (113). Thus effective antigen concentration also appears to influence CD4⁺ T cell subset development.

We would now look at some immunomodulators that act to either upregulate, downregulate or even alter the quality of the cellular immune response developed.

8. Immunomodulators affecting CMI

Thymopentin is a synthetic thymic peptide which is licensed for use in the treatment of cancer and immunodeficiency (19). *In-vitro*, it has been shown to induce T cell differentiation markers and promotes differentiated functions such as interleukin production. Thymopentin enhances IL-2, TNF- α and IFN- γ production but reduces IL-4 secretion from lymphocytes of mice (121). The results therefore indicate that immunopotentiating activity of Thymopentin is due to preferential upregulation of Th1 cells.

Inosine pranobex is an inosine-salt complex which is used as an antiviral immunomodulatory drug (19). It has been shown to increase cellular immune defence. It induces T cell differentiation and promotes functions such as proliferation, cytotoxicity and interleukin production. It also promotes macrophage, granulocyte and eosinophil function. *In-vivo* these actions translate into enhanced responses in infected or cancer bearing individuals.

Morein et al in 1987 reported an alternative vaccine vehicle - the immunostimulating complex (ISCOMS) (27). Quil-A, a saponin extracted from *Quillaia saponaria*, in presence of cholesterol can form micelles with a typical cage like structure called ISCOMS. These have shown to increase humoral and systemic DTH responses to a large number of antigens (27). ISCOMS can stimulate CD8⁺ MHC I restricted Tc cells against non-infectious antigens. At least two distinct routes exist for the degradation of foreign antigens. The lysosomal pathway which leads to triggering of CD4⁺ T cells and the cytosolic pathway, which leads to triggering of CD8⁺ T cells. It was assumed that only infectious agents such as viruses could enter the cytosolic pathway. The capability of ISCOMS to reach cytosol has been explained by the ability of saponins to intercalate into cholesterol membranes (22, 27).

Cyclosporin A (CsA) and FK506 are powerful suppressers of immune system, especially of T cells (68). Ultrastructural examination and DNA electrophoresis of 24h CsA treated thymocytes show chromatin condensation, cellular shrinkage and nuclear fragmentation in oligonucleosomal fragments that are characteristic of apoptosis (122). Liu et al demonstrated that CsA and FK506 inhibit T cell antigen receptor initiated signal transduction pathways that lead to transcription of early activation genes (55). This includes

the IL-2 gene transcription required for transition from a resting G0 state to G1 phase of cell cycle. CsA and FK506 are protein drugs that become active when they bind to endogenous intracellular receptors, the immunophilins. Complexes between CsA and its immunophilin, cyclophilin and between FK506 and FK506-binding protein (FKBP) mediate both therapeutic and toxic effects of the drug. Primary target of both CsA-cyclophilin and FK506-FKBP, is a Ca^{++} , calmodulin dependent protein phosphatase calcineurin (122). Calcineurin is a key enzyme in T cell signal transduction cascade following TCR activation (55). Interaction of drug-immunophilin complexes with calcineurin and inhibition of its phosphatase activity leads to inhibitory effect of the drugs on expression of genes encoding T cell growth factor IL-2.

Apart from action of CsA as a nontoxic inhibitor of immune response, Bretscher and Havele have reported a novel immunomodulating activity of CsA (123). They showed that CsA could either inhibit or facilitate the induction of DTH depending on the class of immune response that would be generated in the absence of the drug.

In T cells, activation of genes for both IL-2 and its receptor determines the progression of the cell from G0 to G1 phase of the cell cycle. G1 to S phase is then regulated by IL-2. Rapamycin, which is a powerful suppresser of T cells, has no effect on early cytokine synthesis by T cells but it inhibits T cell response to IL-2 required for transition from G1 to S phase (68, 124). Rapamycin also binds to FKBP, but it inhibits IL-2/IL-2R mediated cellular proliferation. One of the early events following interaction of IL-2 with its receptor is the hyperphosphorylation of 40S ribosomal protein which is mediated in part by p70⁵⁶ kinase. FKBP-rapamycin inactivates basal and receptor augmented activity of p70⁵⁶k (68).

L156,602 which is a C5a receptor antagonist is shown to have a DTH-specific suppressive effect. It suppressed TNP-sheep RBC induced DTH while it enhanced antibody production against sRBC (125).

Santos et al have shown that iron may play a regulatory role in process of T cell recognition and T cell activation by selectively downmodulating T cell molecules known to be involved in these processes. Iron has been shown to downregulate expression of CD4 and CD2 molecules on the surface of T lymphocytes (29).

Galdiero et al showed that porins from *S.typhimurium* induce the release of IL-4 and IFN- γ by human lymphocytes and release of TNF- α , IL-1 and IL-6 by monocytes (16).

There are many immunomodulators that act on macrophages and NK cells. In fact, majority of modulators act on these cells. Agents that act on macrophages can alter their antigen presenting function and/or their microbicidal property. Various modulators altering antigen presenting functions (like MHC density, IL-1 secretion) have been described in previous section (section 2). We would now consider some modulators that affect the effector function of macrophages and NK cells.

ST789 which is a synthetic compound characterised by an amino acidic group joined to the N9 position of hypoxanthine ring has been shown to have immunomodulatory properties (126). This drug has been reported to protect immunosuppressed mice from microbial infections and tumour growth, and to restore mitogen-induced proliferation of splenocytes from immunosuppressed mice. It can markedly augment the sRBC phagocytosis by peritoneal cells and can potentiate cytotoxic activity and cytokine release by peritoneal macrophages.

A peptide isolated from *Viscum album* (plant - Iscador) has been reported to have cytotoxic and tumour reducing activity (26). Administration of the peptide was found to produce increased NK cell activity in normal and tumour bearing animals. There was an increase in antibody forming cells in the spleen and antibody titres were increased in animals treated with the peptide. Peptide could also stimulate ADCC.

Ethanol intoxication has been associated with bacterial pneumonia and tuberculosis. Bermudez et al showed that ethanol impairs the capacity of pulmonary macrophages to produce superoxide anion and TNF (127). Exposure to ethanol compromises macrophage's ability to respond to stimulation with TNF and GM-CSF, and kill an intracellular pathogen, *Mycobacterium avium*.

Patulin, a fungal metabolite has been shown to exert immunosuppressive effects on cellular and humoral immune systems (128), when its effect on murine peritoneal macrophages was studied. It was shown that phagocytosis and phagosome-lysosome fusion was diminished thus impairing microbicidal activity.

Various other modulators have been examined for their ability to act on macrophages and NK cells. Polysaccharopeptide from a mushroom, *Coriolus versicolor* has been shown to

posses antitumour activity by increasing RNOI, ROI and TNF production by macrophages (129). Carbamazepine has been shown to increase NK cell activity and phagocytosis and killing properties of macrophages (130). A synthetic immunomodulator acridine trihydrochloride induces macrophages to release IFN- α which subsequently activates NK cells (131). Similar results have been shown by neem oil (25). Activation of human NK cells and monocytes has also been shown by cisplatin (132). Aflatoxins have shown a depressive effect on the function of macrophages (133). They impair phagocytosis, intracellular killing and spontaneous superoxide production by macrophages. Compounds like Poly A:U and Poly I:C are interferon inducers and promote macrophage and NK cell mediated viral resistance and in some cases resistance to tumour challenge (19). IFN- γ inducers have also been shown to affect antigen-presenting functions of non-professional APCs. Reports from our laboratory have shown that non-responder state of mice following i.p. immunisation with *M.vaccae* could be corrected by treatment of mice with Poly I:C prior to immunisation (134). Poly I:C is known to enhance expression of MHC glycoproteins on APC. Thus it could improve the antigen presentation by peritoneal cells for *M.vaccae* antigen.

Endothelial adhesion molecules play an important role in tissue recruitment of leukocytes in inflammatory conditions. Endothelial cells express some adhesion molecules constitutively, however others are only expressed in response to localised concentration of cytokines produced during an inflammatory response. Gold sodium thiomalate has been shown to inhibit cytokines TNF, IL-1 and IL-4 stimulated expression of vascular cell adhesion molecule-1 and E-selectin but not intercellular adhesion molecule-1 on endothelial cells (135). Thus Gold sodium thiomalate has a direct effect on endothelial adhesion molecule expression which may contribute to its anti-inflammatory activity. Some plant alkaloids also exhibit anti-inflammatory properties (136). They show inhibitory effects on inflammatory mediators like histamine, platelet activating factor, IL-1 and prostaglandins. Galdiero et al showed that porins from *S.typhimurium* can induce rat paw oedema, that was not due to activation of complement (15). The inflammatory activity was due to release of histamine from rat peritoneal cells.

Immunomodulators can also act on complement. Various microbial enzymes have been shown to activate complement cascade (24), while there are other substances like chondroitin sulphate that show anticomplementary activity (137).

9. Antigenic structure as T cell immunogen

Antigenicity can be defined as the ability of a particular molecular structure to be recognised by antibody and/or TCR molecules. However immunogenicity is the ability of a molecule to induce an immune response (138). The molecular properties of antigen and the way in which these properties ultimately contribute to immune activation are central to our understanding of immune system. To understand how antigenic structure is related to immunogenicity for T cell response, factors like antigen processing, antigen presentation and physicochemical constraints on T cell target antigens have to be considered.

Classical studies by Rosenthal and Shevach (139) demonstrated that T cell recognition of antigen requires that antigenic peptides be displayed within the cleft of a self MHC molecule on the membrane of APC. This requirement is called self MHC restriction. Here T cells interact both with antigen and with self MHC molecules. In general $CD4^+$ Th cells are MHC class II restricted and $CD8^+$ Tc cells are MHC class I restricted. Formation of peptide-MHC complexes requires that protein antigens be degraded into peptides by a sequence of events called 'antigen processing'. Degraded peptides then associate with MHC within the cell interior and the peptide-MHC complexes are transported to membrane where they are displayed. This is called 'antigen presentation'.

a. Antigen Processing

It has been shown that T cells recognise a non-native form of the antigen (140). Conversion of antigen from a native to a non-native form has been termed antigen processing. Class I and class II molecules associate with peptides that have been processed in different intracellular compartments. MHC class I bind peptides derived from endogenous antigens that are available within the cytoplasm of the cell. Class II MHC molecules bind peptides derived from exogenous antigens that are internalised by phagocytosis or endocytosis and processed within endocytic pathway.

Endocytic processing pathway

First step in processing is binding of soluble antigen to APCs. This occurs via non-specific, non-covalent interactions with poorly characterised structures on the cell surface. Antigen in the immune complexes can also bind specifically through receptors for Fc and C3. Internalised antigen moves from endosomes to lysosomes encountering hydrolytic enzymes in each compartment. Endosomes contain a unique collection of more than forty acid dependent hydrolases including proteases, nucleases, glycosidases, lipases, phospholipases

and phosphatases. Within the compartment of endocytic pathway antigen is degraded into oligopeptides of about 10-18 residues which can bind to MHC class II molecules.

Early evidence for antigen processing and presentation was given by experiments of Ziegler and Unanue (141). They observed that Th cell activation by bacterial protein antigens was prevented by treating APCs with paraformaldehyde prior to antigen exposure. However if APCs were allowed to ingest the antigen and then were fixed with paraformaldehyde 1-3 h later, Th cell activation still occurred. During that interval of 1-3 h, APCs had processed the antigen and had displayed it on the membrane in a form able to activate T cells. Treatment of the APC with agents such as ammonium chloride or chloroquin also abrogates the processing and presenting capacity of the treated cells. These agents increase the pH of the lysosomal compartment. Requirement of proteinases for processing was shown by the fact that it can be inhibited by protease inhibitors like leupeptin (142).

Protein antigens are very heterogeneous in nature. Different enzymes can produce different antigenic fragments. Whether such antigenic fragments are recognised will depend primarily on the ability of the fragments to associate appropriately with MHC II molecules, although the TCR repertoire may also play a role (143).

Not all antigens are required to be fragmented before they can be recognised. In case of smaller peptide antigens, denaturation (unfolding) is all that appears necessary for the antigen to be presented by fixed or chloroquin treated APCs (144).

Cytosolic processing pathway

Endogenous antigens such as those produced by viruses replicating within a cell, are degraded within the cytoplasm into peptides that can associate with class I MHC molecules. This degradation is carried out by proteasomes. Proteasome particle which is protease complex has been shown to participate in digestion of cytosolic proteins (145). Class I mediated antigen processing is generally not affected by interference with endocytic pathway, however it is affected by inhibitors of protein synthesis.

b. Antigen Presentation

Presentation involves association of antigenic peptides with MHC class I or class II and movement of peptide-MHC complexes to the cell membrane. Like other proteins, MHC molecules are synthesised on polysomes within rough endoplasmic reticulum (RER), while antigenic peptides are generated in either endocytic or cytosolic processing pathway. Class I

molecules appear to bind antigenic peptides within RER, whereas class II molecules do not. In both cases however, binding of peptides helps to stabilise the association between MHC chains.

Since APCs express both class I and class II MHC molecules, some mechanism must exist to prevent class II molecules from binding to the same set of antigenic peptides as the class I molecules. Jones et al (146) discovered in 1978 that class II antigens were non-covalently associated with a molecule of 31 kDa which they designated the 'invariant chain', because of its lack of polymorphism. Invariant chain was found to be expressed in all cell types expressing class II, and induction of class II by lymphokines was in general, accompanied by induction of invariant chain (147). This protein interacts with peptide binding cleft of class II molecule, preventing any endogenously derived peptides from binding to the cleft while class II is in RER. Invariant chain also appears to be involved in folding of class II α and β chains, their exit from RER and subsequent routing of class II molecules to endocytic processing pathway (146). As class II molecules enter endocytic pathway, the low pH and proteolysis causes release of invariant chain. Subsequent antigenic peptide binding to class II maintains the stability of MHC II. Once the peptide is bound, peptide-class II complex is transported to plasma membrane.

Endogenous antigens are degraded in cytoplasm. Townsend concluded that there must be a special transport protein for peptide translocation required to deliver peptides of cytosolic origin to ER for class I assembly (148). This pump is a member of ABC (for ATP binding cassette) family of proteins and is now called as TAP (transporter associated with antigen processing) (149). It is an ATP-powered transmembrane pump that is located in the ER membrane. Genes coding for proteins that are members of ABC transporter family, are located in the chromosomal region where histocompatibility-related genes are clustered (148).

Peptide-MHC I complexes are then transported from RER through golgi complex to plasma membrane.

The ultimate outcome of processing and presentation in case of antigen processed by class II pathway is a complex of α -chain, β -chain with antigenic peptide brought on the surface of APC. In case of class I pathway, it results into a complex of heavy chains, β_2 microglobulin, and antigen brought on the surface of APC for presentation to TCR.

c. Physicochemical constraints on T cell target antigens

As mentioned earlier, native antigen has often to be denatured and fragmented for recognition by T cells. It has become apparent that this reflects the requirement of T cells that antigen is processed in a form that can interact appropriately with MHC proteins, and subsequently interact with T cell receptor. Thus, T cell antigenicity, in contrast to B cell antigenicity, in which tertiary structure of antigen is important, appears to be related to primary structure, secondary structure (150, 151), the local geometry of a peptide segment, its hydrophobicity and charge properties (152, 153). Tc cells recognise antigens on MHC class I background and bacterial antigens are presumably not recognised by them. Th cells which are important for induction of CMI, recognise antigen on class II background, and antigenicity for T cells will be discussed with regard to this population.

As we know, T cells do not interact with or recognise, an entire immunogenic molecule; instead, lymphocytes recognise discrete sites on macromolecules called epitopes, or antigenic determinants. Epitopes are immunologically active regions of an immunogen that bind to specific membrane receptors for antigen on lymphocytes. The epitopes recognised by T cells are in many cases, different from antibody reactive determinants on the same molecule and are quite often, fewer (154).

As demonstrated by various experiments, many T cell epitopes remain intact in small peptide segments of the antigen, hence it is possible to synthesise peptides of different sizes in order to investigate the requirements for activity. This is elegantly shown by Berzofsky's work on sperm whale myoglobin (3). Reactivity of T cells of B.10 D2 (H-2^b) mice were studied with synthetic peptides of sperm whale myoglobin. T cell clones reactive with a determinant centred around a lysine at position 140 were tested (in a proliferation assay) for their ability to respond to different synthetic peptides of myoglobin. The results showed that whilst Lys-140 was known to be recognised by these T cells, other aminoacids (Lys-133, Glu-136 and Lys-145) were also important for antigenicity of this epitope. The importance of these amino acid residues was thought to be for stabilising the peptide in correct conformation for interacting with the TCR and/or the class II MHC.

Similar studies were done by Schwartz et al (151). They tested the amino acid requirements for an epitope recognised by a pigeon cytochrome c specific T cell clone from B.10 A (H-2^a) mice. The epitope critical for interaction with TCR and two different epitopes important for class II MHC interaction were identified. The minimum size requirement for T cell proliferation was seven critical amino acids. However addition of further residues to the

N-terminus led to a marked increase in stimulatory potency of the peptides. And it was found that these extra residues assumed α helical configuration. Subsequent measurement of various peptides to ascertain their percentage helical configuration have shown that antigenicity does correlate with increased α -helical content of longer peptides.

MHC-class II molecule is thought to have at least one cleft for binding peptides (155). Like a lock and key, the class II cleft might accept only peptides of an α -helical or other fixed shape. The cleft might be hydrophobic (156) in which case α -helical amphipathicity would help to organise the α -helical configuration at the amphipathic interphase between the cleft and the surrounding water.

However, the binding of a peptide to the cleft in an MHC molecule does not appear to have the kind of fine specificity exhibited in the interaction of antibody and epitope. Instead a given MHC molecule can selectively bind a variety of different peptides.

The set of specificities that predominate after immunisation with the native protein is clearly not equal to the total potential epitope on an antigen. Certain sequences or even individual residues of the proteins are presented in an immunodominant manner. These sequences are the ones to which most of the immune response is directed (3). Several mechanisms can explain immunodominance. Various types of experiments have suggested that MHC plays a significant role in determining which T-cell epitopes in a given antigen would be immunodominant in a given individual. This was shown in the experiments of Sette and Grey (157). They analysed 14 synthetic peptides, representing overlapping sequences of the entire length of an immunogenic protein. Of these, three were shown to activate Th cells, and each of these three peptides was shown to bind MHC class II molecule. Another view is that special structural properties intrinsic to certain regions of protein make these regions immunodominant. Immunodominance of a given region is also thought to depend on regulatory mechanisms of host, including tolerance to structures resembling self, immune response genes, specificity of T cell help etc.

d. T cell target antigens of *Salmonella*

There are some limitations in the present understanding of immunology of Salmonellosis. Immunity even after an active infection is short lived (158), carrier state among typhoid patients is not uncommon (159) and contradictory results have been obtained concerning the efficiency of killed typhoid vaccines (160).

It is now generally accepted that the only effective mechanism of controlling infection by facultative intracellular parasites is CMI. Thus induction of CMI is of prime importance for protection and recovery from *Salmonella* infections.

Present knowledge of antigenic relationships of *Salmonella* is based on Kauffman and White scheme (6). In this scheme, various species of *Salmonella* are grouped in different serogroups, based on somatic antigens or LPS and proteinic flagellar antigens, in both phases. Various workers have shown that the *Salmonella* antigens involved in CMI are proteinic in nature (161, 162). This rules out the somatic antigens, which by their LPS nature, could never be the candidates for T cell target. Flagellar antigens being proteinic in nature, could be involved in CMI; however following studies rule out the possibility.

1. Studies done by Collins and Mackaness (5) involving DTH response to culture filtrates of different *Salmonella* in mice immunised with live, sub-lethal doses showed that a good DTH response could be elicited in immunised mice when culture filtrates of *S.pullorum*, an aflagellate strain of *Salmonella* was used.
2. Nair and Kamat (8) have studied the DTH response to the sonicate of *S.gallinarium* in mice immunised with sub-lethal doses of live *S.enteritidis*. Since *S.enteritidis* is an aflagellate, it could be concluded that the flagellar antigens are not involved in CMI. In this study it could be argued that the observed DTH cross reactivity is due to presence of identical somatic antigens in two species of *Salmonella* employed in the study. The same study has also shown that the sonicate of *S.paratyphi* C which belongs to group C elicited good DTH response in *S.enteritidis* (group D) immunised mice. Both the species in spite of being from different serogroups i.e. having different somatic antigens, showed a strong cross reaction when DTH was used as a test parameter.

As observed in the above data, extensive cross reactivity between *Salmonella* has been reported when CMI is used as test parameter (8). Tiwari and Kamat (9) have shown cross reactivity between *Salmonella* serogroups using DTH as the parameter. Even protection studies using an assay dependent upon generation of activated macrophages by T cell antigen interaction showed that in *S.paratyphi* B immunised mice, not only *S.paratyphi* B sonicate but cross reacting *S.poona* antigens could generate activated macrophages (8, 9).

Thus existing knowledge of inter and intra group cross reactions which is based on Kauffman and White scheme, seems to have no relevance as far as CMI is concerned. As ubiquitous, *Salmonella* are contaminants of food items, their relevant antigens may in sub-

clinical infection cause cross reactivity with enteric fever causing bacteria and be an important mode of acquired immune status of the host. Therefore study of target antigen/s of CMI is important while considering cross reactivity between different *Salmonellae*.

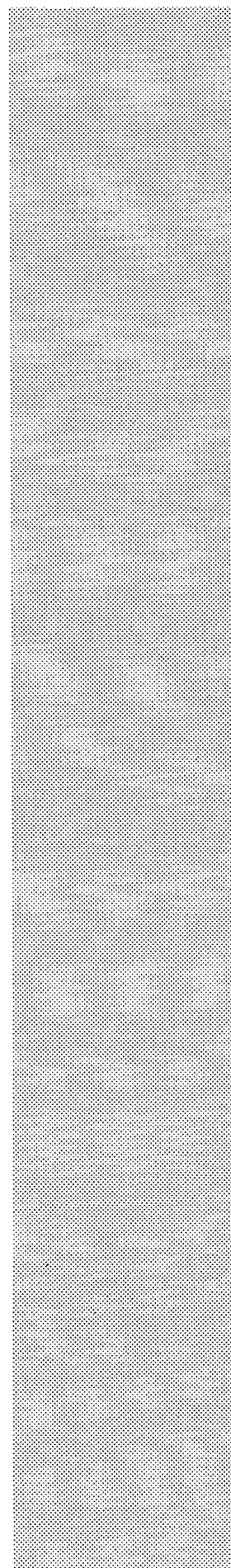
Earlier in a separate study from other group, an antigen capable of eliciting *in-vivo* and *in-vitro* T cell responses in mice immunised i.d. with *S.typhi* was purified from sonicate of *S.typhi*. The purification process yielded a protein having molecular weight of 65 kDa. Its pI was 7.1-7.2. The antigen could elicit DTH response in mice immunised with *S.typhi* and could also induce lymphoproliferation of primed spleen cells. It also showed extensive cross reactivity between *Salmonella* of different groups (163). From its properties it appeared to be related to a family of heat-shock proteins.

