

**STUDIES ON T CELL SUBSET DIFFERENTIATION
FOLLOWING IMMUNISATION BY DIFFERENT
ROUTES WITH MYCOBACTERIUM VACCAE**

**THESIS SUBMITTED
UNIVERSITY OF BOMBAY**

**FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY**

**SUBMITTED BY
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**UNDER THE GUIDANCE OF
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**Studies on T cell subset differentiation following
immunisation by different routes with
*Mycobacterium vaccae***

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submitted to
The University of Bombay
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IN
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by
Mangala Laxman Sawant

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Signature of the Research Guide

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INTRODUCTION

The immune system which provides protection against invading microorganisms has two major effector arms namely innate immunity and adaptive immunity. Innate immunity is due to phagocytic cells such as neutrophils, monocytes, macrophages and non-phagocytic cells such as NK cells. Soluble mediators such as complement also play an important role in innate immunity. On the other hand, the T and B lymphocytes constitute the mediators of adaptive immunity as they can specifically recognise individual pathogens. However, both the innate and adaptive arms of the immune system are finely tuned with each other as there is a constant interaction between the two. For example, macrophages take up antigens and present them to T lymphocytes while the activated T lymphocytes in turn secrete soluble factors, the cytokines, which further activate the phagocytes to effectively destroy the internalized pathogen. In other interactions, antibodies produced by B lymphocytes, help the phagocytes internalize the pathogen by the process of opsonization. Consequently the immune response to infectious organisms is made up of both innate and adaptive components.

The potential range of adaptive immune responses to an antigen is very extensive. Activated B cells can produce various isotypes of immunoglobulin, each with the potential for multiple effector functions. For example, IgM and IgG2 bind and activate complement and can thus participate in complement mediated lysis or phagocytosis of target cell. IgG1 and IgG3 bind to Fc receptors on phagocytic cells such as macrophages and neutrophils and thus opsonize cells or particles. IgE can arm eosinophils to attack parasites.

T cells of the adaptive immune system also have specialized effector functions. CD4⁺ T cells involved in inflammatory responses such as delayed type hypersensitivity release various cytokines which participate in the inflammatory response predominantly by activating macrophages. NK/LAK cells and macrophages can be activated to attack tumor cells and participate in antibody directed cellular cytotoxicity (ADCC). Cytotoxic T cells can attack host cells infected with intracellular pathogens, preventing spread of viral and other relevant infections by releasing cytokines like γ -IFN for activating macrophages.

Although immune responses to pathogens may include several of these mechanisms, most pathogens would best be dealt with by a limited response including primarily those facets of innate immunity which are most suited to remove the offending organism. For instance, in case of viral infection an effective immune response needs to include both a cytotoxic mechanism which can destroy infected cells and circulating antibody to prevent new cells from becoming infected as well as NK cell activity for killing virus infected cells and secretion of γ -interferon for controlling virus replication. The accompanying delayed type hypersensitivity response which is also tissue destructive, is accepted as an unavoidable inconvenience.

The effector mechanisms have to plan for following different contingencies,

1. Infection by organisms easily controlled by neutrophilic phagocytes such as pneumococci - in this situation, the useful adaptive immune mechanism would be opsonic antibody such as IgG2. Cellular immunity if generated would be wasteful and can cause unnecessary tissue damage.
2. Infection by organisms generating exotoxins such as *Clostridia*, *Corynebacterium diphtheriae* - here again the adaptive immune mechanism would be antibody. The cellular immunity would be undesirable.
3. Infection by facultative intracellular bacteria that can resist destruction by naive normal macrophages - here the most useful adaptive immunity would be cellular immunity with delayed hypersensitivity response and mechanism for activating macrophages by lymphokines such as γ -interferon secreted by T cells. In this situation antibody has a limited usage. Large amount of antibody such as IgE would be wasteful and harmful.
4. Virus infections - with a role for diverse adaptive mechanisms already alluded to.
5. Parasitic infection - where most useful adaptive mechanism would be IgE antibody, with its concomitant unavoidable allergic manifestations. In this situation an extremely efficient cellular immunity would cause unnecessary tissue damage and therefore would be undesirable.

In view of this, expansion of the entire spectrum of adaptive immune mechanisms on exposure to antigen is not likely to be beneficial to the host in most situations.