A number of studies show that OMPs extracted from *Salmonella* can induce protective immunity (164). Immunisation with OMPs has been shown to induce protection to subsequent challenge with *Salmonella* (165, 166). Results suggest that OMPs are good antigens interacting efficiently with both arms of host immune systems which could play a role in providing protection against the disease. These results indicate that OMPs could behave as T cell target antigens.

Following aims and objectives were set up for the present study:

1. To study OMPs of *Salmonella typhi* as targets of T cell recognition.
2. To study immunomodulatory properties of OMPs of *S.typhi*.

Materials and Methods



1. Micro-organisms

Salmonella typhi Ty2 (O:9, 12, VI H:d) was employed for detailed study of antigen involved in CMI. Following organisms were selected for study of cross-reactivity of OMP of *S.typhi* Ty2.

Salmonella paratyphi A (O:1, 2, 12 H:a)

Salmonella paratyphi C (O:6, 7, H:c, 1, 5)

Salmonella typhimurium (O:1, 4, 5, 12 H:i, 1, 12)

Salmonella enteritidis (O:1, 9, 12 H:g, m)

Salmonella gallinarium (O:1, 9, 12 H:-)

The strains were obtained from National Collection of Type Culture (NCTC), Colindale Avenue, London. A tube containing lyophilised culture was taken and opened with the help of an ampoule cutter under sterile conditions. 0.5 ml of peptone water was transferred into the tube with a sterile Pasteur pipette and the material suspended. The suspension was transferred to 4 ml peptone water, and was incubated at 37°C for 3 h. At the end of incubation period the revived culture was streaked on nutrient agar slants and incubated at 37°C for 18 h. Slants were preserved at 4°C till further use.

Bacterial inoculum was transferred into liquid broth (167) (Appendix I.1) and incubated at 37°C for 18 h on shaker. Cells were killed by irradiation, (1 Mrad γ -radiation in a Co⁶⁰ chamber) and the growth was harvested by centrifugation (12,000 $\times$ g for 10 min) and suspended in a minimal volume of medium.

M.vaccae was kindly supplied by Dr. J.L. Stanford of Middlesex Hospital, London. The strain was maintained on Lowenstein Jensen's medium slants and cultivated on the Doub and Youman's medium (168) (Appendix I.2). A loopful of bacterial inoculum was gently transferred onto the surface of the liquid medium and incubated at 37°C for 10-12 days. The flasks containing the growth in the pellicle form were gently shaken so as to get the growth to the bottom, excess medium was discarded and the growth harvested in minimal volume of medium. Cells were killed by γ -radiation (2.4 Mrad).

2. Animals

a. Mice

8-10 week old Swiss-White, Balb/c and C3H/HeJ mice obtained from National Institute of Health, USA, and maintained at Cancer Research Institute, Bombay were used for studies in inbred animals. 8-10 week old Swiss White mice obtained from National Institute of Health, USA and maintained in the animal colony of Haffkine Biopharmaceutical Corporation Ltd., Bombay were used for studies in random bred animals. Prior to immunisation, all mice were tested for DTH response to the sonicate of *S.typhi* and/or *M.vaccae*. Only those animals that did not show any response were used in the experiments.

b. Rabbits

Adult albino rabbits weighing 1.5-2 Kg were purchased from Haffkine Biopharmaceutical Corporation Ltd., Bombay and were used for raising antisera.

3. Immunisations

a. For induction of cellular immunity against *Salmonella*

A vaccine containing killed *S.typhi* Ty2 was prepared. Overnight growth was killed by γ -radiation (1 Mrad). Bacterial cells were harvested and washed thrice in phosphate buffered saline (PBS) (Appendix I.3). Pellet was suspended in PBS to give 2×10^9 cells/ml by comparing with Brown's opacity tubes. Mice were immunised intradermally (i.d.) with a single dose of 10^8 *S.typhi* cells in 0.05 ml PBS.

b. For induction of cellular immunity against *M.vaccae*

A vaccine containing killed *M.vaccae* was prepared. 10 day old culture in the medium of Doub and Youman's was killed by γ -radiation (2.4 Mrad). Bacterial cells were harvested and washed 3 times in PBS. One ml of packed cell volume was made to 100 ml with sterile PBS, to give 2×10^9 bacilli/ml. Required number of bacilli (10^8) were suspended in 0.5 ml for intraperitoneal (i.p.) immunisation and in 0.05 ml for i.d. immunisation.

4. Preparation of Sonic extract

Overnight growth of *S.typhi* Ty2 in 250 ml nutrient broth was killed by irradiation. Bacterial cells were harvested and washed with PBS. 3 ml packed cells were suspended in 15 ml PBS and sonicated in a sonifier (Branson, B-30-20 kHz with 50% duty cycle) for 45 min

keeping the tube constantly on ice. Sonicate was centrifuged at $1,00,000 \times g$ (Sorvall ultracentrifuge-OTD combi) for 1h to remove cell debris and to reduce LPS content. Supernatant was filtered through 0.22 mm membrane filter (Millipore, USA) and its protein content was estimated by the method of Lowry et al (Appendix II.1) (169). It was then aliquoted and stored at -20°C .

For preparing *M.vaccae* sonicate, cells in Doub and Youman's medium were killed by irradiation. Cells were washed and sonicated in the same manner.

5. Preparation of outer membrane protein fraction from *S.typhi*

OMP fraction was prepared by the method of Nakae et al (170). Cell envelopes were prepared by sonicating (20 kHz, 50% duty cycle) washed *S.typhi* cells (6-7 g wet weight) in 50 mM Tris-hydrochloride buffer (Tris-HCl), pH 6.8, (Appendix I.4) for 4 min. Sonic extract was centrifuged at $750 \times g$ for 10 min to get rid of large fragments of cell constituents and unbroken intact cells. The supernatant was then centrifuged at $30,000 \times g$ for 30 min and the pellet washed 2 times with Tris-HCl buffer. Pellet was later suspended in 2% SDS. It was kept at room temperature for 30 min and centrifuged at $40,000 \times g$ for 30 min. Washed pellet was dialysed extensively against distilled water. It was then suspended in a minimum volume of 5 mM Tris-HCl, pH 7.4 (Appendix I.5) and was digested with lysozyme (Sigma - 100 $\mu\text{g/ml}$ final concentration). Digestion was carried out at 37°C for 2 h in a water bath with intermittent shaking. Protein concentration of the lysozyme treated sonicate was estimated and then subjected to papain digestion. Papain (Sigma) was added in the proportion of 1 mg enzyme per 15 mg protein. 0.1 M L-cystein hydrochloride (Sigma) was also added to the digestion mixture in the proportion of 0.5 ml of L-cystein to 30 ml of mixture. Reaction was allowed to proceed at 37°C for 2 h in a water bath with intermittent shaking. Digestion was stopped by adding 0.13 M iodoacetamide (Sigma) in the proportion of 0.2 ml for every 10 ml reaction mixture and by transfer of the flask to a chilled ice bath. Treated material was centrifuged at $1,00,000 \times g$ for 1 h to remove cell debris and to reduce LPS content. Supernatant collected was OMP preparation. It was then dialysed against PBS for 1.5 h, and protein estimation of the preparation was done by the method of Lowry.

6. Preparation of porin fraction from *S.typhi*

Porin fraction was prepared as described by Nikaido (186). Cell envelopes were prepared from *S.typhi* Ty2 cells and treated with 2% SDS as described earlier. This SDS-insoluble material containing peptidoglycan associated porin was resuspended in 2 ml of

NaCl buffer (50 mM Tris-HCl, pH 7.7, 0.4 M NaCl, 1% SDS, 5 mM EDTA, 0.05% 2-ME, 3 mM NaN₃) (Appendix I.14) and extracted at 37°C for 2 h. The suspension was centrifuged at $1,00,000 \times g$ for 1 h at 20°C. The supernatant collected was porin preparation. It was dialysed extensively against PBS for 5-6 h with frequent changes. Protein estimation of the preparation was done by the method of Lowry (169).

7. Preparation of LPS

LPS was prepared by phenol-water extraction method as described by Westphal (171). Bacterial pellet (10 g net weight) was suspended in 80 ml distilled water. To this suspension, 130 ml of 75% phenol was added (75% phenol prepared by adding 30 ml of distilled water to 100 g of liquefied phenol). Mixture was incubated at 65-68°C in a water bath for 30 min with occasional shaking. It was then centrifuged at 2000g for 30 min. Two phases, aqueous and phenol were separated with bacterial pellet at the interphase. Upper aqueous phase was collected. Remaining phenol layer and bacterial pellet were washed with 50 ml distilled water. It was incubated and centrifuged as earlier, and upper aqueous phase was combined with the earlier collected material. This fluid containing LPS was then dialysed for 3 days against distilled water, and later centrifuged at 100,000g, for 2h. Pellet containing LPS was suspended in 2-3 ml distilled water and lyophilised.

For use in assays, LPS was resuspended in distilled water. LPS in solution was estimated by Thiobarbituric acid method (172) (Appendix II.2).

8. Isoelectrofocusing procedures

a. Analytical Isoelectrofocusing (IEF)

It was done using a standard procedure (173). A 0.5 mm thick polyacrylamide gel was prepared on a gel bond film (6.0 cm $\times$ 12.0 cm) with 7.5% polyacrylamide in distilled water containing 2% ampholine (Sigma) of appropriate pH range. Strips of Whatman filter paper number 1 soaked in appropriate anolyte and catholyte were placed directly on the gel at the respective electrode ends. Platinum wire electrodes were placed directly in contact with filter paper strips and prefocusing was carried out on a Multiphore electrophoresis unit (LKB) at 5 mA current for 30 min. 10 μ l of sample containing 2 mg/ml of protein was applied on a Whatman filter paper number 1 strip of 5 $\times$ 10 mm size and kept at the cathodal end of the gel. Focusing was carried out at constant power of 3 W, maximum voltage of 2000 V and maximum current of 6 mA, at 4°C for heat dissipation from the gel. After 60 min, pH

gradient was checked with a surface pH electrode (LKB, Pharmacia). The gels were fixed overnight in a solution containing 50 part methanol : 38 parts distilled water : 12 parts glacial acetic acid, and 0.5 ml/lit of formaldehyde. Gels were then stained with a silver stain by the procedure of Blum et al (174).

b. Preparative IEF on agarose

Preparative IEF was done by techniques described earlier (173). 20 ml of 1% agarose (Sigma, IEF grade, type VIII, <0.02 EEO), containing 12.5% (w/v) sucrose and 2% (v/v) Ampholine (Sigma) of appropriate pH range was layered on to a gel bond film of 7.5 cm × 7.5 cm size and cooled to solidify. Filter paper strips (Whatman, number 17) dipped in appropriate anolyte and catholyte solutions were placed at either sides of the gel at respective electrode ends. Prefocusing was carried out at 15 mA current for 45 min. A trough of the size of 0.5 cm × 6.5 cm was cut 5 mm from the cathodal end and 1 ml of sample containing 6 mg/ml protein was added in it. Electrofocusing was carried out at constant power of 5 W keeping maximum current at 15 mA and maximum voltage at 1500 V, for 2-2½ h, after which the current gradually came down. Entire electrofocusing was carried out at 4°C. pI of the gel was checked by a surface electrode, and pH gradient formation was also confirmed.

9. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

This was done employing the method of Laemmli (187). 10% solution of polyacrylamide was prepared in the resolving buffer (Appendix I.15). SDS was added to a final concentration of 1%. A 8.5 cm × 7 cm × 0.75 mm thick vertical resolving gel was prepared by pouring the slurry between an Aluminium Oxide plate & a glass plate separated by a 0.75 mm thick spacer. A separate 5% Polyacrylamide solution was prepared in stacking gel buffer containing 1% SDS (Appendix I.15) and the solution was poured to form a layer of stacking gel 1.5 cm × 8.5 cm × 0.75 mm above the resolving gel. Ten sample channels were prepared on top of the gel with a comb.

10µl of samples containing 1-5µg of protein in sample buffer (Appendix I.15) containing 2% SDS and 5% 2-Mercaptoethanol were loaded on the gel. Both upper and lower reservoirs of the electrophoresis tank were filled with reservoir buffer (Appendix I.15) containing 1% SDS. Electrophoresis was carried out at 25 mA constant current for about 1h (till Bromophenol blue is just off the bottom of the gel). This gel was either used for blotting on NCM or was fixed and then stained by silver staining technique of Blum et al (174).

10. Preparation of antigen adsorbed nitro-cellulose membrane (NCM) particle suspension

a. Electroblotting of OMP bands from the IEF gel

OMP preparation was subjected to preparative agarose IEF. The focused gel was then blotted onto an NCM (Sigma) employing Midget Multiblot Electrophoresis transfer unit (LKB, Pharmacia).

Sheets of thin (Whatman number 1) and thick (Whatman number 17) filter paper of 7.5 cm × 7.5 cm were cut and soaked along with synthetic sponges in Tris-glycine transfer buffer (Appendix I.6). Focused agarose gel was also soaked in transfer buffer for 30 min for equilibration. A thin wet filter paper sheet was placed gently on top of the gel, starting from one end to the other, taking care not to entrap air bubbles between the two. A glass rod was gently rolled over it to ensure removal of air bubbles. After this, a thick wet filter paper was placed over it. Now the gel was turned upside down and the supporting gel bond film was gently stripped off. An NCM of the size same as the agarose gel (7.5 cm × 7.5 cm) was cut and placed over the inverted side of the gel without entrapping air bubbles. A sheet each of thin and thick filter paper were placed on top of NCM and gently pressed. Then on either side of the assembly, one each of thin and thick sponges were placed and the sandwich was fitted into the plastic cassette for electroblotting. The cassette was closed and inserted into the vertical transfer chamber, so that NCM faced the anodal chamber. The transfer chamber was filled $\frac{3}{4}$ th with the transfer buffer. The vertical chamber was kept on a magnetic stirrer for the uniform mixing of transfer buffer during transfer. Electrophoresis was carried out at 80 mA and 4 W keeping V_{max} 215 for 2½ h (for SDS-PAG, transfer was carried out at 30 mA constant current and 8 V overnight at 10°C). At the end of the run, the NCM was washed 3 times with PBS-Tween 20 (Appendix I.8) for 5 min each and the gel was stained with Amido Black B.

b. Preparation of suspension of NCM adsorbed bands

This was done as described by Abou Zeid et al (175). Stained bands were cut into small pieces and taken into a test tube to which Dimethyl Sulphoxide (DMSO, Sigma) (250 µl/20 mm² area of NCM) was added and allowed to dissolve for 1 h at room temperature. Suspension was mixed on a vortex mixer to ensure complete dissolution. Then the dissolved NCM particles were reprecipitated by addition of carbonate buffer (Appendix I.9) (250 µl/20 mm² area of NCM) and centrifuged at 10,000g for 10 min at 4°C. The supernatant was discarded and the precipitate was resuspended in minimum volume of RPMI 1640. This

preparation was titrated for its ability to support lymphoproliferation of spleen cells of mice primed i.d. with *S.typhi*. 100 µl of the optimum dilution of the suspension was employed in all the assays.

11. Cyanogen Bromide treatment

LPS solution and OMP preparation were treated with Cyanogen Bromide (CNBr) as described by Cahnman (176). OMP preparation (5 mg/ml) in 0.0175 M phosphate buffer, pH 6.3 (Appendix I.11) or LPS preparation (5 mg/ml) in the same buffer were mixed with an equal volume of CNBr (Sisco Research Lab, Bombay, 1.5 mg/ml in 0.6 M HCl.) Reaction mixture was incubated at room temperature for 4 h in a tightly closed container with continuous shaking. At the end of the incubation period, the digest was dialysed for 1 h at 4°C against 0.0175 M phosphate buffer, pH 6.3 (Appendix I.11) and then overnight at 4°C against PBS.

12. Estimation of nitrite

Nitrite (NO_2^-), an oxidised product of Nitric oxide (NO) was used as an indicator for Nitric oxide synthesis. Nitrite in the culture supernatants was measured by the colorimetric method of Ding et al as described by Isobe and Nakashima (177). 100 µl of culture supernatants were incubated in duplicates with equal amount Griess reagent (1% Sulphanilamide, 0.1% naphthyl ethylene diamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. For calculation of nitrite concentration sodium nitrite was employed as a standard. Absorbance readings were taken at 550 nm with a microplate ELISA reader (Titretek Multiscan).

13. Delayed type hypersensitivity (DTH) test

DTH test was carried out by the method of Grey and Jennings (178). A 0.03 ml volume of the antigen of the test organism containing 30 µg protein was injected into the hind foot pad of mice. The other foot pad received the buffer employed for test antigen. Measurements of foot pad thickness were taken before, 24 h and 48 h after injection. Differences in the values of foot pad thickness measured after injection and before injection were calculated and designated as foot pad swelling (FPS). The difference between FPS value for test antigen and the FPS value for the corresponding buffer simultaneously tested in the same animal constitutes the foot pad enlargement (FPE). A set of non-immune naive mice of the same strain were also simultaneously tested. The results were expressed as

corrected foot pad enlargement (CPE) which is the difference between FPE of test and the control mice simultaneously tested. The results were analysed by Student's 't' test by comparing the CPE values with zero.

14. Hypersensitivity reaction to OMP/LPS

Hypersensitivity reaction to OMP/LPS of *S.typhi* was tested by injecting different amounts of OMP/LPS in 0.03 ml in the hind foot pad. The other foot pad received the buffer employed for dissolving OMP/LPS. Measurements of the foot pad thickness were taken before, 24 h and 48 h after injection. Differences in the values of foot pad thickness measured after injection and before injection was FPS. The difference between FPS value for OMP/LPS and the FPS value for the corresponding buffer constitutes the measure of hypersensitivity induced and was designated as FPE. Mice were then sacrificed and the foot pads were excised for histological examination.

15. Lymphoproliferation assay

This assay was carried out employing standard microculture technique (179). Normal or immunised mice were sacrificed by cervical dislocation, their spleens removed and a single cell suspension was prepared in cold wash medium (RPMI 1640). Cell suspension was spun at $400 \times g$ for 10 min. Cell pellet was suspended in wash medium, approximately 3 ml for each spleen. Cell suspension was then loaded on Ficoll-Hypaque (density: 1.083 ± 0.001 , Sigma). Approximately 1.5 ml Ficoll was taken per spleen and it was loaded with double the volume of cell suspension. It was centrifuged at $400 \times g$ at room temperature for 20 min. Buffy layer of mononuclear cells at the junction was gently aspirated with a Pasteur pipette and delivered into a centrifuge tube with 10 ml cold wash medium and centrifuged at $400 \times g$ for 10 min. The pellet was washed twice more. Cell viability was estimated by Trypan blue exclusion test and the viable cell count taken. The cell pellet was then suspended at 2×10^6 cells/ml in culture medium, RPMI 1640 containing 5% Foetal calf serum (FCS, Biological Laboratories, Israel), 2 mM glutamine (Sigma) and 1% Antibiotic-Antimycotic (AA) (Sigma). Each well of microculture plate (Nunc, Denmark) received 2×10^5 cells and the antigen. Final volume of the culture well was 200 μ l. Culture medium of control wells did not contain antigen. All test and control cultures were set up in quadruplets. They were then incubated in a humidified incubator (CO_2 incubator, Flow Laboratories) with 5% CO_2 at 37°C . Cultures were pulsed 120 h later with 1 μCi of ^3H labelled thymidine (BARC, India, Sp. act. 6.5 Ci/mM) for a period of 16 h. They were then harvested on 3 mm Whatman filter

paper discs of 20 mm diameter and were air dried. Dried discs were transferred to 10% trichloroacetic acid (TCA) for 5 min, followed by 3 washes for 2 min each with distilled water and finally a methanol wash for 5 min. Discs were dried and then transferred into scintillation vials containing 5 ml scintillation fluid (Appendix I.13). Counts were taken in a scintillation counter (LKB) and results were expressed as counts per min (CPM). Degree of stimulation was calculated as stimulation index (SI), which is the ratio of CPM of cells with to without antigen.

16. Separation of T cells

A spleen cell suspension was made as described above. Mononuclear cells were separated and the cell suspension was loaded on to a preactivated nylon wool column and non-adherent cells were eluted by the method of Julius et al (180) as described below.

Nylon wool (Leucopak, Fenwel Lab, USA) was soaked in running tap water for 20 h. It was then soaked in double distilled water for 2 days at room temperature, with frequent changes. Washed wool was teased and soaked in RPMI 1640 with 5% FCS and 1% AA at 37°C for 16 h with 3 changes. It was again rinsed with 4 volumes of distilled water. This detoxified, washed nylon wool was packed in siliconised syringes. For a 10 ml syringe approximately 0.8 g (dry weight) nylon wool was packed. The syringe with the wool was sterilised by autoclaving. It was later held vertically in an incubator at 37°C. It was gently washed with 10 volumes of medium containing 1% AA. Later, 1 ml suspension of 5×10^7 lymphoid cells were overlayed gently on the column and allowed to enter into the wool. After 20 min incubation, culture medium at 37°C was delivered on top of nylon wool and the column eluted very slowly in 2-3 bed volumes of culture medium at the rate of 6-10 drops a min. The non-adherent cells were collected, chilled, washed once and suspended in the culture medium and viability estimated by Trypan blue exclusion.

17. Preparation of peritoneal cell suspension

The mice were given an i.p. injection of 6 ml wash medium and then killed by chloroform anaesthesia. Their abdomen was massaged gently and within 5 min. of killing, the contents were aspirated by a sterile Pasteur pipette. Aspirates of several mice were pooled, cells were washed in culture medium once and viability estimated by Trypan blue exclusion.

18. Irradiation of spleen and peritoneal cells for use as APCs

Spleen and peritoneal cells were harvested from mice as described earlier and suspended at a density of 10^6 to 5×10^6 cells/ml in culture medium containing 5% FCS. The cell suspension was subjected to 800 rad γ -radiation in a Co^{60} source. Irradiated cells were washed once in wash medium and used as APCs.

19. Rabbit antibody against 65 kDa antigen of *M.vaccae*

This was done by the method described by Khetan et al (181). Lysozyme papain digested sonicate of *M.vaccae* (800 μl of sample containing 10-12 mg protein) was subjected to preparative agarose IEF employing ampholine of pH range 3.5 to 5. 0.05 M H_2SO_4 , pH 1.5 was used as anolyte and 0.4 M HEPES, pH 5.2 was used as catholyte. The visible precipitin band at pI 4.3 was cut. Bands from 3 gels were pooled together and crushed in a glass tube with glass piston to a fine particle size. To this mixture 2 ml of FIA (Sigma) was added and a homogenous emulsion prepared by grinding with a tight fitting glass piston. A rabbit weighing 2.5 kg was depilated and the antigen preparation was injected i.d. at multiple sites on the back. Immunisation was repeated two more times at 10 days interval. 7 days after the last immunisation the rabbit was test bled from ear artery, blood collected and the serum separated. The antiserum was tested against *M.vaccae* sonicate and 65 kDa antigen by agarose gel diffusion method of Ouchterlony (182) and the antibody content was titrated by the same method. When a sufficiently high titre was obtained (1:16 or above), the rabbit was bled out, serum separated, decomplimented, aliquoted in 1 ml aliquots and stored at -20°C .

IgG fraction was obtained from this antiserum by Ammonium Sulphate precipitation (Appendix II.4).

20. Rabbit antibody against peptide of pI 4.85 from OMP preparation of *S.typhi*

OMP preparation from *S.typhi* Ty2 was subjected to preparative agarose IEF employing ampholines of pH range 3.5-6. 0.1 M H_3PO_4 , pH 2.4 was used as anolyte and 0.1 M NaOH, pH 11 was used as catholyte. After completion of the run, pH of the gel was checked by surface electrode and a 2 cm gel strip was cut from the bottom of the gel and stained with Coomassie Brilliant Blue. The stained band always corresponded to pI 4.85. Using the stained gel as template the band on unstained gel was cut at pI 4.85. Bands from 3 gels were pooled together and crushed in a glass tube with glass piston. The mixture was mixed with FIA and rabbit immunised as described previously.

21. Assay for anti-65 kDa antibody levels

a. Titration of anti-65 kDa antibody levels

Antibody levels against 65 kDa antigen of *M.vaccae* were assayed by Sandwich ELISA technique.

Design of the assay: The solid phase (ELISA plate wells, Nunc, Denmark) was coated with rabbit antibody against 65 kDa antigen to capture the 65 kDa antigen from *M.vaccae* sonicate. This complex was then exposed to test mouse serum samples (and positive and negative controls). The anti-65 kDa antibodies in test mouse sera were detected by using an antimouse IgG specific peroxidase conjugate.

Reagents:

- a. Coating antibody: IgG fraction of rabbit antiserum against 65 kDa antigen of *M.vaccae* in coating buffer (Appendix I.9).
- b. Source of 65 kDa antigen: Lysozyme-papain digested *M.vaccae* sonicate in PBS.
- c. Positive control serum: Serum of mice immunised with 65 kDa antigen of *M.vaccae* in FIA given 3 times at one week interval by the i.d. route.
- d. Anti-65 kDa antibody containing known standard mouse antiserum: In our initial assays for standardisation, serum of mice given 3 i.p. immunisations with 10^8 *M.vaccae* cells generated a reasonably good anti-65 kDa antibody response. This serum was employed as a source of standard.
- e. Normal mouse serum.
- f. Anti-mouse IgG specific HRP conjugate (Sigma): Affinity isolated goat antimouse IgG (heavy chain specific) antibody coupled to horse radish peroxidase (Appendix II.5).
- g. Substrate solution: OPD - 4 mg/10 ml, H_2O_2 - 2 μ l (30%)/10 ml substrate buffer (Appendix I.10).
- h. Dilution buffer: PBS-T20 (Appendix I.8).
- i. Washing solution: Saline-T20 (Appendix I.7).
- j. Coating buffer: Carbonate-Bicarbonate buffer, pH 9.6 (Appendix I.9).

Procedure:

- a. Wells of ELISA plates (12 × 8 Nunc) received 100 µl of IgG fraction (100 µl/ml) of rabbit antiserum against 65 kDa antigen. The plates were incubated overnight at 4°C.
- b. Next day, the well contents were discarded and well surfaces were blocked with 2% gelatine in coating buffer at 37°C for 1½ h.
- c. Plates were washed 5-6 times with washing solution, saline T-20 on an ELISA washer (Organon, Teknika, miniwasher) and 100 µl of lysozyme papain digested *M.vaccæ* sonicate (1 mg/ml) was added to the wells and incubated at 37°C for 2 h.
- d. Wells were then washed 5-6 times and 100 µl of different dilutions of test mouse serum samples were added to the wells. Appropriate controls i.e. positive control serum, standard anti-65 kDa antibody containing mouse serum at 1:8 and 1:64 dilutions, PBS-T-20 as negative control and normal mouse serum at 1:8 dilution were also added to separate wells and the plate was incubated at 37°C for 1½ h:
- e. The plate was washed 5-6 times and 100 µl of antimouse HRP conjugate (1:10,000 dilution) was added to the wells and incubated for 1 h at 37°C.
- f. The plate was again washed 5-6 times and 100 µl of substrate solution was added to the wells and incubated at room temperature in the dark for 10-15 min. After colour development, the reaction was stopped with 2.5 M HCl and read at 492 nm.

Calculations:

Day to day variations in absorbance values were observed in spite of using same standards. Hence the absorbance of test serum was expressed as a multiple of a single source of standard mouse serum having reasonable amount of anti-65 kDa antibody. Thus the results of antibody titre of serum samples done on different occasions were normalised with respect to the same standard.

$$\text{Antibody titre (AU/ml)} = \frac{\text{A492 of test serum sample}}{\text{A492 of standard mouse serum}} \times \frac{\text{Dilution of test serum sample}}{10}$$

Antibody titre was expressed as absorbance units (AU)/ml.

b. Determination of major isotype of anti-65 kDa antibody

The above procedure was repeated except in the step 'e'. Instead of anti-mouse IgG-HRP conjugate, anti-mouse IgG1 or IgG2a conjugates were employed. Results were expressed as ratio of AU/ml with the 2 conjugates.

22. Assay of macrophage microbicidal activity

Assay of macrophage microbicidal activity of peritoneal cells (PCs) was carried out employing the technique of Blanden et al (183).

Preparation of antibody for opsonisation

Antiserum against whole sonicate of *S.typhi* was raised by immunisation of adult albino rabbits. The sonicate containing 1 mg/ml protein was mixed with an equal volume of FIA and thoroughly emulsified. The rabbit was immunised and serum collected as described earlier.

One ml of a suspension in PBS of viable *S.typhi* Ty2 containing $2-4 \times 10^5$ cells was added to 9 ml of PBS containing 20% heat inactivated rabbit anti-*S.typhi* antiserum for opsonisation. After thorough mixing on a vortex, the culture was incubated at 37°C for 1 h with vigorous shaking every 15 min. At the end of 1 h the bacterial culture was thoroughly mixed and an aliquot was set aside to determine the exact bacterial count. 0.5 ml of opsonised suspension containing approximately $1-2 \times 10^4$ *S.typhi* cells was injected i.p. into each mouse. After exactly 5 min., which was time allowed for the phagocytosis of the injected bacteria by the macrophages in PCs, the mice were sacrificed by cervical dislocation. 2 ml PBS containing 2% anti-*S.typhi* antiserum, 10% new-born calf serum (NBCS) and 20 IU of heparin (Biological E. Ltd.) were injected i.p. into each mouse and PCs were aspirated. PCs from a number of mice were pooled in siliconised glass centrifuge tubes and kept on ice to prevent killing of phagocytosed bacteria. PC suspension was then spun at $110 \times g$ for 5 min at 10°C, the supernatant which contained unphagocytosed bacteria was again stored in cold and later subjected to a viable count to compute the number of extracellular bacteria. PCs were then washed twice and suspended in PBS containing 12% NBCS at a concentration of 6×10^6 cells/ml after counting viable PCs in Trypan blue. One ml aliquots of this suspension were then incubated at 37°C for varying periods of time and one aliquot was kept on ice immediately. After required time intervals, the aliquots were removed from the incubator and 0.1 ml of Saponin (Sigma) to a final concentration of 2.5% was added to lyse the PCs. The lysates were then appropriately diluted and plated out on

MacConkey's agar to determine the number of viable bacteria at each time point. The percentage viability of the *Salmonellae* at each time point was calculated using the number of organisms injected as 100%.

23. IL-1 assay

Soluble IL-1 was estimated by mouse thymocyte costimulation assay described by Grey et al (184).

Thymuses were dissected from 3-5 week old C3H/HeJ mice and a single cell suspension of thymocytes was made in RPMI 1640 with 1% AA and 10% FCS. Cell suspension was washed three times with RPMI 1640. Each well of microtitre plate received 80 μ l of suspension (1.5×10^6 cells per well) and 120 μ l of culture medium containing 100 μ l of test culture supernatant and 20 μ l of 10% PhA-M (Gibco). The culture were set in triplicates. Plates were incubated at 37°C in 5% CO₂ for 48 h and then pulsed with 3H labelled thymidine (1 μ ci/well) for 16 h and incorporation of label assayed.

24. Generation of IFN- γ rich supernatant

For stimulation of PCs to induce Nitric oxide synthesis, IFN- γ is required (185). C3H/HeJ or Swiss White mice were immunised i.d. with 10^8 *M.vaccae* cells. One week later, their spleen cells were harvested and cultured for 48 h with 20 μ g/ml *M.vaccae* sonicate as antigen. Supernatant was harvested and used as a source of IFN- γ . On an average, the IFN- γ content of such culture supernatant was approximately 1,000 IU/ml as estimated by microplate ELISA employing commercial kit supplied by Genzyme (USA).

25. Absorption of the sonicate of *S.typhi*

Lysozyme papain-digested sonicate of *S.typhi* was mixed (1:1 v/v) with insolubilised gel of rabbit antiserum (Appendix II.6) and incubated at 4°C for 1 h. The mixture was centrifuged at $2000 \times g$ for 10 min and supernatant was collected. The absorption was done 4 times.

26. Agarose gel diffusion

Agarose gel diffusion was done by the method of Ouchterlony and Nilsson (182). 1% agarose (high EEO) in 0.15 M NaCl was poured on to a 7.5 cm $\times$ 2.5 cm glass slide to give a gel layer of 2-3 mm. Wells, 5 mm in diameter were punched in a three way system with a distance of 13 mm between any two wells. These were filled with antiserum or antigen under

study. After the precipitin lines were developed, the slides were washed, dried and stained for immunoprecipitates with CBB.

27. Staining Procedures

a. Silver staining of IEF or SDS-PAGE gels for proteins by the technique of Blum et al (174)

Fixation: The gels were fixed in ten times the volume of fixative solution (Appendix I.16.a) overnight.

Washing: Gels were washed in ten times the volume of 50% ethanol in distilled water for 20 min. This was repeated three times.

Pre-treatment: Gels were pre-treated with ten times the volume of Sodium thiosulphate (Appendix I.16.a) for exactly 1 min.

Washing: Gels were rinsed in twenty times the volume of distilled water for 20 sec. This was repeated three times.

Silver Impregnation: Gels were treated with ten times the volume of Silver nitrate solution (Appendix I.16.a) for 20 min on a shaker.

Washing: They were then rinsed in twenty times the volume of distilled water for 20 sec. This was repeated three times.

Developing: Gels were developed in ten times volume of developer solution (Appendix I.16.a) for 5 min. (until the bands developed)

Washing: Gels were then washed with twenty times the volume of distilled water for 2 min. This was repeated two times.

Termination of development: Staining was stopped by soaking the gel in ten times the volume of destainer solution (Appendix I.16.a) for 10 min.

Washing: Gels were then washed in ten times the volume of 50% methanol for 20 min and dried.

b. Coomassie Brilliant Blue (CBB) R-250 staining for immunoprecipitates

Fixing: Gels were fixed in ten times the gel volume of 5% trichloroacetic acid in distilled water for 15 min.

Staining: Gels were immersed in ten times the volume of 0.5% CBB-R-250 prepared in 'destainer' solution (Appendix I.17.b) for 30 min.

Destaining: Slides were washed repeatedly in 10 times the volume of 'destainer' (Appendix I.17.b).

c. Amido Black B (ABB) staining of NCM

Staining: The NCM was immersed in ten times the volume of 0.05% ABB prepared in 'destainer' solution (Appendix I.17.c) for 10 min.

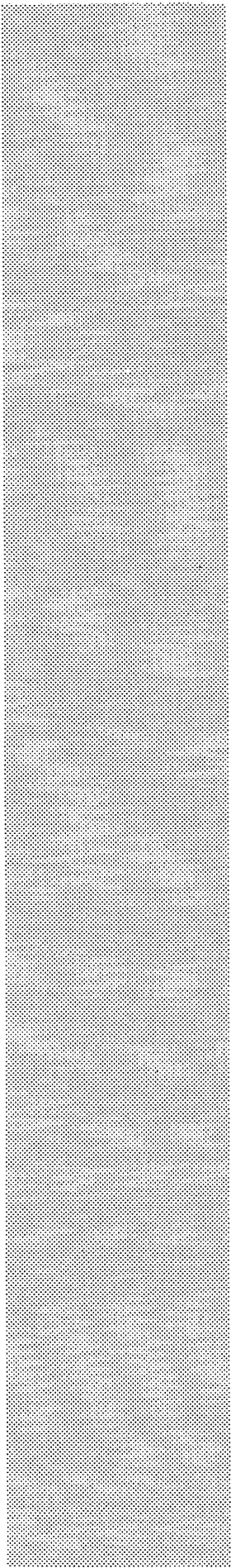
Destaining: NCM was washed repeatedly in ten times the volume of destainer (Appendix I.17.c).

28. Immunoperoxidase staining of NCM blot

This was done as described by Harlow and Lane (1988).

- a. NCM blot was washed with PBS 3-4 times for 10 min each.
- b. Non-specific binding sites on NCM were blocked with 3% BSA in PBS overnight at 4°C.
- c. The blot was washed with PBS 3-4 times for 10 min each.
- d. Blot was then dipped in 1:1,000 dilution of rabbit antibody against peptide of pI 4.85 from OMP preparation, in PBS with 0.25% BSA and 0.1% Tween-20 at 37°C for 2 h.
- e. Blot was washed with PBS containing 0.1% Tween-20 3-4 times for 10 min each.
- f. Blot was then placed in anti-rabbit HRP conjugate (1:10,000 dilution) for 1.5 h at room temperature with mild agitation.
- g. Blot was washed with PBS 3-4 times for 10 min each.
- h. The blot was developed using H₂O₂-diaminobenzidine (DAB) (2 µl of 30% H₂O₂ and 5 mg DAB per 10 ml PBS). After 5 min the reaction was stopped by removing H₂O₂ by rinsing with PBS.

Results



I. Immunomodulatory properties of OMPs

A. Immunomodulatory properties affecting afferent arm of immune response

1. OMPs affect immune response of mice to an unrelated antigen

Effect of OMPs of *S.typhi* on immunogenicity of *M.vaccae* was studied. Two groups of Swiss White mice (15 per group) were injected with 250 µg or 1 mg of OMP preparation per mouse by i.p. route. 72h later, they were immunised i.d. with a vaccine containing 10^8 killed *M.vaccae* cells. 72h later OMP dose was repeated. A third group of 15 mice received i.d. immunisation with *M.vaccae* without any OMP treatment, and a fourth group received OMP treatment without i.d. priming. One week after immunisation, DTH responses of mice of all the four groups were assayed with the sonicate and 65 kDa antigen of *M.vaccae*. Following DTH tests the mice were bled through the retro-orbital plexus. Blood of 5 mice identically treated and immunised was pooled. Thus 3 pools per group were obtained, and the serum separated. ELISA tests for anti-65 kDa antibody titre were done on each serum pool. Reason for employing 65 kDa antigen of *M.vaccae* as the test antigen is that it has been shown to be the major immunodominant antigen of mycobacteria (181). Major isotype of the anti-65 kDa antibody was also assayed by ELISA.

Treatment of mice with both 2 mg and 500 µg of the OMP preparation enhanced their DTH responses to *M.vaccae*, when compared with the responses of untreated mice. OMP treatment also increased anti-65 kDa titres by ten fold (Table 1).

Even though it was a primary immunisation, major fraction of the anti-65 kDa antibody was IgG1. On treatment with OMP, there was a greater rise in the IgG2a fraction. The IgG2a:IgG1 ratio of the anti-65 kDa antibody of the OMP treated mice was twice that observed in mice without treatment (Table 1).

2. Comparison of the effects of OMP and LPS treatment on immunogenicity of *M.vaccae*

OMP preparation can be contaminated with LPS; and LPS has been shown to have direct effect on macrophages and B cells (17). One may argue that the observed effects of OMP are due to contaminating LPS. Therefore it was necessary to ascertain if the immunomodulatory effects of the OMP preparation were due to its LPS content.

Table 2. DTH responses of OMP or LPS treated mice to *M.vacc*

Treatment before i.d. priming with <i>M.vacc</i>	DTH, Mean 48h CPE ± SE (mm)
None	0.27 ± 0.04
OMP - 500 µg	0.40 ± 0.04
LPS - 10 µg	0.11 ± 0.01
LPS - 40 µg	0.18 ± 0.01
LPS - 200 µg	0.14 ± 0.03
LPS - 500 µg	0.05 ± 0.01

Swiss White mice were treated with an OMP or LPS preparation, 72h before and 72h after an i.d. priming with 10⁸ killed *M.vacc*. One week later, DTH was tested to *M.vacc* sonicate.

Table 3. OMPs neutralise the suppressive effect of LPS

Treatment before i.d. priming with <i>M.vacc</i>		DTH, Mean 48h CPE ± SE (mm)
OMP	LPS	
-	-	0.29 ± 0.03
500 µg	-	0.40 ± 0.04
-	10 µg	0.19 ± 0.02
-	20 µg	0.18 ± 0.04
500 µg	10 µg	0.34 ± 0.03
500 µg	20 µg	0.27 ± 0.03

Swiss White mice (5 per group) were treated with a preparation of OMP, LPS and a mixture of OMP and LPS in two equally divided doses 72h before and 72h after an i.d. priming with 10⁸ killed *M.vacc*. DTH responses of mice were tested one week after immunisation.

Swiss White mice (5 per group) were injected with OMP preparation (500 µg per mouse) or different amounts of LPS preparation (10-500 µg per mouse) by i.p. route in two equally divided doses given 72h before and 72h after an i.d. immunisation with 10^8 killed *M.vaccae*, and one week later, DTH tests done.

As observed before, OMP treatment of mice enhanced their DTH responses to *M.vaccae*. In contrast, the LPS treatment suppressed the DTH responses of the mice to *M.vaccae* (Table 2). Therefore it was included that the observed immunomodulatory effects of OMP could not be due to the contaminating LPS.

3. OMP treatment neutralises the suppressive effect of LPS treatment on immunogenicity of *M.vaccae*

Outer membranes of *Salmonella* contain both LPS and OMP. As both the molecules showed the modulatory properties, they can be expected to influence the immune response during a natural infection. In view of this we thought it would be interesting to investigate if the two influence the effects of one another.

Groups of Swiss White mice (5 per group) were injected different amounts of the OMP or LPS preparations again delivered in two equally divided doses by the i.p. route. Two groups received a mixture of 500 µg of the OMP and either 20 µg or 10 µg of the LPS preparation. As before, the mice were given various treatments 72h before and 72h after an i.d. immunisation with 10^8 killed *M.vaccae*, and the DTH was tested one week later.

Results show that as before OMP treatment enhanced and LPS suppressed the DTH responses of mice to *M.vaccae*. But when the OMP preparation was given along with LPS, the suppressive effect of the latter was neutralised (Table 3). Conversely, LPS also neutralised the enhancing effect of OMP.

4. OMPs improve antigen presenting capacity of peritoneal cells

We have shown earlier that OMP treatment enhanced the T-helper responses of mice to an unrelated antigen. It needed to be examined if this was due to improvement in the efficiency of APCs. For this, we needed a cell whose antigen presenting capacity was earlier reported to be poor so that improvement in antigen presentation can be appreciated easily. So we decided to use peritoneal cells (PC), whose capacity to present antigen in a lymphoproliferative assay has been reported to be poor (189).

Table 4. Lymphoproliferative response of i.d. primed splenic T cells induced by different APCs

APC	Mean CPM ± SE	SI
Spleen cells	10453 ± 776	6.5
PC, naive	578 ± 23	1.2
PC, OMP treated	17072 ± 521	9.5

Balb/c mice were immunised with 10^8 killed *M.vaccae* by i.d. route. One week later their spleens were removed and T cells separated. 10^5 i.d. primed T cells were mixed with 10^5 different APCs and cultured with 20 µg/ml *M.vaccae* sonicate and a standard lymphoproliferative assay was performed.

- CPM: Counts per minute
- SI: Stimulation index
- PC: Peritoneal cells

Naive Balb/c mice were given an i.p. injection of 0.5 ml of an OMP preparation containing 500 µg of protein and 72h later the PCs were harvested. PCs were aspirated from normal Balb/c mice also. Spleens of these animals were then removed and single cell suspension made. These various cell suspensions were given 800 rad γ -irradiation in a Co⁶⁰ source for use as APCs.

A separate set of Balb/c mice was immunised i.d. with 10⁸ killed *M.vaccae*. One week later their spleens were harvested, single cell suspension made and passed over nylon wool column to separate T cells. 10⁵ i.d. primed T cells were cultured with 10⁵ syngenic, irradiated spleen cells, naive PCs or OMP treated PCs as APCs, and 20 µg/ml *M.vaccae* sonicate, and a standard 6 day lymphoproliferation assay was performed.

Spleens cells supported excellent antigen specific lymphoproliferation. Naive PCs as reported earlier were poor in inducing proliferation. However, the OMP treated PCs supported a good lymphoproliferative response which was comparable to the response obtained with spleen cells as APCs. Hence it was concluded that OMP treatment improves antigen presenting efficiency of PCs (Table 4).

Since OMPs of *S.typhi* have been reported to induce tissue inflammation in non-immune naive mice (15), and since they could also improve antigen presentation, it was decided to investigate if stimulation with OMP induces IL-1 secretion.

5. OMPs enhance IL-1 secretion by the PCs

PCs were harvested from naive C3H/HeJ mice. The strain was selected for two reasons. The strain does not respond to LPS (73) and it is the appropriate strain to be used in thymocyte co-stimulation assay for IL-1. PCs were cultured in RPMI 1640, with 10% FCS at 10⁶ cells/ml (2 × 10⁵ cells per well) in microtitre culture plates. One group of cultures was stimulated with 5 µg/ml OMP and the other with 25 µg/ml OMP incorporated in the medium. 36h later supernatants were harvested and assayed for IL-1 by thymocyte co-stimulation assay.

Spleen cells of normal C3H/HeJ mice were also cultured with 5 µg/ml ConA and 36h later, supernatants were harvested as a source of standard IL-1 for use in thymocyte co-stimulation assay.

Table 5. OMP induces IL-1 synthesis by PC

Stimulus	Mean CPM ± SE	SI
PhA only	1981 ± 408	3.8
PhA + ConA sup	7375 ± 71	14.7
PhA + PC sup[A]	2040 ± 328	4.1
PhA + PC sup[B]	5790 ± 325	11.5
PhA + PC sup[C]	6304 ± 637	12.6

PCs were harvested from naive C3H/HeJ mice and cultured with 5 µg/ml or 25 µg/ml OMP for 36h. Supernatants were harvested and assayed for IL-1 by thymocyte costimulation assay. As positive control, supernatant of ConA stimulated spleen cell cultures were used.