Therefore, the immune system has evolved regulatory mechanisms to restrict the adaptive immune responses in a given situation, so that a fruitful interaction with innate immune mechanisms takes place, with elimination of the pathogen and minimum damage to the host tissues. Thus when one examines immune responses in experimental or clinical situations, they appear heterogeneous.

This heterogeneity of immune responses is more evident in clinical situations such as mycobacterial and parasitic infections. Leprosy and tuberculosis represent classical examples of distinct patterns of immune responses to the same pathogenic agent in different individuals. The polar forms of lepromatous and tuberculoid leprosy as well as the unreactive and reactive polar cases of tuberculosis exhibit differential generation of humoral and cellular responses respectively (1,2). In tuberculoid leprosy, cellular immunity is expanded greatly, whereas in lepromatous leprosy, there is a marked predominance of humoral immunity and poor cellular response.

Thus the hallmark of an effective immune system is a mechanism for regulating the adaptive immune response so that it develops in such a way so as to be effective in eliminating the pathogen in an economical manner without being extremely deleterious. As the CD4⁺ T cells play a central role in regulation of immune responses to most antigens they form the basis for heterogeneity in immune responses. It has been established now, that the major mechanisms for such regulation involves the development of helper T cell subsets exhibiting polarized behaviour by secreting distinct patterns of cytokines.

Experimental evidence for this polar differentiation, was first obtained from the studies on murine CD4⁺ T cell clones, carried out by Mosmann *et al* (3). It was shown that CD4⁺ T cells can be divided into two subsets-Th1 and Th2 - with distinct and non-overlapping patterns of cytokine secretion (4,5,6). Th1 cells produce IL-2, γ -IFN whereas Th2 cells secrete IL-4, IL-5, IL-6 and IL-10. It has been shown that Th1 cells mediate delayed type hypersensitivity (DTH) (7). γ -IFN produced by Th1 activates macrophages and induces increased expression of MHC antigens on the same (8-11).

IL-4 which is secreted by Th2 cells induces B cell proliferation (12), enhances IgG1 and IgE production (13) and induces increased expression of MHC antigens on B cells (14-16). These functional differences between Th1 and Th2 cells suggest that they are involved in cellular and humoral immune responses respectively. There is a third phenotype Th0, which is characterized by secretion of both γ -IFN and IL-4, the signature lymphokines of Th1 and Th2 respectively, alongwith other cytokines such as IL-2, IL-5, IL-6 and IL-10. Latest evidence supports the view that Th1 and Th2 cells develop from Th0 which could be the common post thymic precursor for the two phenotypes (17).

Recent studies in T cell differentiation reveal that naive CD4⁺ T cells do not exhibit any polar behaviour (18). When these precursors are activated by antigenic or mitogenic stimulation they make only IL-2 and no other cytokine. When they are allowed to differentiate into effectors by further exposure to antigen or mitogen, they display one of the phenotypes such as Th1, Th2 or Th0. The memory CD4⁺ T cells seem to show Th1 of cytokine profile; however this issue remains debated.

It follows therefore that the polar behaviour of CD4⁺ T cells is the consequence of differentiation of naive cells into effectors following encounter with the antigen. Various factors which regulate their differentiation into Th0, Th1 or Th2 have been studied and could be classified as belonging to three categories namely, nature of antigen, antigen presenting cells and cytokines (17,19).

The above information was obtained from experiments employing clones of CD4⁺ T cells. However, how the various factors known to induce differentiation to one or the other pathway are brought into play in a clinical situation has remained a subject for active research. In some conditions such as Leishmaniasis, considerable information has been generated through animal experimental models. In many others there is very little information available.

In polar forms of leprosy, selective expansion of Th1 or Th2 subsets has been observed. however the underlying mechanism for the selective Th subset expansion in

these conditions are not well understood. There is a necessity of experimental animal models to study the Th cell subset differentiation in mycobacterial infections. A murine model showing differential generation of cellular immune response depending on route of immunisation was reported earlier (20-23). It was decided to employ this animal model to investigate if this model also offered a distinct dichotomy in cellular and humoral responses against mycobacteria as a consequence of immunisation by different routes. Further, in case such a dichotomy was observed, it was decided to investigate the regulatory mechanism operating in this *in-vivo* model.

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REVIEW OF LITERATURE

1. Role of T cell subsets in infectious diseases

1a. Role of cellular and humoral immunity in control of infections

The immune response to pathogens consists of various defence mechanisms which are often referred to as the effector mechanisms. The two major effector mechanisms active in the immune response to any pathogen are humoral and cell mediated immunity. By and large, humoral immunity plays a major role against extracellular pathogens whereas the cell mediated immunity comes into play against intracellular pathogens. (24)

Humoral immunity is due to the soluble factors such as antibody, complement and various acute phase proteins which serve as opsonins. Complement activation results into generation of peptides with functions such as - 1) opsonisation 2) chemotaxis 3) induction of inflammation and 4) damage to the cell wall/membrane of the inducing microorganisms thus resulting into lysis of the cell.

Antibodies when bound to a pathogen, act as a flexible adapter between phagocytes and complement and thus facilitate phagocytosis. Phagocytosis is most effective in presence of both complement and antibody. Antibodies to surface proteins or capsules of microorganisms block attachment of bacterium to the host cell thus preventing the invasion. They also block binding of toxin molecule to its target cell and neutralize the locally acting toxins. By coating a virion particle, antibody disturbs the interactions between virus and host cell thus preventing further penetration and multiplication of viruses. Complement assists in neutralisation by coating the virus or by lysing their lipid membranes. Thus antibody along with complement can offer protection against various bacterial and viral infections.

However, there are adverse effects of antibodies such as it may strip off or block viral antigens on the cell surface so that the infected cell escapes destruction by cytotoxic cells. It may also increase the rate of infection of cells bearing Fc receptors by stimulating these cells to take up the virus. The major limitation of humoral immunity is that many intracellular pathogens are resistant to phagocytic destruction.

Microorganisms belonging to the genera *Mycobacterium*, *Salmonella*, *Brucella*, *Listeria* and *Legionella* are facultative intracellular parasites. They can survive and even multiply within the phagosomes of a macrophage (25). They can live either in the

phagosome or even in the cytoplasm^f. Eventhough macrophages are known professional phagocytes particularly well equipped to engulf, kill and degrade invading microorganisms, organisms of the above genera can effectively resist killing after phagocytosis and make the hostile intracellular milieu their habitat. The tubercle and leprosy bacilli are both Mycobacteria, which preferentially replicate in professional phagocytes such as macrophages, with the former being a facultative and the latter an obligate intracellular pathogen (26).

These intracellular pathogens can survive and replicate within normal macrophages by escaping the normal lysosomal destruction either by preventing a phagosome - lysosome fusion (27) or by existing free in the cytoplasm (28) rather than in a pinocytic vesicle. *M. leprae* is an ideal example of the latter phenomenon (27). Three distinct, probably complementary mechanisms have been proposed to account for phagolysosome fusion inhibition.

- i) Bacterial release of polyanionic cell wall components, especially sulphatides (28).
- ii) cAMP has been suggested to inhibit the lysosomal fusion and release of enzymes into phagosomes. The cAMP may be synthesized by the organism or the macrophage may be induced to do so (29).
- iii) Release of ammonia (30).

Therefore, resistance against such bacterial parasites predominantly depends on the ability of host to mount a cell mediated immune response (CMI). The cell mediated immune responses of importance in these infections consist of generation of effector T lymphocytes that can activate macrophages (31-36). The effector T cells are generated during the course of active cell mediated immunity which can recognise the antigen and secrete certain lymphokines such as gamma ineterferon (γ -IFN), which in turn activates the macrophages converting them into potent killers. The need for such activated macrophages (AM ϕ) was first demonstrated by Lurie (37). The activated macrophage differ from normal ones in their morphological, biochemical and functional characteristics. They are larger, attach more rapidly and avidly to glass and plastic and spread more extensively on the substrate (38). They display characteristic undulating plasma membrane (39); contain a greater number of mitochondria, lysosomes, ribosomes and more extensive Golgi apparatus (39). These activated macrophages manifest an increased total protein synthesis, increased ATP, increased lysosomal enzymes (40) and hexose monophosphate shunt activity and increased oxygen consumption and thus increased speed of

phagocytosis (41). The functional change in activated macrophages is exhibited in their increased ability to destroy pathogens (42,43). Most of the killing occurs intracellularly after the pathogen has been enclosed in the phagocytic vesicle. Once inside the vacuole, the ingested particle is immediately attacked in various ways, all aimed at killing and digesting the foreign particle.