A separate set of C3H/HeJ mice were sacrificed, thymuses removed and single cell suspension made. Thymocytes were cultured in a medium containing 1% PhA (w/v) and 50% supernatant (v/v) of PCs stimulated with OMP or spleen cells stimulated with ConA.

- ConA sup: Supernatant of spleen cells cultured with 5 µg/ml ConA
- PC sup[A]: Supernatant of PC cultured without OMP
- PC sup[B]: Supernatant of PC cultured with 5µg/ml OMP
- PC sup[C]: Supernatant of PC cultured with 25µg/ml OMP

Table 6. FPE response of non-immune mice to LPS of *S.typhi*

Amount of LPS/foot pad	Mean 48h FPE ± SE (mm)
100 ng	0.12 ± 0.01
1 µg	0.36 ± 0.04
2 µg	0.85 ± 0.12
5 µg	1.16 ± 0.06
10 µg	1.35 ± 0.05

Different amounts of LPS were injected in the foot pads of naive Swiss White mice and FPS was assayed at 24h and 48h after injection, and FPE was calculated.

FPS: Foot pad swelling

FPE: Foot pad enlargement: Difference between 48h FPS induced by LPS and PBS simultaneously injected in the hind foot pads of mice

Supernatant of PCs cultured without any OMP in the medium showed no IL-1 activity. The supernatant of PCs treated with OMP showed significant IL-1 content indicating that production of IL-1 by PCs is induced on treatment with OMP (Table 5).

B. Immunomodulatory properties affecting efferent arm of immune response

1. OMPs induce hypersensitivity in foot pads of naive mice

Ten non-immune naive Swiss White mice were given an injection of 0.03 ml of an OMP preparation containing 30 µg protein, in the left hind foot pad. The other foot pad received PBS. The foot pad thickness was measured before, and 24h and 48h after injection. Foot pad swelling (FPS), i.e. the difference between the 24h and 48h foot pad thickness and the foot pad thickness before injection was calculated. Specific foot pad enlargement (FPE) was calculated by subtracting FPS value for PBS from the FPS value for the OMP preparation, simultaneously tested in the two foot pads of the same mouse.

OMPs induced a sizeable FPE in naive mice (1.18 ± 0.02 mm at 24h and 0.37 ± 0.03 mm at 48h). The mice were then sacrificed and their foot pads biopsied. The histological examination of the foot pads revealed acute inflammation with infiltration by a pleomorphic infiltrate rich in macrophages and lymphocytes; significant neutrophilic component was also seen in the exudate.

2. Minimum hypersensitivity inducing dose of LPS; LPS content of OMPs

LPS is known to induce hypersensitivity reaction in naive mice (17). So, it may be argued that hypersensitivity reaction of OMP preparation could be due to contaminating LPS. To check this possibility, first a minimum hypersensitivity inducing dose of pure LPS was found out.

Different amounts of LPS in 0.03 ml volume were injected into hind foot pads of naive Swiss White mice. Foot pad thickness was measured before, 24h and 48h after injection. FPE was calculated as before.

It can be seen that minimum hypersensitivity inducing dose of LPS is 1 µg per foot pad (Table 6). LPS content of the OMP preparation was then assayed by thiobarbituric acid method (Appendix II.2) and different amounts of the OMP preparation were injected into the hind foot pads of naive Swiss White mice (4 per group). FPE was calculated as before.

Table 7. Comparison of protein and LPS content of different amounts of the OMP preparation vis-à-vis its ability to induce FPE in naive mice

Dilution of OMP preparation	Amount of OMP/foot pad	Amount of LPS inadvertently injected/foot pad	Mean 48h FPE ± SE (mm)
Undiluted	60 µg	< 1.68 µg	0.46 ± 0.04
1:4	15 µg	< 0.42 µg	0.35 ± 0.06
1:8	7.5 µg	< 0.21 µg	0.41 ± 0.01
1:16	3.75 µg	< 0.105 µg	0.26 ± 0.01

Different dilutions of OMP preparation (2 mg/ml) were made. 0.03 ml of each dilution was injected into the hind foot pads of naive Swiss White mice. Foot pad thickness was measured before and 48h after injection and FPE calculated. LPS content of the OMP preparation was assayed and amount of protein and LPS injected (per foot pad) with each dilution was calculated.

Table 8. CNBr treated OMP preparation cannot induce FPE in naive mice but can induce DTH in *S.typhi* primed mice

OMP/LPS per foot pad	Mean 48h FPE ± SE (mm) (Naive mice)		DTH Mean 48h CPE ± SE (mm) (Primed mice)	
	Untreated	CNBr Treated	Untreated	CNBr Treated
OMP - 60 µg	0.33 ± 0.03	0.20 ± 0.02	0.21 ± 0.01	0.26 ± 0.01
OMP - 15 µg	0.24 ± 0.02	0.13 ± 0.01	0.23 ± 0.01	0.29 ± 0.01
OMP - 7.5 µg	0.23 ± 0.02	0.09 ± 0.01	0.22 ± 0.02	0.29 ± 0.03
OMP - 3.75 µg	0.23 ± 0.02	0.08 ± 0.01	0.17 ± 0.02	0.23 ± 0.01
LPS - 5 µg	0.83 ± 0.03	0.75 ± 0.01	ND	ND
LPS - 1 µg	0.53 ± 0.01	0.49 ± 0.01	ND	ND
LPS -100 ng	0.16 ± 0.01	0.15 ± 0.05	ND	ND
LPS - 50 ng	0.11 ± 0.01	0.09 ± 0.01	ND	ND

OMP and LPS were treated with CNBr and injected into the hind foot pads of naive Swiss White mice and mice primed i.d. with 10⁸ killed *S.typhi*. Foot pad thickness was measured before, 24 h and 48 h after injection and FPS, FPE, and CPE calculated. FPE is the difference in the 48 h FPS induced by LPS/OMP and PBS simultaneously injected in the hind foot pads. CPE is the difference in the FPE of primed and naive mice simultaneously tested and is an indication of DTH response.

Table 9. Proliferative response of naive spleen cells to OMPs

OMP per well	Mean CPM ± SE
-	501 ± 57
500 ng	615 ± 25
1 µg	553 ± 44
10 µg	432 ± 51
20 µg	327 ± 25
50 µg	431 ± 35

Spleen cells of naive Balb/c mice were cultured with different amounts of OMP preparation and a 72h lymphoproliferation assay was performed.

Results (Table 7) show that the OMP preparation induced FPE in amounts that contained LPS less than 100 ng per foot pad. This implies that hypersensitivity reaction exhibited by OMP preparation was not due to its LPS content.

3. Cyanogen Bromide (CNBr) treatment of OMP destroys its ability to induce FPE

CNBr breaks methionine-lysine bonds in protein resulting into its fragmentation. However it has no effect on LPS. So we studied the effect of CNBr treatment on the ability of pure LPS and OMP preparation to induce FPE.

OMP preparation and LPS were treated with CNBr as described by Cahnman (176). Different amounts of untreated and treated LPS and OMP preparations were injected into the foot pads of naive Swiss White mice (5 per group). Foot pad thickness was measured before, 24h and 48h after injection and FPE calculated as before.

A separate set of Swiss White mice was immunised i.d. with 10^8 killed *S.typhi* cells and DTH response tested with different amounts of untreated and CNBr treated OMP preparations (5 mice per group). CPE was calculated by subtracting mean FPE values of naive mice from FPE values of the primed mice.

It can be seen that in case of naive mice CNBr treatment of OMP preparation decreased the FPE induced by it, whereas the treatment had no effect on LPS induced FPE. This implies that proteins in the OMP preparation are responsible for FPE and not the contaminating LPS. However, as T cells recognise processed peptides and not the native protein, CNBr treatment of OMP did not destroy its ability to induce DTH response in *S.typhi* primed mice (Table 8).

4. OMPs do not behave as T or B cell mitogens or superantigens

Superantigens can induce polyclonal T cell activation. Similarly T or B cell mitogens also can cause polyclonal activation. The stimulated T or B cell clones can secrete cytokines that can be expected to induce FPE in naive mice. Therefore the OMP preparation was tested for superantigen or mitogen-like properties.

Spleen cells of normal naive Balb/c mice were cultured with different amounts of the OMP preparation and a 72 h proliferation assay was performed.

Results show that OMP preparation did not induce proliferation of naive spleen cells (Table 9).

Table 10. OMP treatment enhances microbicidal activity of PCs

Time after injection of <i>S.typhi</i>	% Killing	
	Naive mice	Mice treated with OMP
5 min	17.1	52.5
10 min	18.1	52.6
20 min	18.3	52.7
35 min	18.7	52.8

Normal Swiss White mice were treated with 500 µg of an OMP preparation by the i.p. route. 72 h later the mice were challenged i.p. with $2-4 \times 10^4$ live *S.typhi* cells. 5 min later their PCs were harvested and microbicidal assay was performed.

Table 11. OMP stimulates NO production by the peritoneal cells

Treatment	Nitrites (µ moles)/10 ⁶ cells ± SE
None	22.0 ± 1.0
OMP - 5.0 µg/ml	39.5 ± 0.5
OMP - 10.0 µg/ml	38.0 ± 1.0
IFN-γ - 250 IU/ml	52.5 ± 0.5
OMP - 5 µg/ml + IFN-γ - 250 IU/ml	44.5 ± 0.5

PCs from naive C3H/HeJ mice (10⁶ per well) were cultured with different amounts of OMP preparation incorporated in the medium, at different concentrations. PCs stimulated with IFN-γ (250 IU/ml) were used as positive control. 20 h later supernatants were harvested and nitrite levels in them estimated.

Table 12. OMP and LPS show no synergism in inducing nitrite production by PCs

Treatment	Nitrites (μ moles)/ 10^6 cells $\pm$ SE	
	Without IFN- γ	With IFN- γ
None	17.0 $\pm$ 1.3	39.5 $\pm$ 0.6
OMP - 5 μ g/ml	38.5 $\pm$ 0.6	41.5 $\pm$ 0.3
OMP - 10 μ g/ml	39.5 $\pm$ 0.3	42.0 $\pm$ 0.3
LPS - 100 ng/ml	34.5 $\pm$ 0.3	40.0 $\pm$ 1.4
LPS - 1 μ g/ml	37.0 $\pm$ 0.3	42.0 $\pm$ 1.2
OMP - 10 μ g/ml + LPS - 100 ng/ml	37.0 $\pm$ 0.6	43.5 $\pm$ 0.3
OMP - 10 μ g/ml + LPS - 1 μ g/ml	38.0 $\pm$ 0.7	43.0 $\pm$ 0.8
Indomethacin*	37.0 $\pm$ 1.3	94.0 $\pm$ 5.1

PCs from naive Swiss White mice (10^6 per well) were cultured with different amounts of OMP, LPS or a mixture of OMP and LPS, with IFN- γ (250 IU/ml) or without IFN- γ incorporated in the medium. Indomethacin, an agent known to enhance nitrite levels was employed as a positive control. 20 h later supernatants were harvested and nitrite levels in them estimated.

* Swiss White mice were treated i.p. with 6 doses of 50 μ g each of indomethacin at 12 h intervals. 12 h after the last dose, PCs were harvested and cultured with or without IFN- γ .

5. OMP treatment enhances microbicidal activity of PCs

Normal healthy Swiss White mice were given an i.p. injection of OMP (500 µg per mouse). 72h later they were challenged with $2-4 \times 10^4$ live *S.typhi* Ty2 cells by the i.p. route. After exactly 5 min, which was the time allowed for the phagocytosis of the bacteria by the macrophages in PCs, the mice were sacrificed, their PCs harvested and microbicidal assay was performed by employing a technique of Blanden et al (183).

PCs of normal non-treated mice killed only 17.1% of the challenge Salmonellae in 5 min and the killing increased to 18.7% on incubation up to 35 min. The killing potential of the peritoneal macrophages was significantly enhanced by treatment with the OMP preparation. The OMP-treated PCs killed 52.5% of the challenge Salmonellae in 5 min. As with naive PCs, treated PCs also did not show any increase in microbicidal activity on further incubation for 35 min (Table 10).

6. OMP treatment enhances nitric oxide production by peritoneal macrophages

PCs were harvested from naive C3H/HeJ mice. These were cultured in microtitre plates at 5×10^6 cells/ml (10^6 cells per well) in RPMI 1640 containing 10% FCS. The PCs were stimulated with different amounts of an OMP preparation incorporated in the culture medium. As a positive control, the PCs were stimulated with IFN-γ (250 IU/ml), an agent known to enhance NO production (185), incorporated in the medium. The cells were cultured at 37°C for 20h, supernatants were harvested and nitrite levels assayed as an index of NO production.

Results show that even unstimulated PCs produced nitrites. Stimulation with OMP and IFN-γ increased the nitrite production. No synergism was observed between OMP and IFN-γ in stimulation of nitrite production (Table 11).

Above study of NO production was carried out in C3H/HeJ mice. Since these animals are LPS non-responders, study of synergism between LPS and OMP with respect to NO production could not be carried out. So a similar experiment was carried out in Swiss White mice to study synergism. Indomethacin, a prostaglandin blocker, an agent known to enhance NO production by PCs (191) and IFN-γ, were employed individually and in combination as positive controls.

As before OMP induced secretion of NO by PCs, however no synergism was observed between OMP and LPS in inducing NO production (Table 12). Since large amount



Figure 1 SDS-PAGE profile of crude OMP preparation, porin preparation and porin-depleted OMP preparation

50 μ l of the three types of OMP preparations containing 100 μ g/ml protein were mixed with equal volume of 2x sample buffer containing 4% SDS and 10% 2ME. The mixture was held in boiling water bath for 2 min. 10 μ l of each was loaded on 10% SDS-PAGE. Electrophoresis was carried out at 25 mA constant current. The gel was fixed and stained by silver staining technique.

- Lane A: Molecular weight marker (lysozyme).
Other light band seen is an impurity
- Lane B: Molecular weight marker (BSA)
- Lane C: Porin preparation.
Mainly a broad band corresponding to molecular weight 35-40 kDa is seen. Minor bands of higher molecular weight aggregates are also seen
- Lane D: OMP preparation from which porins were extracted.
Porin extraction is incomplete
- Lane E: Crude OMP preparation
- Lane F: CNBr-treated Porin preparation

of NO could be produced by indomethacin treated PCs, the conclusion regarding absence of synergism was considered to be a reality.

7. Study of peptide composition of OMP

In order to study the heterogeneity of OMP preparation, SDS-PAGE of same was carried out, several peptide bands were observed ranging from low to high molecular weights (Fig. 1). Outer membranes of gram negative bacteria contain porins, which are the major OMPs, low molecular weight lipoproteins, Omp A and some minor proteins (190). In order to investigate the nature of the peptide responsible for immunomodulatory activity, two separate preparations were made. One containing only porins and the other an OMP preparation from which porins were depleted by salt extraction. Porin preparation was made as described by Nikaido (186). Cell envelopes were prepared, and extracted with NaCl extraction buffer to make a porin preparation (see methods). SDS-PAGE analysis of the porin preparation revealed no low molecular weight peptides; only porins of 35-40 kDa were present. Small amounts of higher molecular weight aggregates (higher than 40 kDa) were also seen. The cell envelopes from which porins were extracted by NaCl buffer were then subjected to lysozyme, papain treatment in an attempt to obtain porin-free OMP. SDS-PAGE analysis of this preparation, however revealed all the bands present in crude OMP including porins (Fig. 1), indicating that the NaCl extraction procedure was not exhaustive.

8. Characterisation of the peptide/s with immunomodulatory properties

Three groups of Swiss White mice (5 per group) were injected with either 500 µg of crude OMP preparation or 500 µg of a porin preparation or 500 µg of a preparation of porin-depleted OMP by the i.p. route. 72h later they were immunised i.d. with 10^8 killed *M.vaccae*. A fourth group of 5 mice received i.d. priming alone. One week after immunisation, DTH responses of mice from all four groups were assayed. Later, the mice were bled through their retro-orbital plexus, serum separated and antibody titres against 65 kDa antigen of *M.vaccae* were assayed by ELISA.

Purified porin preparation, lacking low molecular peptides showed the immunomodulatory effects i.e. enhancement of DTH and anti-65 kDa antibody response, of the OMP preparation. Since salt extraction procedure was not very efficient in removal of porins, sizeable porins were retained in the envelopes. As a result OMP prepared from porin-extracted cell envelopes also showed immunomodulatory effect. Since porin preparation lacking low molecular weight peptides showed immunomodulatory properties, it was

Table 13. Immunomodulatory property of OMP resides in porin fraction

Treatment before immunisation with <i>M.vaccae</i>	DTH, Mean 48h CPE ± SE (mm)	Antibody response AU/ml
None	0.23 ± 0.01	165 ± 8
Crude OMP preparation	0.34 ± 0.01	984 ± 33
Porin preparation	0.39 ± 0.01	1310 ± 42
Porin-extracted OMP preparation	0.35 ± 0.01	ND
CNBr-treated Porin preparation	0.25 ± 0.02	176 ± 24

Different groups of Swiss White mice (5 per group) were injected with 500 µg of either crude OMP preparation, porin preparation, porin-depleted OMP preparation or CNBr-treated Porin preparation by i.p. route 72 h before an i.d. immunisation with 10⁸ killed *M.vaccae*. One week later the DTH response of mice was tested against *M.vaccae* sonicate. The mice were then bled through their retro-orbital plexus and antibody titres against 65 kDa antigen of *M.vaccae* were assayed by ELISA.

Table 14. Hypersensitivity inducing peptides are porins

Amount/foot pad	Mean 48h FPE ± SE (mm)
Crude OMP preparation - 7.5 µg	0.31 ± 0.02
Crude OMP preparation - 3.75 µg	0.27 ± 0.01
Porin Preparation - 7.5 µg	0.41 ± 0.01
Porin Preparation - 3.75 µg	0.35 ± 0.02
Porin-extracted OMP Preparation - 7.5 µg	0.38 ± 0.02
Porin-extracted OMP Preparation - 3.75 µg	0.31 ± 0.01
CNBr-treated Porin preparation - 7.5 µg	0.15 ± 0.02
CNBr-treated Porin preparation - 3.75 µg	0.07 ± 0.01

Different amounts of crude preparation, porin fraction, porin-depleted preparation of OMP and CNBr-treated Porin preparation were injected into the hind foot pads of naive Swiss White mice. Foot pad thickness was measured before, 24h and 48h after injection. FPE was calculated as before.

Table 15. Porins from different *Salmonella* and *E.coli* affect immune response of mice to *M.vaccae*

Source of porin given before immunisation with <i>M.vaccae</i>	DTH Mean 48 h CPE $\pm$ SE (mm)	Antibody response AU/ml
-	0.23 $\pm$ 0.01	165 $\pm$ 8
<i>S.enteritidis</i>	0.35 $\pm$ 0.01	ND
<i>S.typhimurium</i>	0.36 $\pm$ 0.01	1246 $\pm$ 99
<i>S.paratyphi</i> A	0.33 $\pm$ 0.01	555 $\pm$ 37
<i>S.paratyphi</i> C	0.33 $\pm$ 0.02	ND
<i>E.coli</i>	0.30 $\pm$ 0.02	548 $\pm$ 17

Different groups of Swiss White mice (5 per group) were injected 500 μ g of a porin preparation made from different *Salmonella* or *E.coli* by the i.p. route. 72 h later i.d. immunisation with *M.vaccae* was given. One week later, DTH response against *M.vaccae* sonicate and the serum anti-65 kDa antibody titre were assayed.

Table 16. Porins of different *Salmonella* and *E.coli* induce hypersensitivity in naive mice

Source of porins	Mean 48 h FPE $\pm$ SE (mm)
<i>S.enteritidis</i>	0.32 $\pm$ 0.01
<i>S.typhimurium</i>	0.34 $\pm$ 0.03
<i>S.paratyphi</i> A	0.29 $\pm$ 0.01
<i>S.paratyphi</i> C	0.30 $\pm$ 0.02
<i>E.coli</i>	0.26 $\pm$ 0.02

30 μ g of porin preparations made from different *Salmonella* and *E.coli* were injected into hind foot pads of naive Swiss White mice. Foot pad thickness was measured before and 48 h after injection and FPE was calculated.

concluded that the property resided in the porin fraction of OMP (Table 13). To confirm that the porins were capable of immunomodulatory activities, porin preparation was treated with CNBr to fragment the porins. The SDS-PAGE very clearly demonstrated the fragmentation, as indicated by absence of 35-40 kDa band in CNBr treated porin preparation (Fig. 1). Such a preparation failed to enhance DTH and anti-65 kDa antibody responses against *M.vaccae* (Table 13). It was therefore concluded that Porins were capable of the above immunomodulatory properties.

9. Characterisation of peptide/s causing hypersensitivity in naive mice

Two doses of crude OMP, porins and porin-depleted OMP (3.75 and 7.5 µg) in a volume of 0.03 ml were injected into the hind foot pads of naive mice and 48 h FPE measured. All the preparations caused FPE (Table 14). Since porin preparation devoid of low molecular weight peptides could induce FPE, it was concluded that FPE inducing property resided in the porins. Further, CNBr-treated porin preparation failed to induce FPE in naive mice (Table 14), and SDS-PAGE analysis revealed absence of 35-40 kDa band. Thus, the hypersensitivity inducing activity of porins was confirmed.

10. Comparison of immunomodulatory properties of porins from different *Salmonella* and *E.coli*

Five groups of Swiss White mice (5 per group) were injected with 500 µg of a porin preparation made from *S.enteritidis*, *S.typhimurium*, *S.paratyphi* A, *S.paratyphi* C and *E.coli* respectively by the i.p. route. 72 h later they were immunised i.d. with 10^8 killed *M.vaccae*. A sixth group of mice received i.d. priming without any prior porin treatment. One week after immunisation, DTH and antibody responses against *M.vaccae* were assayed as before.

Results show that porins from all *Salmonella* species and *E.coli* showed similar enhancement in DTH and anti-65 kDa antibody responses against *M.vaccae* (Table 15).

The ability of Porin preparations made from these different organisms to induce hypersensitivity reaction in naive mice was also studied.

Five groups of Swiss White mice (5 per group) were injected with 0.03 ml of porin preparation containing 30 µg of protein made from *S.enteritidis*, *S.typhimurium*, *S.paratyphi* A, *S.paratyphi* C and *E.coli* respectively in hind foot pads. 48 h FPE was measured as before. Results show that porins from all *Salmonella* species and *E.coli* elicited hypersensitivity response (Table 16).