The proposed killing mechanisms fall into two categories - oxygen dependent and oxygen independent. Oxygen dependent killing is due to two most significant effectors - the reactive oxygen intermediates (ROIs) and hydrogen peroxide (44,45). The ROIs such as superoxide anions (O_2^-), singlet oxygen (1O) and hydroxyl radicals (OH) all are potent microbicidal agents. The combination of peroxide, myeloperoxidase and halide ions constitutes a potent halogenating system leading to halogenation of bacterial proteins, aldehyde formation and cleavage of polypeptide chains due to removal of lipids eg. arachidonic acid from bacterial cell membranes. The end effect of all these toxic moieties is degradation of bacterial cell constituents. Another oxygen dependent attack mechanism is accumulation of lactic acid and hydrogen ions in the cell during metabolic burst due to glycolysis which leads to rapid drop in pH inside the phagocytic vacuole. Within 3 minutes of particle uptake, the pH in the vacuole drops from normal physiological pH i.e. 7 to 6 and within 7 minutes reaches the value of 4. In addition, this low pH facilitates action of many enzymes emptied into the vacuole, since their pH optima lie between 4.5 and 5.0. Furthermore, the generation of hypochlorous (OCl^-) ions is observed to be efficient antimycobacterial activity.

The oxygen independent killing mechanisms include peroxidases, eg. myeloperoxidase and catalase, lysozymes eg. muraminidase, lactoferrin eg. lactotransferrin, hydrolytic enzymes eg. β -glycerophosphatase, β -glucuronidase and cationic proteins such as phagocytin, leukin and cathepsin G (44).

Generation of reactive nitrogen intermediates (RNIs) by nitric oxide pathway is most recently discovered killing mechanism. In this the enzyme nitric oxide synthase gives rise to nitric oxide which is toxic for microorganisms and tumor cells. Tetrahydrobiopterin is needed as a cofactor in the reaction (46). The pathway is activated initially by γ -IFN, a product of T cells and then optimally triggered by another T cell / M ϕ product, the tumor necrosis factor, TNF- α . This mechanism enables murine macrophages to kill mycobacteria (47).

It has become increasingly clear that lymphocytes collaborate with macrophages in the process of recovery from and subsequent resistance to, disease caused by facultative intracellular parasites (35,36,48). The ability of effector T cells to attract macrophages to the site of infection appears to be one of their most important functions in bacterial infection. In a series of elegant experiments, Mackaness, Blanden and Collins have demonstrated (35,49) the importance of T cell mediated monocyte recruitment and T cell triggered macrophage activation in the recovery from a number of bacterial infections such as Tuberculosis, Salmonellosis, Listeriosis and Brucellosis.

Patterson and Youmans (50) have demonstrated that spleen cells of animals immunised with *M. tuberculosis* when cultured with virulent *M. tuberculosis* bacilli *in-vitro*, produced a dialysable substance that enhanced the ability of macrophages to digest homologous intracellular bacteria. γ -IFN is the potent stimulator of macrophages amongst the products of T cells. All the metabolic changes occurring in the so called 'activated macrophages' that are stimulated by T cells, can be induced by treating normal macrophages with γ -IFN. When any foreign antigenic material can not be degraded, T cells accumulate and release various lymphokines, this leads to the aggregation and proliferation of macrophages, and the characteristic appearance of a nodular mass called granuloma. This granuloma isolates the focus of infection. Thus T cells play dual role in granuloma formation, by causing both accumulation and activation of macrophages. Such granuloma are characteristic of infections with organisms such as *M. tuberculosis* and *M. leprae*. Analysis of the phenotype of T cells in the granulomatous foci indicate that CD4⁺ T cells are located at the centre of the reaction and CD8⁺ T cells are around the periphery suggesting that CD4⁺ T cells are of prime importance in antigenic recognition and accumulation of other lymphocytes and macrophages. This indicates that CD4⁺ T cells must be playing a role of prime importance in cell mediated immunity against mycobacterial infections.

1b. Role of CD4⁺ T cells in immunity against infections

CD4⁺ T helper cells play several central roles in the humoral and cell mediated immune responses, consisting of a number of effector and regulatory functions. While some of these functions are mediated directly through cell to cell contact, many of them

are carried out by secreted lymphokines (51). By interacting with antigen presenting cells bearing antigenic peptides associated with MHC class II, they determine which antigen or even which antigenic epitope in particular, is to be recognised (52).

Once the CD4⁺ T cells recognise the antigen, they initiate differentiation and conversion to effectors of themselves as well as of other components of the immune system.

They induce activation, proliferation and differentiation of B cells to plasma cells and 'help' produce the antibody. They are also required for differentiation of cytotoxic T cells into functional effectors. This is also the subset of T cells involved in activation and accumulation of phagocytes, leading to inflammatory reactions such as DTH.

CD4⁺ T cells also secrete an array of lymphokines which exert positive or negative regulatory effects in autocrine or paracrine fashion on various cell populations (51). The type of effector mechanism chosen during an immune response is of critical importance to the host and therefore is subject to precise and complex regulation. The most important function of Th cells is in selection of suitable effector mechanisms against the pathogen. This is achieved by Th cells through an array of cytokines which may direct the immune response towards a particular effector mechanism (52).

This becomes more clear in the context of mycobacterial infections such as Tuberculosis and Leprosy.

1c. CD4⁺ T helper cell responses in mycobacterial infections

Mycobacteria being intracellular pathogens, preferentially inhabit the professional phagocytes i.e. macrophages. *M. tuberculosis*, the causative agent of Tuberculosis and *M. leprae*, the causative agent of Leprosy are facultative and obligate intracellular parasites respectively (26).

However, both Leprosy and Tuberculosis do not exist as single clinical entities but represent themselves as a wide spectrum of clinical and immunological manifestations, exhibiting different patterns of immune responses to the same pathogenic agent in different individuals (1,2). For example, lepromatous leprosy and tuberculoid leprosy belong to two poles of the spectrum with predisposition to humoral and cellular

immunity respectively.

The tuberculoid leprosy (T.T.) is paucibacillary, self-limiting disease with lesions which are restricted to skin and nerves. Individuals with tuberculoid leprosy show low levels of antibodies but good CMI which results in the killing and clearing of bacilli in tissues with concomitant tissue destruction.

In contrast to this, the lepromatous leprosy (LL) is the disseminating, multibacillary type requiring long term chemotherapy. It is associated with high levels of antibodies and poor cell mediated immunity resulting into a selective cellular unresponsiveness to *M. leprae* antigens (1) leading to widespread lesions of skin, peripheral nerves, upper respiratory tract, eyes etc. It is characterised by massive bacillary infiltration of the tissues and bacteraemia but minimal tissue destruction.

Thus in both the cases even though there is tissue destruction, complete killing and clearing of bacilli in the host does not take place,

Presence of T helper cell response is evident from good CMI in tuberculoid leprosy. However in lepromatous leprosy also there are high IgG antibody levels which in turn indicate the presence of T cell 'help'. Thus even though Th cells are activated in both TT and LL, they produce totally different effector mechanisms, i.e. cellular and humoral immunity respectively.

Immunological studies of chronic carriers of lepromatous leprosy revealed that these forms did not show total absence of T cell response. CD4⁺ T cells were present and antibody response was T-dependent indicating that T cell help for antibody response is available but T cell help for CMI is not available thus indicating that CD4⁺ T cells exist in two subpopulations with varied functions.

2. CD4⁺ T cell subsets

2a. Different types of CD4⁺ T cell subsets

Recent studies of murine CD4⁺ T cell clones have demonstrated two major subsets depending on their profile of cytokine secretion which are designated as Th1 and Th2 (3,4,5). Th1 clones produce IL-2, γ -IFN, TNF- β and IL-12 but not IL-4, IL-5, IL-6, or IL-10, while Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 but not γ -IFN (6). This

distinction between Th1 and Th2 clones, based on the array of secreted lymphokines, is useful in as much as it correlates with functional differences between the cell subsets as well. IL-2 appears to be autocrine growth for TH1 cells, whereas IL-4 mediates the autocrine growth factor of Th1 cells (53-56). It has been reported that Th1 but not Th2 cells mediate delayed type hypersensitivity (DTH) (7). γ -IFN produced by Th1 cells has been reported to activate macrophages and to induce increased expression of MHC antigens on these cells (8,9). IL-4 secreted by Th2 cells has been reported to induce B cell proliferation in resting B cells (10-13) and to enhance MHC class II expression on B cells (14-16). Th1 cells promote IgM and IgG2a responses while Th2 cells promote responses with selective production of IgG1, IgE and IgA isotypes (57-61).