Table 17. DTH responses against whole sonicate and OMP preparation in mice primed i.d. with *S.typhi*

Stimulating antigen	Naive mice 48 h FPE	Immunised mice 48 h FPE	CPE
Sonicate	0.08 ± 0.01	0.32 ± 0.04	0.24
OMP preparation	0.35 ± 0.02	0.60 ± 0.06	0.25

Table 18. Lymphoproliferative responses against whole sonicate and OMP preparation in mice primed i.d. with *S.typhi*

Stimulating antigen	Lymphoproliferative response CPM ± SE (mm)	SI
Sonicate	15510 ± 451	12.3
OMP preparation	10543 ± 937	8.4

Two groups of Balb/c mice were immunised i.d. with 10^8 killed *S.typhi*. One week later DTH response of one group was tested with whole sonicate of *S.typhi* and that of other group was tested with OMP preparation. FPE responses of naive mice to the sonicate and the OMP preparation were also tested simultaneously. Later the mice were sacrificed and a standard lymphoproliferative assay was performed employing the same antigens.

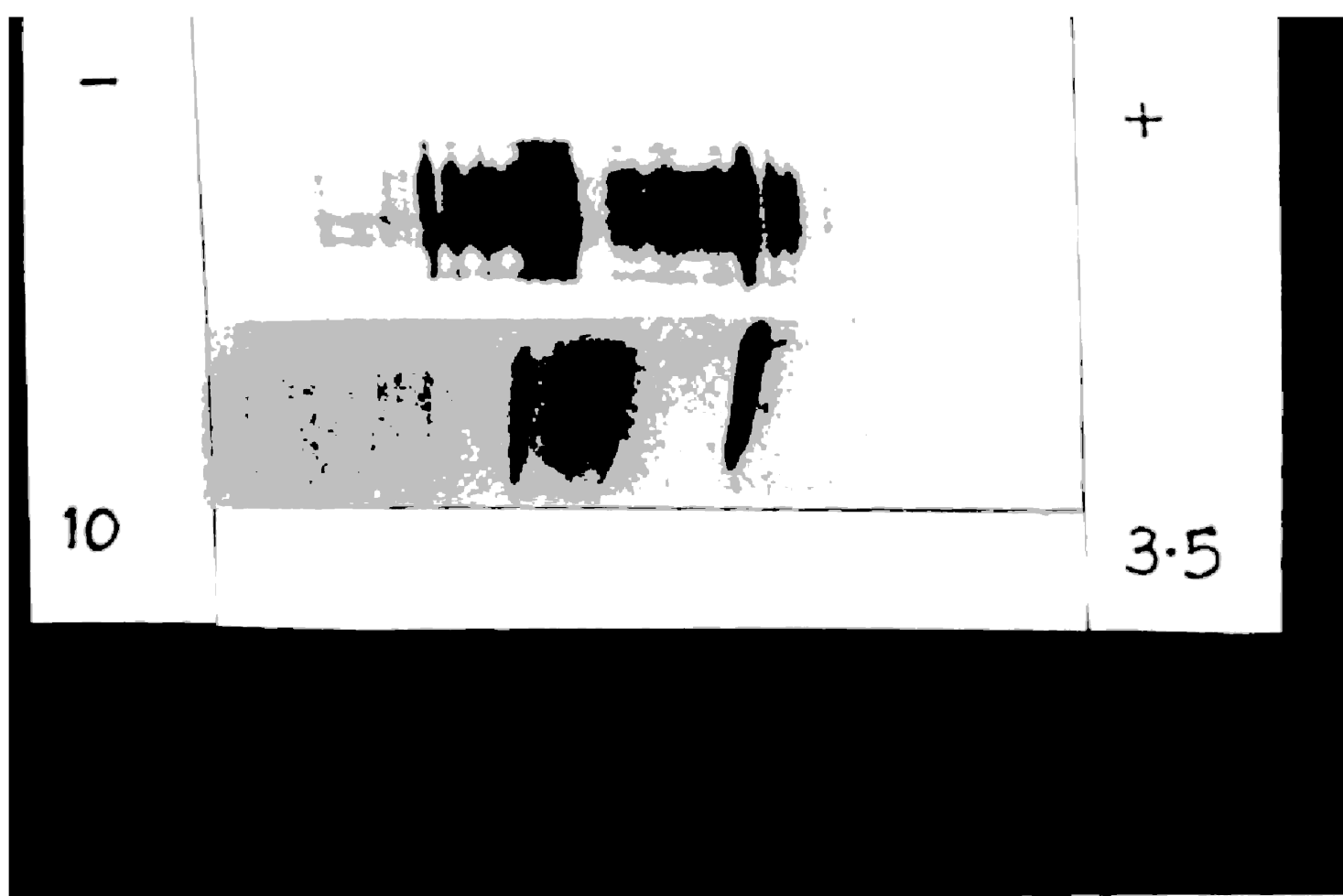


Figure 2. Analytical IEF profiles of whole sonicate and OMP preparation of *S.typhi*

10 μ l sample containing 2 mg/ml protein was loaded on a 7.5% PAG containing 2% ampholine of pH range 3.5-10. Electrophoresis was carried out at 3 W constant power for 60 min. After completion of the run, pH readings of gel surface were taken at 0.5 cm distance. Gel was fixed and stained by silver staining technique.

Upper gel: Profile of whole sonicate of *S.typhi*

Lower gel: Profile of OMP preparation of *S.typhi*.

pI of 4 bands seen were 5.2, 5.1, 4.85 and 4.5

II. OMPs as targets of T cell recognition

1. OMPs are recognised by T cells of mice-immunised i.d. with *S.typhi*

Two groups of Balb/c mice (5 per group) were immunised i.d. with 10^8 killed *S.typhi*. One week later DTH response of one group was tested by injecting 0.03 ml of whole sonicate of *S.typhi* containing 30 µg protein in the hind foot pad. DTH response of the other group was similarly tested by injecting 0.03 ml of an OMP preparation of *S.typhi* Ty2, containing 30 µg protein. Responses of normal non-immune naive mice were also simultaneously tested with sonicate of *S.typhi* and OMP preparation.

The mice were then sacrificed and a lymphoproliferation assay was performed on spleen cells employing the same two antigens.

Results show that the whole sonicate did not induce any hypersensitivity reaction in naive mice and gave a good DTH response in mice primed i.d. with *S.typhi*. OMP preparation showed hypersensitivity reaction in naive mice. However, FPE increased further in mice that were immunised with *S.typhi* and the CPE of 0.25 was obtained which was comparable with the CPE obtained with the whole sonicate (Table 17).

It can also be seen that whole sonicate and OMP preparation induced good proliferative responses of *S.typhi* primed spleen cells, which were comparable (Table 18). Therefore it was concluded that OMPs behave as T cell target antigens.

2. Identification of T cell target antigen in OMP preparation

To identify which peptides in the OMP preparation are recognised by T cells of mice primed with *S.typhi*, we used lymphoproliferation assay as the test parameter. Identification was carried out employing IEF. Analytical IEF was undertaken with 10 µl of OMP preparation containing 2 mg/ml protein. It was loaded on the cathodal end of a 7.5% polyacrylamide gel containing 2% w/v ampholine of pH range 3.5-10. 0.5 M acetic acid, pH 1.5 was used as the anolyte and 0.5 M NaOH, pH 11 was used as the catholyte. After completion of focusing, the gel was stained with silver stain by the method of Blum et al (174).

Four peptide bands were seen having pIs 5.2, 5.1, 4.85 and 4.5 (Fig. 2).

As peptide bands were seen in the pI range of 3.5-6, Preparative IEF was carried out using ampholines of the same pH range. One ml of OMP preparation containing 6 mg protein was subjected to IEF on an agarose gel with 2% (w/v) ampholine (pH 3.5-6). After

Table 19. Lymphoproliferative response of i.d. primed spleen cell to different peptides in OMP preparation

pl of peptide	Mean CPM ± SE
4.5	4030 ± 27
4.85	7095 ± 268
5.2	1406 ± 154
Only NCM	1448 ± 493
NCM - OMP	13433 ± 495

Balb/c mice were immunised i.d. with 10^8 killed *S.typhi*. After one week, their spleen cells were harvested and a standard 6 day lymphoproliferation assay was performed. As antigens 1:20 dilution of suspensions of NCM-adsorbed OMP (NCM-OMP) and each NCM-adsorbed peptides were used.

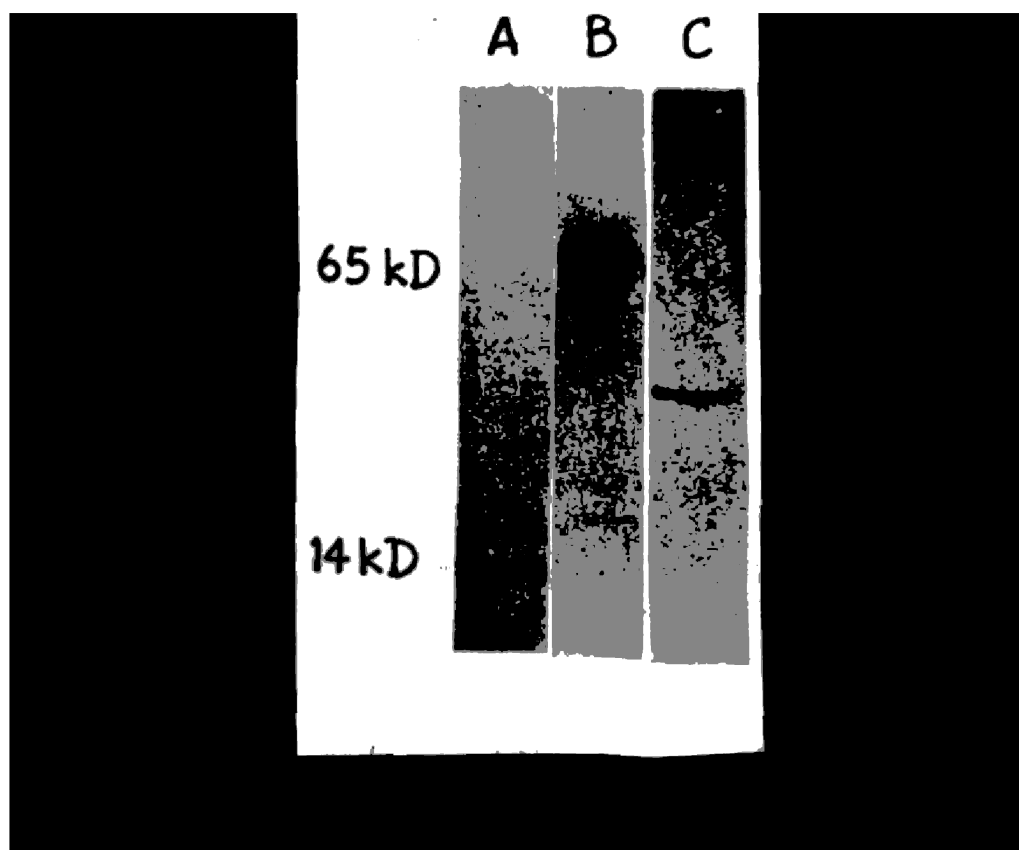


Figure 3 Molecular weight determination of peptide of pI 4.85

50 μ l of OMP preparation of *S.typhi* containing 100 μ g protein was mixed with 50 μ l of 2x sample buffer with 4% SDS and 10% 2ME. After keeping in boiling water bath for 2 min, 10 μ l of sample was loaded on a 10% SDS-PAGE. BSA and lysozyme were also run as molecular weight markers. After completion of electrophoresis, gel was electroblotted on to NCM. Lanes of molecular weight markers were cut and stained with ABB and the remaining blot was subjected to immunoperoxidase staining employing antiserum against peptide of pI 4.85.

Lane A: Molecular weight marker (lysozyme)

Lane B: Molecular weight marker (BSA)

Lane C: OMP preparation.

Single band corresponding to molecular weight 35-40 kDa seen

completion of electrofocusing, the agarose gel was electroblotted onto NCM. The blotted NCM was stained with Amido Black B. Three bands of pI 5.2, 4.85 and 4.5 were seen. These bands were then cut. Unfractionated OMP preparation was also dot blotted on NCM (NCM-OMP). The cut bands and NCM-OMP were dissolved in DMSO, reprecipitated and suspended in RPMI-1640 as described by Abou Zeid et al (175).

Balb/c mice were primed i.d. with *S.typhi*. One week later spleens were harvested, single cell suspension made, and a lymphoproliferation assay performed with different dilutions of NCM-OMP and each NCM adsorbed peptide preparation in RPMI 1640. Maximum counts were obtained with 1:20 dilution of all the preparations.

Results show that peptides with pI 4.85 and 4.5 supported lymphoproliferation of primed spleen cells. However, among them, peptide with pI 4.85 was better in supporting proliferation (Table 19).

3. Molecular weight of peptide with pI 4.85

50 µl of OMP preparation of *S.typhi* containing 100 µg protein was mixed with 50 µl of 2x sample buffer containing 4% SDS and 10% 2 mercaptoethanol. Mixture was held in boiling water bath for 2 min. 10 µl of the sample was loaded on a 10% PAG and electrophoresis was carried out at 25 mA constant current. Standard BSA and lysozyme were also run as molecular weight markers. After completion of the run the gel was electroblotted on to NCM. The lanes of BSA and lysozyme were cut and stained for proteins with Amido Black B. Antigen detection on the blot was carried out by immunoperoxidase staining (188). Non-specific sites on the membrane were blocked and the blot was then treated with an appropriate dilution of rabbit antibody against peptide of pI 4.85 (1:1,000). Bound antibodies were detected using an anti-rabbit HRP conjugate.

A single band corresponding to molecular weight 35-40 kDa could be seen (Fig. 3).

4. Study of immunodominance of peptide with pI 4.85

Five Balb/c mice were primed i.d. with 10^8 killed *S.typhi*. One week later their spleens were harvested, single cell suspension made and a lymphoproliferative assay performed with 20 µg/ml of following antigens.

1. Whole sonicate of *S.typhi*.
2. Crude OMP preparation.

Table 20. Peptide with pI 4.85 is an immunodominant antigen of *S.typhi*

Antigen	Mean CPM ± SE	SI
Whole sonicate	7584 ± 496	8.5
Lysozyme - papain digested sonicate	6805 ± 187	7.6
OMP preparation	5929 ± 205	6.6
Absorbed sonicate (sonicate devoid of peptide of pI 4.85)	3023 ± 423	3.4

Balb/c mice were primed i.d. with 10^8 killed *S.typhi*. One week later a standard lymphoproliferative assay was performed by culturing whole spleen cells of these mice with different antigens.

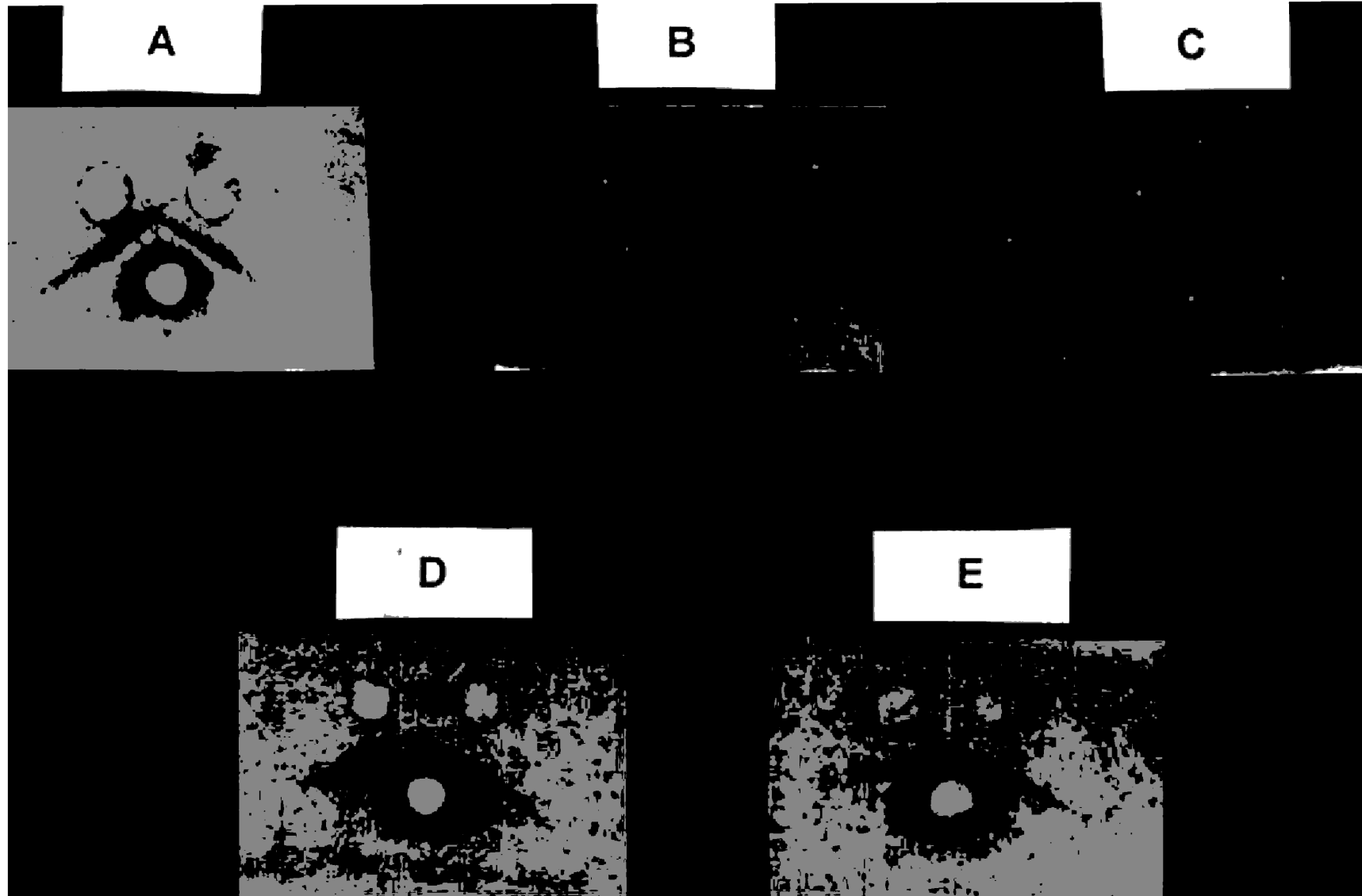


Figure 4 Analysis of serological cross-reactivity of peptide of pI 4.85

Cross-reactivity was studied with OMP preparations of different species of *Salmonella* employing antiserum raised against peptide of pI 4.85 from OMP preparation of *S. typhi*.

Bottom wells in all panels:	Antiserum against peptide of pI 4.85 from OMP preparation of <i>S. typhi</i>
Top left well in all panels:	OMP preparation of <i>S. typhi</i>
Top right well in panel A:	OMP preparation of <i>S. paratyphi</i> A
Top right well in panel B:	OMP preparation of <i>S. paratyphi</i> C
Top right well in panel C:	OMP preparation of <i>S. typhimurium</i>
Top right well in panel D:	OMP preparation of <i>S. gallinarium</i>
Top right well in panel E:	OMP preparation of <i>S. enteritidis</i>

3. Lysozyme papain digested *S.typhi* sonicate.

4. Lysozyme papain digested *S.typhi* sonicate devoid of peptide of pI 4.85. This was prepared by exhaustive absorption of lysozyme papain digested sonicate on immobilised rabbit serum against peptide of pI 4.85 (Appendix II.6).

Results showed that proliferative response of primed T cells to absorbed sonicate was considerably abrogated as compared to that of lysozyme-papain digested *S.typhi* sonicate. This implies that peptide of pI 4.85 is an immunodominant antigen recognised by the cells primed with the whole bacterium (Table 20).

5. Serological polymorphism of peptide with pI 4.85 in different *Salmonella* species

Serological cross-reactivity was checked by simple agar gel diffusion technique described by Ouchterlony and Nilsson (182). OMP preparations belonging to *Salmonella* species of different serogroups were individually tested in a three well system employing anti-serum raised against peptide of pI 4.85 and OMP preparation of *S.typhi*.

In organisms belonging to group D viz. *S.gallinarium* and *S.enteritidis*, the precipitin line representing peptide of pI 4.85 showed complete identity with the antigen of *S.typhi*. However in species belonging to other groups viz. *S.paratyphi* A, *S.paratyphi* C, and *S.typhimurium*, the peptide showed partial identity with the corresponding antigen of *S.typhi* forming a distinct spur (Fig. 4). This indicates presence of genus-specific and group-specific determinants on the antigen.

6. Cross-reactivity studies on OMP preparation using lymphoproliferation as a recognition parameter

Antigenic relationships in *Salmonella* as described in Kauffman and White scheme (6) are based on somatic or 'O' antigens and flagellar or 'H' antigens. Nair and Kamat (8) have shown that they are not likely to be target antigens in CMI in *Salmonella* infections. Other reports from our laboratory (9) have also demonstrated that there is extensive cross-reactivity across serogroup limits when CMI is used as a test parameter. As our OMP preparation is a T cell target antigen, we wanted to investigate if it shows cross-reactivity among different serogroups.

Five groups of Balb/c mice (5 per group) were immunised i.d. with 10^8 killed cells of five different species of *Salmonella*, viz. *S.typhi* Ty2, *S.gallinarium*, *S.enteritidis*, *S.paratyphi* A and *S.paratyphi* C. One week later, a standard lymphoproliferative assay was

Table 21. Lymphoproliferative response of spleen cells of mice primed i.d. with different Salmonellae to OMPs of *S.typhi*

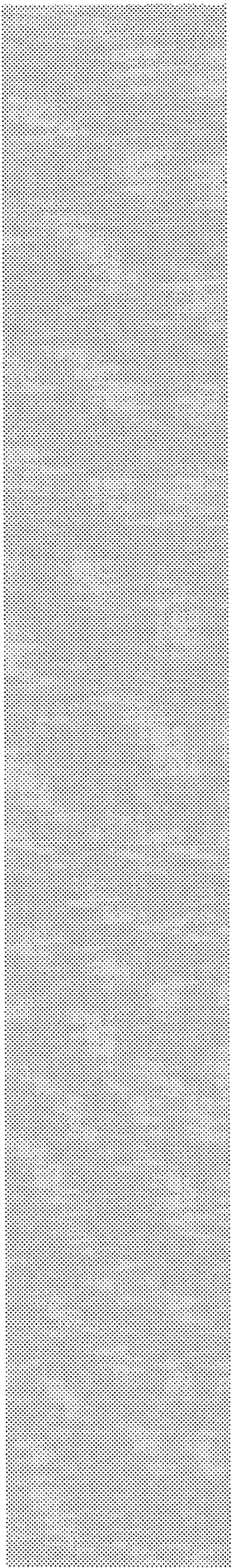
Immunogen	Mean CPM $\pm$ SE, (SD)	
	Sonicate	OMP preparation of <i>S.typhi</i>
<i>S.typhi</i> Ty2	12969 $\pm$ 463 (6.1)	11189 $\pm$ 955 (5.3)
<i>S.gallinarium</i>	13095 $\pm$ 1174 (6.4)	9345 $\pm$ 789 (4.6)
<i>S.enteritidis</i>	18735 $\pm$ 1266 (5.7)	18708 $\pm$ 327 (5.7)
<i>S.paratyphi</i> A	15309 $\pm$ 1343 (7.3)	9827 $\pm$ 823 (4.7)
<i>S.paratyphi</i> C	18270 $\pm$ 1712 (7.8)	13291 $\pm$ 743 (5.3)

Balb/c mice were immunised i.d. with 10^8 killed cells of different *Salmonella*. After one week a standard lymphoproliferative assay was performed by culturing spleen cells of primed mice with 20 μ g/ml of either the whole sonicate or the OMP preparation made from *S.typhi*.

performed by culturing 2×10^5 whole spleen cells of these mice with OMP preparation made from *S.typhi* Ty2.