These functional differences between Th1 and Th2 cells suggest that they are involved in cellular and humoral immunity respectively.

The Th1 and Th2 phenotypes were originally defined as very distinct cytokine secretion patterns that were expressed by large numbers of independently derived T cell clones. However, there are a number of other cytokine secretion phenotypes displayed by T cell clones in tissue culture, some of which are stable and obtained in a number of independent experiments. Most predominant of them is a phenotype known as Th0 which expresses most or all of the cytokines made by both Th1 and Th2 clones (62-64).

Recent evidence now favours the view that Th1 and Th2 cells develop from Th0 cells which could be the common post-thymic precursor for both the phenotypes (17). Experiments in which primary murine Th clones were derived by limiting dilution suggest that many Th cells differentiate first into Th0 cells that express both Th1- and Th2-specific cytokines when restimulated and Th0 clones often differentiate into either Th1 or Th2 clones after additional cycles of antigenic stimulation (65,66). Swain and colleagues have identified, at the population level, a precursor-ThP-(presumably a virgin Th cell) that makes initially only IL-2 but then differentiates into cells making the Th0 cytokine pattern (67). This differentiation pathway has been supported by evidence from human studies which showed that human Th clones specific for antigens to which the donor is not immunised are similar to murine Th0 clones (as they secrete both IL-4 and γ -IFN) whereas most clones for which the donor was hyperimmune, resemble murine Th1 or Th2 clones (68-69).

It is possible, however, that some Th cells are restricted, during thymic maturation, to the development of a Th1 or Th2 cytokine profile. There is, at present, no experimental evidence, to rule out such a possibility, but there is no evidence in support of this alternative either. Thus lymphokine secreting pattern of a particular helper T cell subset determines the functional abilities of the subset and development of these subsets is strictly regulated.

It is precisely this kind of information, that eventually lead to the belief that CD4+ T cells of individual, when stimulated either by immunisation or during the course of infection, can lead to generation of exclusive Th1 or Th2 type of response. Crucial questions such as phenotypes of naive, unactivated and memory CD4+ T cells, and factors that determine selective Th1/Th2 differentiation were then posed by scientists everywhere.

2b. Expression of T subset commitment in precursors, effectors and memory populations

Eventhough significant conceptual advances have been made in understanding of CD4+ T cell subsets and their role in regulation of immune response; little is known about how CD4+ T cell development into phenotypically and functionally distinct subsets takes place. Recent studies suggest that peripheral CD4+ T cell population consists of subsets such as naive precursor, recently activated, effector and memory. These subsets are different developmental stages of CD4+ T cells. However, unequivocal functional or phenotypical characteristics for distinguishing the different developmental subsets of T cells are still not available.

As described earlier, polar functional subsets of CD4+ T cells such as Th1 and Th2 are observed in both clinical and experimental situations following natural or purposeful exposure to antigen. However, it is not yet understood whether this polarization as a phenomenon exists at the precursor level or it appears after activation. Crucial questions which need to be posed are - Do Th1 / Th2 subsets exist as precursors or do they differentiate from a common precursor following activation ? Memory is known to be an important facet of an immune response. Do polar T cell subsets exist in the memory population, too ?

To answer these questions the three populations of T cells i.e. the naive precursor, the effector and the memory cell need to be defined. It is a well established fact that in a naive animal neither effector, nor memory cells, specific for a given antigen are observed. The effector and memory T cells arise only after the animal is exposed to and activated by a specific antigen. Hence differentiation of precursors into effectors and / or memory is a post - activation event and hence the most significant phenomenon in the development of an immune response.

So far as generation of memory and effector cells by a process of differentiation following active immunisation is concerned, a developmental biologist is confronted with the following dilemma.

A memory cell and an effector cell may arise from a precursor by a process of asymmetric division and differentiation. On the other hand, a memory cell may arise from a precursor of separate memory lineage, as has been suggested for B cells (70). So far there is no evidence for separate T cell memory lineage.

In addition to generation of memory and effector, the asymmetry of cell division and differentiation may also result into replicated precursor itself.

Differentiation to memory and effector cell is most likely a multistep process. It is of course presumed that the effectors are specialised cells capable of secreting large amounts of cytokines on stimulation by antigen. Whether the effectors can replicate is a debated issue (70). Memory cells are definitely able to replicate. Some of the multiple stages of differentiation between precursor and effector, probably have mixed functional features such as replication and cytokine secretion. Thus functional and phenotypic characterization of precursor, memory and effector pose a major problem.

Matters are further complicated by the following issues - 1) One does not know the fate of effectors. Are they end cells ? If not are they capable of replication ? What happens to them when the stimulating antigen is completely destroyed and eliminated? 2) It is postulated that memory cells also tend to change into functional state similar to that of a naive precursor, when antigen stimulation is not available.

To define the naive, effector and memory T cells we need to establish the

functional differences, if there are any, between them on the basis of different parameters such as proliferation in response to the antigen and cytokine secretion. Swain and colleagues who are striving to differentiate between these subsets have attempted to define them on the basis of the phenotype, activation state and functional differences (including cytokine secretion and help to B cells).

The naive CD4⁺ T cells are short lived, small, resting (Go) cells that disappear after adult thymectomy (71, 72). There is a dramatic loss of precursor population by 10 wk after adult thymectomy with nearly complete loss after 20 to 25 wk. These precursors can be activated by antigenic or mitogenic stimulation as a result of which they proliferate and secrete lymphokines. However, the only lymphokine they make is IL-2 and no other within first 24h. The precursors proliferate and differentiate by 4 days into effectors (71, 72).

Effectors are large cells (probably in G_s phase) with nucleus showing blastoid features. Along with IL-2, when restimulated, they make much large quantities of diverse lymphokines. The conditions during the 4 day development of effectors determine the nature of the effectors and the lymphokines produced. When restimulated with the same antigen, effectors secrete a wide array of lymphokines such as IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, γ -IFN, TNF etc. depending upon the nature of effectors generated. This response is faster with very short lag period and is enhanced with high levels of cytokines secreted.

According to Swain et al, memory T cells are small, resting (Go) cells, that persist even after adult thymectomy. They respond directly to antigenic or polyclonal stimulation, by diverse lymphokine secretion (before proliferation) with predominant IL-2 component and proliferate. By 4 days they develop into memory effectors which can be restimulated to produce large quantities of diverse lymphokines. Memory population exhibits properties distinct from that of precursor and effector populations such as -

1) they generate a rapid and heightened response to antigenic stimulation with increased levels of diverse cytokines secreted unlike precursors and 2) they have capacity to replicate unlike effectors, nevertheless, they produce cytokines without proliferation.

Various attempts have been made to differentiate the naive, effector and memory

T cell subsets on the basis of phenotypical characteristics also. Phenotypical characterisation has been carried out by using various surface markers such as CD45R, mel-14, Pgp-1 and various adhesion molecules such as LFA-1, CD2, CD11, LFA-3, ICAM-1 etc.

The naive precursors show presence of high mol. wt. isoform CD45R^{hi} (i.e. CD45RA in humans and CD45RB in rats and mice) while the memory T cell carries a low mol. wt. isoform CD45R^{lo} (ie. CD45RO in humans and rats, but no such marker is shown in mice) The effectors probably express the moderate mol. wt. isoform CD45RB (in humans as well as in rats and mice).

The naive precursors are Mel-14 +ve and Pgp-1-low while the effectors are Mel-14 variable and Pgp-1-high (71, 72). The effectors are rather heterogenous regarding expression of Mel-14. In contrast to this, the memory cells are in Mel-14 -ve and Pgp-1-high population. Low affinity IL-2 receptor (CD25) is most recently observed to be exclusively expressed as an activation marker. Naive precursors and memory cells are CD25 -ve and effectors are CD25 +ve.

Swain and colleagues have been successful in defining the functional differences between memory and effectors in terms of their cytokine secretion kinetics based on mRNA studies (71). The two populations ie. memory and primed effector cells respond quite distinctly when they are stimulated with Con A. The memory population synthesizes mRNA for lymphokines with a lag of some 6h, with maximal mRNA levels peaking between 12h and 18h after stimulation. These levels remain high till 44h and secreted lymphokines accumulate in the medium with a kinetics that reflects this RNA level. In contrast, the cultured effector population synthesizes detectable message by 2.5h after restimulation with maximum levels at 7.5h to 13h. The lymphokine mRNA levels are also more rapidly down regulated in the effector cells. This and the striking difference in the pattern and quantities of lymphokine secreted by the two populations - i.e. high IL-2, low γ -IFN, IL-3, GM-CSF by the directly stimulated memory cells and the modest IL-2, extremely high IL-3, IL-4, IL-5, GM-CSF and γ -IFN by the in vitro effector cells underscore the very different properties of these two populations (71).