Results show that OMPs from *S.typhi* induced proliferation of spleen cells from mice that were immunised with other species of *Salmonella*. This implies that OMPs have considerable cross-reactivity among different *Salmonellae* (Table 21). This indicates recognition of genus-specific determinants on the antigen by primed T cells.

Discussion



Immunomodulatory properties of OMPs

Our study revealed several immunomodulatory properties of OMPs of *S.typhi*.

1. Enhancement of humoral and cellular immune responses to an unrelated antigen.
2. Improvement in efficiency of antigen presentation by even non-professional APCs such as peritoneal macrophages.
3. Induction of IL-1 secretion by the peritoneal cells.
4. Induction of hypersensitivity response in naive mice.
5. Enhancement of microbicidal activity of peritoneal macrophages.
6. Induction of Nitric Oxide secretion by peritoneal cells.

OMPs could enhance both humoral and cellular responses to an unrelated antigen. OMP treatment when given before i.d. priming with *M.vaccae* enhanced both DTH and antibody responses against 65 kDa antigen of *M.vaccae*.

Outer membranes of gram negative bacteria contain proteins such as Porins which are approximately 35-40 kDa in size, lower molecular weight lipoproteins, OmpA, some minor proteins and LPS (190). In order to investigate which of them is responsible for immunomodulatory activity, separate preparations enriched in a particular type of component were made. One preparation contained Porins with no low molecular weight peptides and the other was LPS preparation.

Immunomodulatory effects of LPS from gram negative bacterial cell walls are well documented (71). LPS can act on cells directly or via mediators such as cytokines. Also, OMP preparations can be contaminated with LPS. So we studied modulatory effect of pure LPS preparation. When LPS was administered prior to immunisation with *M.vaccae*, it suppressed the DTH response of mice to *M.vaccae*. Since this was contrary to our observation with OMPs, it was concluded that modulatory effect of OMP preparation was not due to LPS. Porin preparation which was devoid of low molecular weight peptides present in crude OMP preparation, had the same modulatory potential as crude OMP preparation. Based on these results it was concluded that immunomodulatory component in crude OMP preparation was Porin.

Major fraction of the anti-65 kDa antibody in the sera of mice receiving i.d. priming with *M.vaccae* was IgG1. In contrast, IgG2a fraction was higher in sera of mice treated with Porins before immunisation. Based on this data it appears that Porin treatment favours Th1 type of response. However, a high antibody titre induced by Porins seemed to suggest a Th2 type of response because efficient antibody generation is associated with IL-4 which is a classical Th2 cytokine (108). But IL-4 switches the response to IgG1 type (50) which is not seen in our experiments. IFN- γ , the product of Th1 cells generally reduces the antibody titres (192). The apparent controversy can be explained by proposing that the B helper activity of CD4⁺ T cells favouring high titre IgG2a response observed in our system could be mediated by IL-2. IL-2 has been shown to favour such a response (193). Cells that secrete high levels of IL-2 on activation with antigen are either naive precursors or memory T cells (40). One could therefore hypothesise that IL-2 producing memory T cell fraction following immunisation with *M.vaccae* increases under the influence of Porins. Enhanced Th1 type of response, particularly DTH observed in our system could be due to IFN- γ produced in addition by NK cells. Stimulation of NK cells to produce IFN- γ has already been reported by OMPs of *S.typhimurium* (16, 194).

In view of the Porin-induced enhancement in responsiveness of mice to immunisation with *M.vaccae*, the effect Porin treatment on antigen presentation by PCs which are poor in antigen presentation (189), was investigated. Porin treatment could improve antigen presenting efficiency of PCs in an *in-vitro* antigen-specific proliferation assay. The antigen presentation by Porin-treated PCs was comparable to that of spleen cells as APCs. The heightened *in-vitro* proliferation of primed T cells by Porin-treated APCs supports the presumption that the number of IL-2 producing cells increases on treatment with Porins.

How Porins achieve this needs to be hypothesised. We have shown that Porins induce secretion of IL-1 by PCs. However, IL-1 has not been shown to enhance IL-2 producing T cell fraction. Naive macrophages can secrete prostaglandins which are known to inhibit T cell responses (195). Since Porins augment T cell response, it is possible that it downregulates secretion of prostaglandins.

Naive T cells after encounter with antigen differentiate into effector and memory cells. Many investigators believe that memory T cells arise by differential outcomes after activation (40). However, Swain et al propose that memory T cells could arise from primary

effector cells (97). The size of an antigen-activated clone capable of responding again by proliferation, is usually restricted by either differentiation to effector cells (that is if we believe that effectors cannot be converted to memory) or by death due to apoptosis (196). If we consider apoptosis as the mechanism for restriction of the size of clone, postulation of Russell needs to be considered (196). He has proposed that cytokine production and T cell death occurs at similar thresholds of stimulation. Therefore, default response of antigen-activated T cells is to produce cytokines and die. This activation-induced death in mature T cells requires expression of functional Fas and Fas-ligand by the activated T cell population (196). Concomitant stimulation of Fas and TCR-CD3 produces death. Since qualitatively similar signals from TCR are responsible for both T cell expansion and T cell elimination in activation-induced death, regulating balance between these opposing effects plays a crucial role in development of response. Russell further proposes that APC could be a principal regulator of these opposing responses. Co-stimulator molecules on APCs such as B7 can open a 'window' between threshold stimulus for cytokine production and T cell death. One could therefore suggest that Porins might bring about changes in the APCs that widen the gap between activation of apoptosis and secretion of cytokines like IL-2. So we studied expression of B7 by PCs after treatment with Porins. However, no significant enhancement in B7 expression was observed (data not shown). It is possible that other adhesion molecules may play a role in this regard.

Death/proliferation also depends on induction of Fas or Fas-ligand by T cells. If their expression is down-regulated due to signals from APCs, death of the cells could be prevented. However, since Porin-treated PCs could enhance proliferative response of primed T cells, direct effect of Porins on expression of Fas/Fas-ligand by T cells is ruled out. Porin-treated APCs may prevent cell death of T cells by preventing Fas-mediated apoptosis by known mechanisms such as up-regulation of bcl-2 (197).

Salmonella have both Porins and LPS. Since both can affect immune response to unrelated antigen, we studied the possibility of the two having synergistic or antagonistic effects in the context of immunomodulatory properties affecting Th cells. Interestingly, we found that Porins could neutralise the suppressive effect of LPS on T cell response. T cell suppression induced by LPS could be due to several causes. LPS stimulates macrophages to secrete prostaglandins and ROI and RNOI synthesis (198). Prostaglandins are known to inhibit T cell responses (195) and secretion of cytokines such as IFN- γ (199) and IL-2 (200), thus shifting the T cell response to Th2 type. RNOI have also been shown to inhibit antigen-

induced T cell proliferation (177) and favour Th2 differentiation (202). In our study PCs were shown to generate NO when stimulated *in-vitro* by Porins. In this regard, the effect of Porins and LPS is similar. So it could be suggested that LPS neutralising effect of Porins is independent of NO synthesis. It is possible that prostaglandin secretion is down-regulated by Porins. LPS is a known B cell mitogen and can induce secretion of IL-10 by them (203) which favours Th2 response (204). Secretion of IFN- γ under the influence of Porins can antagonise this effect. Thus Porins may neutralise the suppressive effect of LPS by multiple mechanisms.

Porins could improve antigen presentation, IL-1 being major mechanism of delivery of help by APC to T cells, and since Porins could also induce tissue inflammation in non-immune mice, we investigated the effect of Porins on IL-1 secretion. We found that production of IL-1 was induced in PCs by Porin treatment.

Porins of *S.typhi* also appear to have major effects on the innate immune mechanisms. It could induce sizeable FPS in naive mice, which could be due to release of inflammatory mediators such as IL-1 and TNF- α by macrophages (16). Evidence for the former has been given. Galdiero et al have reported that secretion of TNF- α by macrophages is increased on treatment with Porins of *Salmonella* (16). They have also shown that the inflammatory effect observed was due to activation of complement and release of histamine (15).

LPS stimulates macrophages to secrete pro-inflammatory cytokines (17). We have shown that up to 1 μ g of LPS per foot pad could induce hypersensitivity reaction in naive mice. So we examined if the hypersensitivity reaction of naive mice to Porin preparation was caused by contaminating LPS. The minimum amount of Porin needed to induce FPS in naive mice contained less than 100 ng of LPS which was not sufficient to induce FPS, implying that the swelling was not due to contaminating LPS.

This issue was addressed in one more fashion. Effect of Cyanogen Bromide treatment, on LPS and Porin-induced FPS was studied. CNBr fragments proteins and has no effect on LPS. FPS induced by CNBr-treated Porins in naive mice was considerably less than that induced by untreated Porins while treatment had no effect on LPS-induced FPS. These results confirm that hypersensitivity caused by Porins is not due to contaminating LPS.

CNBr treatment of Porins was employed to resolve another issue. One may argue that the mice employed in the investigation are not truly naive, non-immune mice but were

naturally primed by the environmental *Salmonella*, common in tropics, particularly in animal colonies. Therefore, the observed hypersensitivity response of the so-called naive mice to Porins could in fact be a DTH response of the T cells recognising the cross-reactive Porins of *S.typhi*. T cell recognition is known not to be affected by fragmentation of proteins, because they recognise fragmented processed peptides. On the other hand, direct hypersensitivity-inducing effect of Porins would be dependent upon intact native protein. DTH and FPS response of *S.typhi* immunised and naive mice respectively was studied by injecting untreated and CNBr treated Porins in their foot pads. FPS of naive mice due to treated Porins was less than that induced by untreated Porins. In contrast, CNBr treatment had no effect on CPE i.e. the DTH induced by Porins in primed mice. This implies that observed hypersensitivity induced in naive mice was not a DTH response.

The ability of Porin preparation to induce FPS in naive mice could be due to its ability to act as T or B cell mitogen/superantigen. But Porin preparation could not induce proliferation of naive spleen cells *in-vitro*. However, absence of mitogenic activity does not completely rule out the possibility of presence of weak superantigens in the preparation.

Enhancement in the microbicidal activity of peritoneal macrophages is another significant effect of Porins on innate immune mechanisms. ROI and particularly RNOI play an important role in affecting intracellular killing of microbial pathogens (185). We have shown that Porins enhance secretion of NO by peritoneal macrophages. IFN- γ along with a second signal can induce NO synthesis from L-arginine (185). TNF acts with IFN- γ as an autocrine or paracrine modulator to elicit the enzymatic pathway converting L-arginine to oxygenated nitrogen derivatives.

We have also shown that LPS and Porins exhibit no synergism in secretion of NO. It may be argued that the observed lack of synergism may be an experimental artefact in which either LPS or Porin might have saturated the capacity of PCs to synthesise NO. The same number of PCs from the same pool on indomethacin treatment could produce almost twice the amount of NO that is produced on treatment with either Porins or LPS. Therefore, the observed effect was considered to be real. Lack of synergism may be due to differential effects of LPS and Porins on prostaglandin synthesis. Milano et al studied the role of prostaglandin E₂ in synthesis of NO in murine macrophage cell line J774 (191). They reported that concentration of prostaglandin between 1-10 ng/ml can stimulate expression of nitric oxide synthase and release of NO while higher concentrations (> 50 ng/ml) are

inhibitory. The doses of LPS employed by us may have induced secretion of large amount of prostaglandins. As a consequence, synergism may not have been observed.

Immunobiological activities of Porins from various gram negative organisms like *Helicobacter pylori* (205), *Fusobacterium nucleatum* (73), *Salmonella typhimurium* (16) have been reported. In view of this, we compared immunomodulatory properties of Porins from some *Salmonella* species and *E.coli*. Porins from all *Salmonella* species tested and *E.coli* could enhance both DTH and antibody responses to *M.vaccae* and could also induce a hypersensitivity response. This implies that Porins derived from various micro-organisms might have similar immunomodulatory activities.

Salmonella belong to a group of facultative intracellular parasites. These organisms resist killing by naive macrophages. Therefore, the only effective mechanism in controlling infection by *Salmonella* is CMI (Th1 type of response). Molecules such as LPS help the organism to evade host defence mechanisms. LPS not only suppresses T cell response but also shifts the response to Th2 type. Therefore it is likely that host response to Porins has evolved so as to counteract the effects of LPS. These antagonistic effects of LPS and Porins would enable the host to mount effective T cell response which is essential for the control of infection.

OMPs as T cell target antigens

Salmonella, being facultative intracellular parasites, CMI is the only major avenue of combat; serum antibodies do not play a significant role in recovery or prevention (4, 5). Therefore, critical antigen/s responsible for CMI need to be identified. Antigens of *Salmonella* have been classified on the basis of their LPS i.e. the somatic or 'O' antigens and their proteinic flagellar or 'H' antigens, as per Kauffman and White scheme (6). However, neither of the two participate in CMI (5, 7, 161). So this scheme has no relevance as far as target antigens recognised by T cells is concerned.

Since OMPs can be good candidates for T cell target antigens due to their amphipathicity, we studied if our crude OMP preparation could act as a T cell target. DTH is an *in-vivo* manifestation of effector T cell response. OMP preparation could elicit a DTH response in mice immunised i.d. with *S.typhi* which was comparable to that elicited by the whole sonicate of *S.typhi*. Lymphoproliferative response of spleen cells of mice primed i.d. with *S.typhi* was also investigated. It was observed that OMP preparation could induce good

proliferation of primed spleen cells. Based on these two parameters it was concluded that OMP preparation behaves as T cell target antigen of *S.typhi*.

The peptide recognised by the T cells of mice primed with *S.typhi* from the crude OMP preparation was identified. It was found to be a peptide having pI 4.85 and a molecular weight of 35-40 kDa. These findings suggest that the protein is a Porin.

Porins are major OMPs of gram negative bacteria that form transmembrane permeability channels which allow diffusion of small hydrophilic molecules (186). Porins of *S.typhi* have been reported to have a molecular weight of 35-36 kDa and pI of 4.8 (186). Various workers have shown that Porins from *Salmonella* can induce protective immunity to salmonellosis (164, 165, 166). Also, Udhaykumar and Muthukaruppan have shown that Porins can elicit a typical DTH response in *S.typhimurium* infected mice (201).

Bacteria usually have a large number of peptides that have the potential to activate T cell clones. However, only certain peptides are immunodominant; i.e. those to which most of the response is directed (3). We studied if peptide of pI 4.85 behaves as an immunodominant peptide when animals are immunised with killed whole bacillus. When proliferation of *S.typhi* primed spleen cells, due to whole sonicate of *S.typhi*, was compared with that of Porin-depleted sonicate, considerable abrogation of proliferation was observed. This implies that Porins behave as immunodominant antigens for T cell recognition when whole organism is used as an immunogen.

Porins also exhibit polymorphism as observed by sero-reactivity studies. The reactivity of the antiserum raised against pI 4.85 Porin of *S.typhi* was studied by Ouchterlony and Nilsson's method (182) of gel diffusion against OMP preparations of other *Salmonella* species. Peptide with pI 4.85 showed only a partial identity with the corresponding antigen from *Salmonella* of other groups, viz. *S.paratyphi* A, *S.paratyphi* C and *S.typhimurium*. However, in case of group D organisms tested, viz. *S.gallinarium* and *S.enteritidis*, peptide with pI 4.85 showed complete identity with *S.typhi*. This data indicates the presence of at least two types of determinants on the antigen, the genus-specific and group-specific.

Tiwari and Kamat (9) have reported a spectrum of cross-reactivity across the boundaries of Kauffman and White groups when CMI was used as reference parameter. *Salmonella* being ubiquitous, antigenic relatedness can lead to cross-protection provided antigens are relevant in terms of protective immunity. Since our antigen behaves as T cell target antigen, we investigated if it shows cross-reactivity among different *Salmonella*.

Lymphoproliferative assays were carried with spleen cells of mice immunised with *Salmonella* belonging to different groups by culturing them with Porin preparation of *S.typhi*. Proliferation was exhibited by spleen cells belonging to mice immunised with all the different *Salmonellae*. This implies that our antigen contains determinants recognised by T cells of mice immunised with different *Salmonellae*. Since proliferative response of spleen cells from mice immunised with different *Salmonella* species was comparable, it may be assumed that genus-specific determinants on Porins are recognised by T cells.

Summary and Conclusions

Immunomodulatory properties of Porins

Porins of *S.typhi* showed immunomodulatory properties affecting both afferent and efferent arms of immune response.

Immunomodulatory properties affecting afferent arm of immune response

Porins enhanced both cellular and humoral responses to an unrelated antigen. Porin treatment of mice enhanced both DTH and antibody responses against 65 kDa antigen of *M.vaccae*.

The isotype of anti-65 kDa antibody favoured by Porin treatment was IgG2a. Therefore it appears that Porin treatment favours Th1 type of response. However, IFN- γ , a Th1 cytokine downregulates antibody response (192) and IL-4, a Th2 cytokine associated with high antibody response, favours IgG1 isotype (50). It was therefore hypothesised that high IgG2a antibody titre observed could be mediated by IL-2. Since large amount of IL-2 secretion is a property of memory Th cells, it was postulated that Th cell memory component might have increased when Porin treatment was given before immunisation. Enhanced memory and Th1 type of response observed may be due to IFN- γ secreted by NK cells under the influence of Porins. Induction of NK cells to secrete IFN- γ due to OMPs has been reported earlier (16, 194).

The proposition that IL-2 producing T cell clones increase on treatment with Porins was also supported by the results of lymphoproliferation experiment. PCs which are poor antigen presenters in a lymphoproliferation assay, could support excellent proliferation of *M.vaccae* primed T cells after treatment with Porins.

Explanation for enhanced proliferative response (enhanced IL-2 producing T cell fraction) when antigen is presented by Porin treated PCs is not obvious.

- We have shown that Porins induce secretion of IL-1 by PCs. However, IL-1 has not been shown to augment IL-2 producing T cell fraction.
- Naive macrophages can produce prostaglandins which downregulate T cell responses (195). Therefore it is possible that prostaglandin synthesis is downregulated by Porins.
- Porins may also increase the number of IL-2 producing T cells on priming with antigen by inhibiting apoptosis that is reported to occur following activation by antigen. As proposed

by Russell, increased expression of co-stimulatory molecules such as B7 on APCs can bring about such an effect (196). But when expression of B7 on PCs was studied, no significant increment in expression was recorded after Porin treatment. It is possible that other adhesion molecules may play a role in this context.

LPS present in gram negative bacterial cell wall is a known immunomodulator (71). We have shown that it suppressed DTH response of mice to *M.vaccae* when administered prior to immunisation. Interestingly, this suppressive effect of LPS on T cell response could be neutralised by Porins. Suppression of T cell response due to LPS could be due to its ability to stimulate macrophages to secrete prostaglandins and NO (198). Both are known to downregulate T cell responses and also favour Th2 differentiation (177, 195, 199, 200, 202). Incidentally, Porin treatment could induce NO synthesis by PCs. It is therefore hypothesised that Porins may downregulate secretion of prostaglandins by macrophages. Porins might also neutralise the effect of IL-10 (which also favours Th2 differentiation (204)) secreted by B cells under the influence of LPS through secretion of IFN- γ .

Immunomodulatory properties affecting efferent arm of immune response

Porins of *S.typhi* also appear to have major effects on innate immune mechanisms. Porins induced hypersensitivity response in non-immune naive mice, which could be due to IL-1 synthesis and/or induction of TNF- α secretion by macrophages as reported earlier (16).

The conclusion that this ability to induce hypersensitivity was independent of LPS was based on two findings: 1. The amount of LPS inadvertently injected into the foot pads as a contaminant of Porin preparation was not sufficient to induce FPS and 2. CNBr-fragmented Porin preparation failed to induce FPS while CNBr treatment had no effect on LPS induced FPS.

Enhancement in microbicidal activity of peritoneal macrophages is another significant effect of Porins on innate immune mechanisms which could be due to their ability to stimulate NO production. We also have shown that LPS and Porins exhibit no synergism in secretion of NO. Lack of synergism may be due to differential effects of LPS and Porins on prostaglandin synthesis. The doses of LPS employed in our assays may have induced secretion of large amount of prostaglandins which can inhibit NO production (191). As a consequence, synergism may not have been observed.

We have also shown that Porins from various *Salmonella* species and *E.coli* could exhibit immunomodulatory properties. This implies that Porins derived from various micro-organisms might have similar immunomodulatory activities.

Porins as T cell target antigens

Porin preparation could elicit a good DTH response in mice immunised i.d. with *S.typhi* and it could also induce lymphoproliferation of *S.typhi* primed spleen cells *in-vitro*. It was therefore concluded that Porin preparation behaved as T cell target antigen.

Identification of peptide recognised by *S.typhi* primed T cells from the Porin preparation yielded a peptide having pI 4.85 and molecular weight of 35-40 kDa.

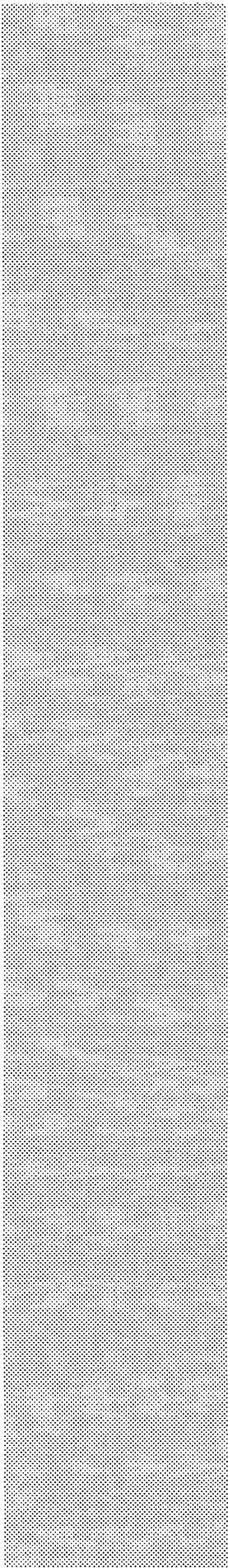
The peptide with pI 4.85 behaved as an immunodominant peptide i.e. most of the proliferative response against *S.typhi* was directed to this peptide when whole bacillus was used as an immunogen.

Serological cross-reactivity of peptide with pI 4.85 was investigated employing the technique of Ouchterlony and Nilsson (182). In gel precipitation analysis it was observed that the peptide showed only a partial identity with the corresponding antigen from *Salmonella* of other groups while it showed complete identity among group D *Salmonella* tested. It was therefore concluded that it had at least two types of determinants, genus and group specific.

Cross-reactivity² was also studied in a lymphoproliferation assay. Spleen cells from mice immunised with *Salmonella* belonging to different serogroups could proliferate in response to Porins of *S.typhi*. It was therefore concluded that the genus specific determinants of Porins were recognised by primed T cells.



Appendices



Appendix I

Formulae and preparation of reagents

1. Liquid Broth

Bacto-tryptone	10.0 g
Yeast Extract	5.0 g
Sodium Chloride	5.0 g
Glucose	1.0 g
Distilled water	1000 ml

The ingredients were mixed and dissolved by keeping in boiling water bath. After cooling, the pH was adjusted to 7.0. The medium was filtered and sterilised by autoclaving at 110°C for 10 min at 10 lb.

2. Doub and Youman's medium

Asparagine	5.0 g
KH_2PO_4	5.0 g
K_2SO_4	0.5 g
Magnesium citrate	1.5 g
Glycerol	20 ml
Distilled water	980 ml

All the ingredients except Magnesium citrate were dissolved in 980 ml distilled water. 20 ml glycerol was added. After thorough mixing, the pH was adjusted to 6.8 to 7.0 by adding appropriate amount of 1 N NaOH. Magnesium citrate was added after

adjusting the pH. The solution was heated if necessary to dissolve Magnesium citrate and then dispensed into tubes or flasks. Medium was sterilised by autoclaving at 15 lb. and 121°C for 20 min.

3. Phosphate buffered saline (PBS) (0.01 M, pH 7.2)

NaCl	8.0 g
K_2HPO_4	1.21 g
KH_2PO_4	0.34 g
Distilled water	1000 ml

4. Tris-HCl buffer (0.05 M, pH 6.8)

Tris	0.606 g
Distilled water to	100 ml

Tris was dissolved in 60 ml distilled water. The pH was adjusted to 6.8 with 1 N HCl. The volume was

then made up to 100 ml.

5. Tris-HCl buffer (0.005 M, pH 7.4)

Tris	0.061 g
Distilled water to	100 ml

Tris was dissolved in 60 ml distilled water, pH was adjusted with 1 N HCl to 7.4, and the volume was

made up to 100 ml.

6. Tris-glycine buffer (0.025 M Tris, 0.192 M glycine, pH 8.3)

Tris	3.03 g
Glycine	14.4 g
Distilled water	800 ml
Methanol	200 ml

Tris and Glycine were dissolved in 800 ml distilled water and then 200 ml methanol was added to it.

7. Saline-Tween-20

NaCl	9.0 g
Tween-20	0.5 ml
Distilled water	1000 ml

NaCl was dissolved in 1000 ml distilled water to which 0.5 ml Tween-20 was added and mixed.

8. Phosphate buffered Saline-Tween-20

PBS (0.15 M, pH 7.2)	1000 ml
Tween-20	0.5 ml

9. Carbonate-bicarbonate buffer (0.05 M, pH 9.6) (Coating buffer)

Na ₂ CO ₃	0.13 g (0.01 M)
NaHCO ₃	0.36 g (0.04 M)
Distilled water	100 ml

10. Sodium citrate buffer (0.1 M, pH 6.0) (Substrate buffer)

Citric acid	0.23 g (0.01 M)
Sodium citrate	2.63 g (0.09 M)
Distilled water	100 ml

11. Phosphate buffer for CNBr treatment (0.0175 M, pH 6.3)

K ₂ HPO ₄	0.01 g (0.0014 M)
KH ₂ PO ₄	0.23 g (0.0161 M)
Distilled water	100 ml

12. O-phenylenediamine dihydrochloride (OPD) Substrate solution for peroxidase assay

4 mg OPD (Sigma) was dissolved in 10 ml Sodium citrate buffer (0.1 M, pH 6.0). 2 µl of 30% H₂O₂ solution was added and mixed gently. Substrate solution was made fresh each time just before use. It was kept in a glass bottle with aluminium wrapping.

13. Scintillation fluid

1,4 bis (5-phenyl oxazolyl) benzene (POPOP)	0.3016 g
2,5 diphenyl oxazole (PPO)	3.968 g
Toluene	1000 ml

PPO and POPOP were dissolved in 800 ml toluene and mixed thoroughly. After complete dissolution, the volume was made to 1 lit with toluene.

14. Salt buffer for extraction of porins (50 mM Tris-HCl, pH 7.7, 0.4 M NaCl, 1% SDS, 5 mM EDTA, 0.05% 2-mercaptoethanol, 3 mM NaN₃)

50 mM Tris-HCl buffer, pH 7.7

Tris	0.606 g
Distilled water to	100 ml

Tris was dissolved in 60 ml distilled water. The pH was adjusted to 7.7 with 1 N HCl. The volume was then made up to 100 ml.

NaCl buffer

NaCl	2.34 g
SDS	1.0 g
EDTA	0.186 g
2-mercaptoethanol	50 µl
NaN ₃	0.02 g
50 mM Tris-HCl to	100 ml

15. Reagents for SDS-PAGE

Gels for SDS-PAGE

Reagents	Resolving gel 10% acrylamide	Stacking gel 5% acrylamide
Acrylamide stock solution	5.0 ml	0.8 ml
Distilled water	6.8 ml	3.6 ml
Resolving gel buffer	3.0 ml	-
Stacking gel buffer	-	0.5 ml
10% SDS	150 µl	50 µl
TEMED	7.5 µl	5.0 µl
10% Ammonium Persulphate (prepared fresh)	50 µl	17 µl

Reagents

a. Acrylamide stock solution:

Acrylamide	30.0 g
Bisacrylamide	0.8 g
Distilled water	100 ml

The two ingredients were dissolved in 50 ml distilled water and the final volume was adjusted to 100 ml with distilled water.

b. Resolving gel buffer (1.875 M Tris-HCl, pH 8.8):

Tris	22.7 g
Distilled water to	100 ml

then made up to 100 ml.

Tris was dissolved in 60 ml distilled water. The pH was adjusted to 8.8 with 5 N HCl. The volume was

c. Stacking gel buffer (1.25 M Tris HCl, pH 6.8):

Tris	15.12 g
Distilled water to	100 ml

then made up to 100 ml.

Tris was dissolved in 60 ml distilled water. The pH was adjusted to 6.8 with 5 N HCl. The volume was

d. Reservoir buffer (0.025 M Tris, 0.192 M glycine, pH 8.3):

Tris	3.03 g
Glycine	14.4 g
SDS	1 g
Distilled water to	1 l

e. Sample buffer (2x):

1.25 M Tris HCl, pH 6.8 (Stacking gel buffer)	5 ml
SDS	2 g
2-mercaptoethanol	5 ml
Glycerol	11.6 ml
Bromophenol blue	10 mg
Distilled water to	50 ml

Preparation of protein samples for SDS-PAGE:

50 µl of samples containing 10-50 µg of protein were mixed with 50 µl of 2x sample buffer. The mixture was then held in a boiling water bath for 2 min, cooled and 10 µl of the sample was loaded on the gel. Samples were stored frozen at -20°C.

16. Reagents for staining procedures

a. Silver staining of proteins bands

i. Fixative solution

Methanol	50 ml
Acetic acid, glacial	12 ml
Formaldehyde, 37%	50 µl
Distilled water	38 ml

ii. Pre-treatment

Sodium thiosulphate	0.02 g
Distilled water	100 ml

iii. Staining solution

Silver nitrate	0.2 g
Formaldehyde, 37%	75 µl
Distilled water	100 ml

iv. Developer

Sodium carbonate	7.5 g
Formaldehyde, 37%	75 µl
Sodium thiosulphate	0.5 mg
Distilled water	100 ml

v. Destainer solution

Methanol	5 ml
Acetic acid, glacial	12 ml
Distilled water	38 ml

b. Coomassie Brilliant Blue - R-250 staining of immunoprecipitates in gels

i. Destainer solution

Methanol	40 ml
Acetic acid, glacial	10 ml
Distilled water	50 ml

ii. Staining solution

Coomassie Brilliant Blue - R-250	0.5 g
Destainer solution	100 ml

c. Amido Black B staining of protein bands on NCM

i. Destainer solution

Acetic acid, glacial	10 ml
Isopropanol	10 ml
Distilled water	80 ml

ii. Staining solution

Amido Black B	0.05 g
Destainer solution	100 ml

Appendix II

Procedures

1. Estimation of protein by Lowry's method

Reagent A-1

Sodium-potassium tartarate	0.2 g
Distilled water	10 ml

10 ml with distilled water.

The tartarate was dissolved in 8 ml distilled water in a volumetric flask and the volume was made to

Reagent A-2

Cupric sulphate	0.1 g
Distilled water	10 ml

made to 10 ml.

Cupric sulphate was dissolved in 8 ml distilled water in a volumetric flask and the volume was

Reagent A-3

Sodium carbonate	2.0 g
Sodium hydroxide (0.1 N)	100 ml

100 ml with the same.

The salt was dissolved in 40 ml of alkali in a volumetric flask and the volume was made up to

Reagent A: (0.5 ml A-1 + 0.5 ml A-2) + 50 ml A-3. This reagent was prepared just before use and the remainder, if any, discarded after the assay.

Reagent B: Phenol reagent (Folin-Ciocaltau's reagent, Sisco Research Laboratory) was diluted 1:2 with distilled water and used.

Standard protein solution: Bovine serum albumin (BSA, Sigma) in the concentration of 200 µg/ml, was used as the standard protein solution.

Assay procedure

A set of clean glass test tubes were taken to which 0.1, 0.2, 0.3, 0.4, and 0.5 ml of standard BSA was added in duplicate. The volume in each tube was made to 0.5 ml with distilled water. Blank of 0.5 ml distilled water was also set up. 0.5 ml of appropriate dilution of test samples were also set up in duplicate.

2.5 ml of reagent A was added to each tube. The contents of each tube were mixed and the tubes were allowed to stand at room temperature for 15 min.

0.25 ml of reagent B was added to each tube with vigorous shaking and the tubes were allowed to stand at room temperature for 30 min.

Optical density (O.D.) readings were taken at 660 nm, adjusted against the distilled water blank. A graph of O.D. against protein concentration was plotted from which the protein concentration of the test samples was calculated.

2. Estimation of LPS

Reagent A

Periodic acid	0.285 g
Sulphuric acid (0.125N)	50 ml

Reagent B

Sodium arsenite	1g
HCl (0.5 N)	50 ml

Reagent C

2 Thiobarbituric acid	0.3 g
NaOH (0.01 N)	50 ml

Standard LPS solution: Lyophilised LPS prepared from *S.typhi* Ty2 by the method of Westphal in a concentration of 2 mg/ml was used as standard LPS solution.

Assay procedure

A set of clean glass tubes were taken to which 0.025, 0.05, 0.1, 0.15 and 0.25 ml of standard LPS solution was added in duplicate. The volume in each tube was made to 0.25 ml with distilled water. Blank with 0.25 ml distilled water was set up. 0.25 ml of appropriate dilution of test samples were also set up in duplicate.

0.25 ml of reagent A was added to each tube. The contents of each tube were mixed and tubes were allowed to stand at room temperature for 20 min. 0.5 ml of reagent B was added and the contents of the tubes were mixed. After 2 min, 2 ml of reagent C was added and the tubes were kept in boiling water bath for 20 min. Tubes were then cooled and O.D.

readings were taken at 548 nm against distilled water blank. A graph of O.D. against LPS concentration was plotted from which LPS concentration of the test samples was calculated.

3. Siliconisation of glassware

The glassware to be siliconised was washed thoroughly and dried. The glassware was rinsed with Sigmacote (Silicon solution, Sigma) by dipping or filling the articles to be coated. They were then drained and air dried. All the glassware for cell culture work was siliconised.

4. Preparation of IgG fraction from serum

The IgG fraction was separated from the whole serum by ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ precipitation method. One volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added slowly to two volumes of serum, with constant stirring at 4°C. After 30 min, the solution was centrifuged at 10,000 g for 30 min. The supernatant was discarded, and the precipitate was suspended in the buffer of choice. The solution was dialysed against the same buffer extensively to remove SO_4^{2-} ions. The absence of SO_4^{2-} ions was tested by BaCl_2 precipitation test. The protein content of the IgG fraction was estimated both by spectrophotometry (extinction coefficient for 1.0 mg/ml solution at 280 nm taken as 1.4) and by Lowry's method.

5. Standardisation of conjugate dilution

The dilution of conjugate to be employed in a given ELISA was standardised as follows:

- a. Solution of required antigen (e.g. mouse IgG for anti-mouse HRP conjugate) was prepared in coating buffer (Appendix I.9) at concentrations 1, 5 and 10 $\mu\text{g/ml}$.
- b. 10 mg/ml BSA solution in coating buffer was also prepared.
- c. 100 μl of each concentration of antigen was added to the wells of ELISA plate (Maxisorp, NUNC) in adequate numbers to titrate various dilutions of the conjugate. 100 μl of the BSA solution was added to another set of wells also in sufficient numbers as controls for the various dilutions of the conjugate.
- d. Plate was covered and incubated in a moist storage box overnight at 4°C.
- e. Next day, the well contents were discarded and the unbound excess coating solution was removed by forcefully inverting the plate several times onto a pad of paper towelling.

- f. All the wells were filled with 2% gelatine in coating buffer, the blocking solution, and incubated at 37°C for 1.5 h.
- g. The plates were washed 5-6 times with saline-T20.
- h. Three suitable dilutions (such as 1:5,000, 1:10,000 and 1:20,000) of the conjugate to be tested, were made in dilution buffer (Appendix I.8).
- i. 100 µl of each conjugate dilution was added to the wells. PBS-T20 was added to a set of wells as conjugate blank and incubated at 37°C for 1 h.
- j. The plate was then washed 5-6 times and 100 µl of the substrate solution (OPD + H₂O₂) (Appendix I.12) was added. The plate was kept in dark at room temperature for 10-12 min.
- k. When optimal colour developed, the reaction was stopped with 50 µl of 2.5 N HCl and absorbence was read at 492 nm in an ELISA plate reader.
- l. A₄₉₂ values were plotted against the dilution of conjugate.

6. Preparation of insolubilised gel of rabbit antiserum

Rabbit serum was insolubilised by addition of 2.5% glutaraldehyde (Sigma) dropwise with shaking to a final concentration of 0.55%. The resulting gel was ground in a glass homogeniser and washed extensively with PBS.

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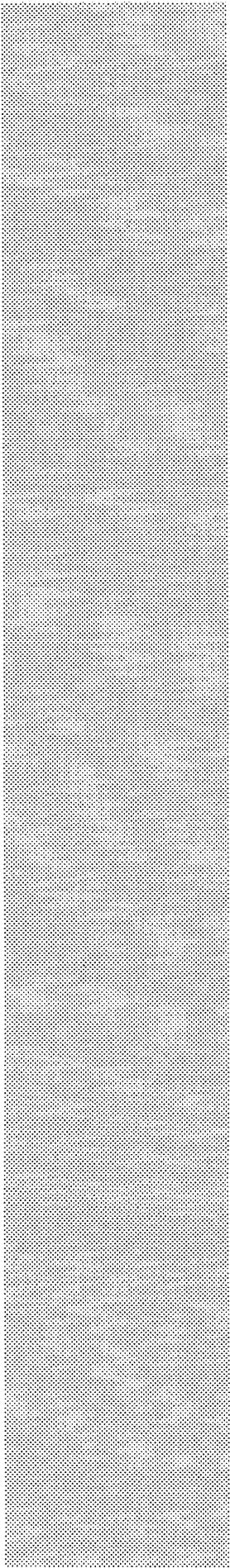
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Synopsis



INTRODUCTION

Proteins are one of the most abundant and diverse class of biologically active molecules encountered by the immune system.

- They act as peptide antigens which are targets of adaptive immune response of the clonally selected T and B cells, and
- They also have direct effect on non-clonally selected immunocompetent cells, such as macrophages, NK cells etc.

Generally proteins are important to the bacteria for various functions such as formation of ion and other channels, flagella, matrix, enzymes and many others. Bacterial proteins come from different loci viz. cytoplasm, cell membrane or cell wall.

Immunological responses directed against surface as well as internal macromolecules of gram negative bacteria are important for the host's defense system (1). But among macromolecules, proteins are primary targets for immune recognition by T cells. Bacteria usually have a variety of surface proteins that play a role in pathogenesis and are major antigens against which host immune response is directed (2).

Among various bacterial infections, Salmonellosis has always been a major problem. *Salmonella* carry two major antigenic systems. O or somatic antigen, which is LPS and H or flagellar antigen which is a protein. But earlier reports (3) have shown that these are not likely to be target antigens of cell mediated immunity. Hence antigenic relationships based on the Kauffmann and White scheme have no relevance as far as target antigens recognised by T cells is concerned.

Many pathophysiologic mechanisms of gram negative bacterial infections are due to endotoxins acting on tissues directly or via mediators like cytokines. However it has been observed that several pathophysiologic properties can also be due to bacterial proteins. Major outer membrane proteins (porins) of *Salmonella typhi* have been shown to induce secretion of cytokines such as IL-1, TNF by non-immune macrophages and γ IFN by NK cells (4). In view of this, porins can have direct effect on accessory cells such as macrophages and dendritic cells. Through cytokines such as IL-1, TNF, porins can also have indirect effects on accessory cells, and can also influence differentiation of antigen activated CD4⁺ T cells to a selective pathway of differentiation. As porins contribute the major component of outer membrane proteins (OMP) of gram negative bacteria (4), studies on OMP's of *S.typhi* were undertaken with the following objectives.

1. To study OMP's as targets of T cell recognition.
2. To study immunomodulatory properties of the OMP's.

MATERIALS AND METHODS

1. Micro-organisms:

Salmonella typhi Ty2 (O:9, 12, Vi H:d)

Salmonella paratyphi A (O:1, 2, 12 H:a)

Salmonella paratyphi C (O:1, 4, 5, 12 H:b, 1, 2)

Salmonella typhimurium (O:1, 4, 5, 12 H:i, 1, 12)

Salmonella enteritidis (O:1, 9, 12 H:g, m)

Salmonella gallinarum (O:1, 9, 12 H:-)

The strains were obtained from National Collection of Type Cultures, London, maintained on nutrient agar slants and cultivated in nutrient broth (5).

Mycobacterium vaccae was kindly supplied by Dr. J.L. Stanford of Middlesex hospital, London. The strain was maintained on Lowenstein Jenson's slants and cultivated on the medium of Doub and Youmans (6).

2. Animals:

8-10 week old Balb/c and C3H/HeJ mice obtained from National Institute of Health, USA, and maintained at Cancer Research Institute, Bombay, were used for studies in inbred animals.

8-10 week old Swiss White mice obtained from National Institute of Health, USA and maintained in the animal colony of Haffkine Biopharmaceutical Corporation Ltd, Bombay, were used for studies in random bred animals. Prior to immunisations, all mice were tested for delayed type hypersensitivity (DTH) response to the sonicate of *S.typhi* and/or *M.vaccae*. Only those animals that did not show any response were used in the experiments.

3. Immunisations:

For induction of cellular immunity against *Salmonella*: Mice were immunised intradermally (i.d.) with a single dose of 10^8 γ -irradiated (see section on preparation of sonic extract) *S.typhi* Ty2 cells in 0.05 ml phosphate buffered saline (PBS).

For induction of cellular immunity against *Mycobacteria*: Mice were immunised with 10^8 γ -irradiated cells of *M.vaccae* in 0.05 ml or 0.5 ml PBS. Former was used for i.d. and the latter for intraperitoneal (i.p.) immunisation respectively.

4. Preparation of sonic extract:

Overnight growth of *S.typhi* Ty2 in 250 ml nutrient broth was subjected to 1 mrad γ -irradiation in a Co^{60} chamber. Bacterial cells were pelleted by centrifugation at 10000 g for 10

min. and washed thrice with PBS. 3 ml packed cells were suspended in 15 ml PBS and sonicated in a sonifier (Branson, B-30 - 20 KHz with 50% duty cycle) for 45 min. keeping the tube constantly on ice.

Sonicate was centrifuged at 100000 g for 1 hr to remove cell debris and to reduce the LPS content. Supernatant was filtered through 0.22 μ m membrane filter (Millipore, USA) and its protein content was estimated by the method of Lowry et al (7). It was then aliquoted and stored at -40° C.

For preparing *M.vaccae* sonicate, the cells in Douv and Youmans medium were subjected to 2.4 Mrad γ -irradiation in a Co⁶⁰ chamber. Cells were washed and sonicated in the same manner.

5. Preparation of outer membrane proteins (OMP) fraction from *S.typhi*:

OMP's were prepared by the method of Nakae et al (8). Cell envelopes were prepared by sonicating (20 KHz, 50% duty cycle) washed *S.typhi* cells (6-7 g wet weight) in 0.05M Tris hydrochloride buffer (pH 6.8) for four min. Sonic extract was centrifuged at 750g for 10 min. to get rid of large fragments of cell constituents and unbroken intact cells. It was then centrifuged at 30000g for 30 min. and washed two times with Tris buffer. The pellet was washed two times with 2% SDS containing buffer, and later dialysed extensively against D/W. Pellet was then suspended in minimum volume of 5mM Tris HCl (pH 7.4) and was digested with lysozyme and papain-cystein. The treated material was centrifuged at 100000g for 1 hr to remove cell debris and to reduce LPS content. Supernatant collected was the OMP preparation. It was then dialysed against PBS for 2 hr. Protein estimation of the preparation was done by the method of Lowry (7).

6. Isoelectric focusing:

Analytical and preparative isoelectric focusing (IEF) was done using standard procedure (9).

7. Preparation of antigen adsorbed nitrocellulose membrane (NCM) particles:

It was done by the method of Abou-Zeid et al (10).

8. Preparation of LPS:

LPS was prepared by phenol-water extraction method, as described by Westphal (11), and it was estimated by Thiobarbituric acid method (12).

9. Cyanogen Bromide treatment:

LPS solution and OMP preparation were treated with Cyanogen Bromide as described by Gross (13).

10. Estimation of Nitrite:

Nitrite was estimated using Griess reagent (21).

Immunological Assays:

1. Delayed type hypersensitivity (DTH) test:

This test was done by the method of Grey and Jennings (14). The results were expressed as corrected foot pad enlargement (CPE).

$$\text{CPE} = \text{Mean foot pad enlargement (FPE) of test mice} - \text{Mean foot pad enlargement of control mice simultaneously tested.}$$

2. Hypersensitivity reaction to OMP:

Hypersensitivity reaction to OMP of *S.typhi* was tested by injecting different amounts of OMP in 0.03 ml in the hind foot pad. Measurements of the foot pad thickness were taken prior to injection and 24 and 48 hr. after injection. Mice were then sacrificed and the foot pads were excised for histopathological examination.

3. Lymphoproliferation assay:

Lymphoproliferation assay was carried out employing standard microculture technique (15).

4. Separation of T cells:

This was done by the method of Julius et al (16).

5. Peritoneal cells:

Mice were given an i.p. injection of 8 ml RPMI-1640 and then killed. Their abdomen was massaged gently and within 5 min. the contents were aspirated. Aspirates of several mice were pooled, the cells washed thrice with RPMI and viable cell number estimated.

6. Irradiation of spleen and peritoneal cells for use as antigen presenting cells(APC):

Spleen and peritoneal cells of mice were subjected to 800 rad γ -radiation in a Co^{60} source. Irradiated cells were washed in RPMI and used as APC's.

7. Assay for antibody levels against 65KDa antigen of *M.vaccae*:

Antibody levels against 65 KDa antigen of *M.vaccae* were assayed by sandwich ELISA technique.

ELISA plate (Nunc, Denmark) wells were coated with rabbit antibody against 65 KDa antigen of *M.vaccae* by incubating with 2.5 µg/well (in 100 µl vol.) of the antibody solution (IgG) overnight at 4° C. Next day well contents were discarded and well surfaces were blocked with 2% gelatin for 1 hr at 37° C. Wells were washed 5-6 times and 100 µl lysozyme-papain digested *M.vaccae* sonicate (1 mg/ml) was added to the wells and incubated for 1 hr at 37° C. Wells were then washed 5-6 times and 100 µl of different dilutions of test mouse serum samples along with appropriate controls (positive control serum and PBS-Tween 20 and normal mouse serum) were added and incubated for 1 hr at 37° C. Wells were washed 5-6 times. 100µl of antimouse HRP conjugate (dilution 1:10000) was added to the wells and incubated for 1h at 37° C. The plate was then washed 5-6 times and 100 µl of substrate solution (TMB - 1 mg/10ml, H₂O₂ - 2µl /10ml) was added to the wells and kept at room temperature in the dark for 10-15 min. The reaction was stopped with 2M H₂SO₄ and read at 450 nm. Antibody levels were expressed as absorbance units (A.U.) per ml.

8. IL-1 assay:

Soluble IL-1 was estimated by mouse thymocyte costimulation assay described by Grey et al (17).

Thymuses were dissected from 3-5 week old C3H/HeJ mice and a single cell suspension of thymocytes was made in RPMI with 5% FCS, at a concentration of 15×10^6 cells/ml. Thymocytes (1.5×10^6 cells/well /100 µl) were dispensed in microtitre plates. Test material such as culture supernatants (100 µl) were added in triplicates to wells with or without PhA-M (Gibco, 20µl of 10% PhA added/well). Plates were incubated at 37° C in 5% CO₂ for 72 hr. 18 hr. before harvesting, they were pulsed with tritiated thymidine (1 µCi/well) and incorporation of label was assayed.

9. Assay of macrophage microbicidal activity:

The assay was done by the method of Blandon et al (18). 0.5 ml of a suspension of pre-opsonised *S.typhi* Ty2 containing approximately $1-2 \times 10^4$ bacteria/ml was injected i.p. into each mouse. After exactly 5 min., mice were sacrificed by cervical dislocation and 2 ml of PBS containing 2% heat inactivated anti-*S.typhi* antiserum, 10% new born calf serum (NBCS) and 20 IU heparin were injected i.p. into each mouse and the peritoneal exudate cells were aspirated. PC from a number of mice were pooled and kept on ice to prevent killing of bacteria by the phagocytes. PC suspension was then spun at 110g for 5 min. at 10° C. Supernatant which contained unphagocytosed bacteria was again stored in cold. PC pellet

was washed twice and finally suspended in pre-warmed PBS containing 12% NBCS at a concentration of 6×10^6 cells/ml. One ml aliquots were then incubated at 37°C for varying periods of time and one aliquot was kept on ice immediately for viable counting. After required time intervals, aliquots were removed from the incubator and 0.1ml saponin, to a final concentration of 2.5% was added to lyse the PC, and lysate was kept on ice. All tubes were then serially diluted and plated out on MacKonkey's agar to determine the viable count.

The percentage viability of the Salmonellae at each time point was calculated using the number of organisms injected as 100%.

10. Generation of γ -Interferon (γ -INF) rich supernatant:

For stimulation of PC to induce Nitric oxide synthesis, γ -INF is required (19). Balb/c mice were immunised i.d. with 10^8 *M.vaccae* cells. One week later, the spleen cells were harvested and cultured for 48 hr with 20 μ g/ml *M.vaccae* sonicate, supernatant was harvested and used as a source of γ -INF. On an average, the γ -INF content of such culture supernatant is approximately 1000 IU / ml.

11. Flow cytometry:

Peritoneal exudate cells from normal and OMP treated Balb/c mice were studied for expression of the co-stimulatory molecule B7/BB1 by Flow cytometry.

The cells were stained with phycoerythrine labeled anti-B7, commercially obtained from Pharmingen. In order to identify the cell type, the cells were also stained with fluoresceine labeled anti-B220 which was also purchased from Pharmingen.

Flow cytometry was performed on FACSCAN (Becton Dickinson) by Dr. K.E. Shroff, in the laboratory of Prof. J.J. Cebra, Department of Biology, University of Pennsylvania, U.S.A.

RESULTS AND DISCUSSION

i. Outer membrane proteins (OMP) as targets of T cell recognition

1. Ability of OMP's to participate in T cell response:

OMP preparation was tested for its ability to participate in a T cell response by testing its ability to elicit DTH response and to induce lymphocyte stimulation.

Balb/c mice were immunised i.d. with 10^8 killed *S.typhi* cells. One week later their DTH response was tested against OMP preparation. Later their spleen cells were cultured with various amounts of OMP's.

DTH and lymphoproliferative responses against OMP preparation
in mice primed i.d. with *S.typhi*.

DTH CPE(mm) $\pm$ S.E.	Lymphoproliferation CPM $\pm$ S.E., S.I.
0.25 $\pm$ 0.03	12672 $\pm$ 2113, . 19.31

As OMP's gave good DTH and lymphoproliferative response, it was concluded that OMP's behave as T cell target antigens.

2. Components of OMP's:

Analytical IEF of OMP preparation was done using ampholines of pH range 3.5 to 10. Four peptide bands were seen in the pH range 3.5 to 6.

3. Purification of T cell target antigen from OMP preparation:

Purification was attempted by IEF. OMP preparation containing 4 mg protein was loaded on an agarose gel with ampholines of pH range 3.5 to 6. After completion of the run, gel was blotted on NCM, which was then stained with amido black B (10).

Three bands were seen having pI 5.2, 4.85 and 4.5. These bands were then cut, solubalised in DMSO and cultured with whole spleen cells of Balb/c mice primed i.d. with *S.typhi*.

Lymphoproliferative response of i.d. primed spleen cells to different peptides in OMP preparation.

pl of agarose band	CPM $\pm$ S.E.
-	647 $\pm$ 98
4.5	4030 $\pm$ 27
4.85	7095 $\pm$ 268
5.2	1406 $\pm$ 154
only NCM	1448 $\pm$ 493
NCM-OMP	13433 $\pm$ 495

Results showed that among OMP's, peptide with pl 4.85 gave the maximum lymphoproliferative response.

4. Cross reactivity studies on OMP preparation:

Different sets of Balb/c mice were immunised i.d. with 10^8 killed cells of either *S. typhi* Ty2, *S.gallinarium*, *S.enteritidis*, *S.paratyphi* A or *S.paratyphi* C. One week later, lymphoproliferative assay was performed by culturing spleen cells of these mice with OMP preparation made from *S.typhi* Ty2.

Lymphoproliferative response of spleen cells of mice primed i.d. with different Salmonellae to OMP's of *S.typhi*.

Organisms used for immunisation	Amount of OMP from <i>S.typhi</i>	
	-	1 μ g / well
<i>S.typhi</i> Ty2	2124 $\pm$ 567	11189 $\pm$ 955
<i>S.gallinarium</i>	2038 $\pm$ 716	9345 $\pm$ 789
<i>S.enteritidis</i>	3268 $\pm$ 112	18708 $\pm$ 3277
<i>S.paratyphi</i> A	2096 $\pm$ 154	9827 $\pm$ 823
<i>S.paratyphi</i> C	2532 $\pm$ 806	13291 $\pm$ 743

Results showed that OMP's have considerable cross reactivity among different Salmonellae.

ii. Immunomodulatory properties of OMP's

(a) Immunomodulatory properties affecting afferent arm of immune response

1. Effect of OMP's on immune response of mice to an unrelated antigen:

Effect of OMP's of *S.typhi* on immunogenicity of *M.vaccae* was studied. One set of Swiss White mice was treated with 250 µg OMP preparation per mouse (injected i.p.) and a second set with 1 mg per mouse. 72 hr. later, they were immunised with 10^8 killed *M.vaccae* cells. 72 hr. later, the OMP dose was repeated. A third set of mice received i.d. priming alone. One week after immunisation, DTH response of mice to *M.vaccae* was measured. Antibody levels against 65 KDa antigen of *M.vaccae* in the serum were also estimated by ELISA.

Effect of *S.typhi* OMP's on DTH response to *M.vaccae*

Treatment	i.d. immunsn with <i>M.vaccae</i>	DTH to <i>M. v</i> sonicate CPE $\pm$ S.E. (mm)
-	yes	0.225 $\pm$ 0.03
OMP - 2 mg	no	0.063 $\pm$ 0.02
OMP - 500 µg	yes	0.400 $\pm$ 0.05
OMP - 2 mg	yes	0.420 $\pm$ 0.06

Results showed that OMP treatment of mice before i.d. immunisation with *M.vaccae* enhanced their ability to mount DTH response.

Effect of OMP treatment on antibody production by mice primed i.d. with *M.vaccae*

Experiment	Antibody response against 65 kDa antigen of <i>M.v</i> estimated by ELISA	
	i.d.immsn with <i>M.vaccae</i>	OMP + i.d. immsn with <i>M. v</i> .
1	253 A.U. / ml	1040 A.U. / ml
2	96 A.U. / ml	1880 A.U. /ml
3	136 A.U. / ml	2154 A.U. / ml

Results showed that OMP treatment of mice before i.d. immunisation with *M.vaccae* increased the anti 65 KDa antibody response.

2. Effect of OMP or LPS treatment on immunogenicity of *M. vaccae*:

OMP preparation can be contaminated with LPS. So to check the possibility that the immunomodulatory effect of OMP preparation was because of LPS, following experiment was

carried out. Swiss White mice were injected different amounts of OMP or LPS by i.p. route in two equal doses given 72 hr. before and 72 hr. after an i.d. immunisation with *M.vaccae*. DTH with *M.vaccae* sonicate was tested one week after immunisation.

DTH response to *M.vaccae* sonicate of mice treated with OMP/LPS

Treatment	DTH to <i>M.vaccae</i> sonicate CPE(mm) ± S.E.
nil	0.267 ± 0.04
OMP - 500 µg	0.399 ± 0.04
LPS - 10 µg	0.109 ± 0.01
LPS - 40 µg	0.175 ± 0.01
LPS - 200 µg	0.142 ± 0.03
LPS - 500 µg	0.047 ± 0.01

As before, OMP treatment enhanced the ability of mice to mount DTH response on i.d. priming with *M.vaccae*. In contrast LPS suppressed the response.

3. Ability of OMP treatment to neutralise the suppressive effect of LPS treatment on immunogenicity of *M.vaccae*:

Following experiment was carried out to check if OMP's and LPS influence effects of one another in the context of immunomodulatory properties. Swiss White mice were injected 2 doses of different amounts of OMP and LPS by i.p. route, given 72 hr. before and 72 hr. after an i.d. immunisation with *M.vaccae*. DTH was tested with *M.vaccae* sonicate, one week after immunisation.

DTH response to *M.vaccae* sonicate of mice treated with OMP/ LPS

Treatment		CPE ± S.E.
OMP	LPS	
-	-	0.292 ± 0.03
500 µg	-	0.399 ± 0.04
-	10µg	0.192 ± 0.02
-	20 µg	0.177 ± 0.04
500 µg	10 µg	0.339 ± 0.03
500 µg	20 µg	0.269 ± 0.03

As before, OMP treatment enhanced and LPS suppressed the DTH response of mice primed i.d. with *M. vaccae*. But when OMP was given along with LPS, OMP corrected the suppressive effect due to LPS.

4. Presentation of antigen by OMP treated peritoneal cells (PC):

It has been reported earlier that PC's are not efficient antigen presenters in a lymphoproliferative assay (20). So we decided to use PC's as APC's to study the effect of OMP on antigen presentation, as improvement in antigen presentation can be demonstrated appreciably in these cells. 10^5 splenic T cells from mice primed i.d. with *M. vaccae* were cultured with 10^5 different types of irradiated (dose - 800 rads) APC's with *M. vaccae* antigen.

Lymphoproliferative response of i.d. primed splenic Tcells and different APC's

APC's used	CPM $\pm$ S.E.	
	without antigen	with antigen
Spleen cells, naive	1608 $\pm$ 199	10453 $\pm$ 776
PC's, naive	497 $\pm$ 18	578 $\pm$ 23
PC's, OMP treated	1890 $\pm$ 271	18129 $\pm$ 4107

Spleen cells are a source of professional APC's, so they supported good lymphoproliferation. Naive PC's did not stimulate T cells. However, PC's that were treated with 500 μ g OMP's 72 hr. before harvesting, supported good lymphoproliferative response. Hence it was concluded that antigen presentation by PC's is enhanced by OMP treatment.

To investigate the mechanism for improvement in antigen presentation by PC's on treatment with OMP's, changes in production of IL-1 and expression of T cell costimulatory molecule B7/BB1 by PC's were studied.

5. Effect of stimulation of PC's by OMP's on IL-1 synthesis:

PC's from naive C3H/HeJ mice were cultured with 5 μ g / ml or 25 μ g / ml OMP's. Also, spleen cells of these animals were cultured with Con A (5 μ g / ml) as a source of known IL-1. 36 hr. later, IL-1 in the culture supernatants was assayed by mouse thymocyte costimulation assay.

IL-1 content of OMP stimulated PC's

Source of supernatant	CPM $\pm$ S.E.	
	- PhA	+ PhA (1 %)
Spleen cells, Con A stimulated	1663 $\pm$ 75	7375 $\pm$ 71
PC's only	875 $\pm$ 162	2040 $\pm$ 328
PC's, OMP treated (5 μ g/ml)	969 $\pm$ 221	5790 $\pm$ 325
PC's OMP treated (25 μ g/ml)	846 $\pm$ 280	6304 $\pm$ 637
-	502 $\pm$ 24	1981 $\pm$ 408

From the results it can be seen that production of IL-1 by PC's is increased on treatment with OMP's.

6. B7/BB1 expression by cells in the peritoneal exhudate:

PC's of Balb/c mice were studied for B7/BB1 expression by Flow cytometry. 4.78% cells were B7 +ve and B220 -ve (i.e. non B cells expressing B7). In case of PC's that were treated with OMP's, the % of these cells increased to 9.10. This indicated that OMP's induced B7/BB1 expression by non B cells in the peritoneal exhudate.

(b) Role of OMP's in efferent arm of immune response

1. Hypersensitivity response of non-immune mice to OMP's of *S. typhi*:

30 μ g of OMP preparation was injected in the hind foot pad of naive Swiss White mice, and foot pad swelling (FPS) measured before, 24 hr and 48 hr after the injection.

Hypersensitivity response of non immune mice to OMP

Mice	24 hr swelling FPS (mm) $\pm$ S.E.	48 hr swelling FPS (mm) $\pm$ S.E.
Naive	1.27 $\pm$ 0.02	0.48 $\pm$ 0.02

OMP's gave a sizable foot pad swelling in naive mice. Histology of the biopsyed foot pad showed the lesion to be infiltrated with neutrophils, lymphocytes and macrophages.

2. Minimum hypersensitivity inducing dose of LPS; LPS content of OMP's:

It may be argued that the hypersensitivity reaction of OMP preparation could be due to contaminating LPS. To check this possibility, following experiments were carried out. First, minimum hypersensitivity inducing dose of LPS was found out. Different amounts of LPS were injected in the foot pads of naive Swiss White mice.

FPS response of non immune mice to LPS of *S.typhi*

Amount of LPS / foot pad	FPE (mm) $\pm$ S.E.
100 ng	0.124 $\pm$ 0.0
1 μ g	0.363 $\pm$ 0.04
2 μ g	0.850 $\pm$ 0.12
5 μ g	1.16 $\pm$ 0.06
10 μ g	1.35 $\pm$ 0.05

It can be seen that minimum hypersensitivity inducing dose of LPS is 1 μ g / foot pad.

Different amounts of OMP were injected in the foot pad of mice and 48 hr FPS was measured. Amount of LPS content of OMP preparation was also assayed.

Comparison of protein and LPS content of different amounts of OMP preparation vis-a-vis its ability to induce foot pad swelling in naive mice

Amount of OMP/foot pad	Amount of LPS inadvertantly injected/foot pad	FPE (mm) $\pm$ S.E.
60 μ g	< 1.68 μ g	0.463 $\pm$ 0.04
15 μ g	< 0.42 μ g	0.356 $\pm$ 0.06
7.5 μ g	< 0.21 μ g	0.413 $\pm$ 0.01
3.75 μ g	< 0.105 μ g	0.263 $\pm$ 0.01

Amount of OMP injected that had LPS content less than 100 ng per foot pad also gave a sizable FPS implying that it was not due to LPS content of OMP.

3. Cyanogen Bromide (CNBr) treatment of LPS and OMP; effect on ability to induce hypersensitivity reaction:

CNBr breaks down methionine-lysine bonds of protein, resulting into fragmentation. However, it has no effect on LPS. OMP and LPS were treated with CNBr and injected in the foot pad of naive Swiss White mice and FPS measured at 48 hr.

FPS response to CNBr treated OMP

Amount of OMP/ foot pad	FPE (mm) $\pm$ S.E.	
	untreated	CNBr treated
60 μ g	0.338 $\pm$ 0.04	0.262 $\pm$ 0.01
15 μ g	0.250 $\pm$ 0.03	0.125 $\pm$ 0.02
7.5 μ g	0.200 $\pm$ 0.03	0.050 $\pm$ 0.03
3.75 μ g	0.163 $\pm$ 0.01	0.038 $\pm$ 0.01

FPS response to CNBr treated LPS

Amount of LPS/ foot pad	FPE (mm) $\pm$ S.E.	
	untreated	CNBr treated
5 μ g	0.825 $\pm$ 0.03	0.750 $\pm$ 0.01
1 μ g	0.525 $\pm$ 0.01	0.488 $\pm$ 0.01
100 ng	0.162 $\pm$ 0.01	0.150 $\pm$ 0.05
50 ng	0.112 $\pm$ 0.01	0.088 $\pm$ 0.01

It is seen that in case of OMP's, foot pad swelling decreased after CNBr treatment but in case of LPS, there was no change; implying that proteins in the OMP preparation were responsible for the hypersensitivity and not LPS.

4. Behaviour of OMP's as T or B cell mitogens:

Hypersensitivity due to OMP's could be due to their action as T or B cell mitogens. To check this, lymphoproliferative assay was performed by culturing whole spleen cells of naive Balb/c mice with different amounts of OMP's.

Proliferative response of spleen cells to OMP

OMP's/well	CPM $\pm$ S.E.
-	501 $\pm$ 57
500 ng	615 $\pm$ 25
1 μ g	553 $\pm$ 44
10 μ g	432 $\pm$ 51
20 μ g	327 $\pm$ 25
50 μ g	431 $\pm$ 35

OMP's did not induce naive spleen cell proliferation indicating that they are not T/B cell mitogens.

5. Effect of OMP treatment on microbicidal activity of peritoneal exudate cells:

Normal healthy Swiss White mice were given an i.p. injection of OMP (500 µg/mouse). 72 hr later, they were challenged with $1-2 \times 10^4$ *S.typhi* by the i.p. route, their PC removed and microbicidal assay performed.

PC of normal mice could phagocytose and kill only 17.1 % of challenge Salmonellae in 5 min. Killing of intracellular organisms increased to 18.27 % on further incubation of PC upto 35 min.

PC of mice treated with 500 µg of OMP's 72 hr. before killing, could kill 52.52 % organisms in 5 min. and upto 52.76 % Salmonellae were killed in 35 min.

Thus it can be said that microbicidal activity of PEC increased on OMP treatment.

6. Effect of OMP treatment on nitric oxide production by peritoneal macrophages:

PC's were harvested from normal non-immune mice. They were cultured with or without γ -INF in presence of either OMP's, LPS or combinations of OMP's and LPS. After 20 hr the supernatants were harvested and nitrite in these supernatants was estimated using Griess's reagent (21).

Nitrite synthesis by PC's treated with different inducers

Treatment	Nitrite $\mu\text{moles}/10^6$ cells $\pm$ S.E.	
	without γ -INF	with γ -INF
PC's only	22 $\pm$ 1	52.5 $\pm$ 0.5
PC + 100 ng / ml LPS	39.5 $\pm$ 0.5	47.5 $\pm$ 0.5
PC + 1 μg /ml LPS	37 $\pm$ 0.0	47.5 $\pm$ 0.5
PC + 5 μg / ml OMP	39.5 $\pm$ 0.5	44.5 $\pm$ 0.5
PC + 10 μg / ml OMP	38 $\pm$ 1	45 $\pm$ 0.0
PC + 10 μg / ml OMP + 100 ng / ml LPS	39.5 $\pm$ 1.5	48.5 $\pm$ 1.5
PC + 10 μg / ml OMP + 1 μg / ml LPS	43 $\pm$ 1	50.5 $\pm$ 0.5

Results show that untreated PC's as well as PC's treated with different inducers produce nitrite in presence of γ -INF. However PC's produce nitrite even without γ -INF on treatment with these inducers which was not seen in case of PC's that were untreated.

CONCLUSIONS

1. OMP's behave as T cell target antigens.
2. OMP's show immunomodulatory properties in both afferent and efferent arms of immune response.
 - OMP's enhance both T cell and antibody responses to unrelated antigens.
 - OMP's increase the ability of macrophages to present antigens by a) Upgradation of B7/BB1 expression b) By making them secrete IL-1 and possibly other lymphokines like IL-6 as reported earlier (4).
 - They induce inflammatory reaction in tissues by their action on macrophages.
 - They enhance microbicidal properties of macrophages and OMP's also induce secretion of nitric oxide by them.



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