Hence, as understood from the available data, the naive CD4 + T cells secrete

only IL-2 and no other lymphokines. On the other hand, the lymphokine secretion by effectors showed either Th1 or Th2 or Th0 pattern (71). In this context, it should be noted that several other recent studies have in fact suggested that cloned, normal Th cells which have been in culture for shorter periods of time do not fall neatly into Th1/Th2 categorisation and that many Th cells in both mouse and man secrete lymphokines characteristics of both Th1 and Th2 lines (71).

The memory population studied by Swain *et al* had a pattern of lymphokine secretion some what like Th1 lines - IL-2 and γ -IFN secretion without IL-4 or IL-5 (71-74). It must be stressed, however, that the pattern of lymphokine secretion by putative memory (i.e. high IL-2 and low quantities of other Th1 lymphokines) is quite different from Th1 cell lines since these often secrete γ -IFN, IL-3 and GM-CSF in high quantities. Nevertheless, few memory cells secreting IL-4 or IL-5 have also been detected, which could possibly represent the Th2 type of memory subset. The authors do not rule out the possibility that a different set of conditions of antigenic stimulation or immunisation will lead to Th2 type of memory CD4⁺ T cell population.

The long term Th cell lines described by Mosmann *et al* may be thought to be like memory population as they are selected by many rounds of restimulation and are continuously perpetuating. However, they are more like the cultured effector T cells as the cells in the cell lines are larger, IL-2 R positive and they synthesize and secrete lymphokines with more rapid kinetics and mRNA up and down regulation characteristic of cultured effector cell rather than resting memory cells.

Hence the consensus is that - 1) memory can not be differentiated into Th1/Th2 eventhough theoretically it is possible and 2) effectors are terminally differentiated into Th1 or Th2 or Th0 type subsets however the issue of terminal differentiation still remains debated.

2c. Selective differentiation of CD4⁺ T helper cell subsets

It is difficult to define and characterize mechanisms involved in the activation of T lymphocytes using cells obtained directly from animals because lymphoid tissues consist of a heterogenous mixture of cells. Hence several *in-vitro* systems including various clonal populations of T cells have been established to identify the factors

regulating the Th cell differentiation. These models involve isolation and stimulation of naive CD4⁺ T cells in the presence of various antigen presenting cells or cytokines or anti-cytokine antibodies, followed by measuring the cytokines produced upon restimulation. Initial studies used polyclonal activators such as anti-CD3, mitogens, as a stimulation for naive CD4⁺ T cells, as the frequency of cells responding to a conventional antigen is low (75, 76). However, recently a more elegant approach was used involving CD4⁺ T cells from TCR transgenic mice (77-80). Because of the high percentage of CD4⁺ T cells bearing the transgenic TCR, this model permits stimulation with a conventional antigen, presumably more accurately reflecting *in-vivo* events. The combination of *in-vivo* and *in-vitro* approaches has been quite fruitful in elucidating mechanisms of CD4⁺ T subset differentiation.

i) Role of cytokines and cells of innate immunity :

Both *in-vivo* and *in-vitro* systems have shown that the cytokines present in the microenvironment during primary stimulation constitute an important influence on naive Th - cell differentiation. This has been most clearly shown with the protozoan parasite *Leishmania major*. Depending upon the inbred mouse strain infected, this parasite causes either a progressive and eventually fatal infection or a self-healing lesion. Studies from several laboratories have shown that the outcome of infection depends upon which CD4⁺ T cell subset is stimulated - Th1 cell development leads to resistance whereas Th2 cells confer susceptibility (81). Endogenous cytokine levels control which subset predominates (81-84). Thus treatment of resistant mice with anti-interferon (IFN)- γ monoclonal antibody (mAb) ablates Th1 cell development and promotes Th2 cell differentiation (85-88). In contrast, treatment of susceptible mice with anti-IL-4 mAb inhibits Th2 cell expansion, leading to Th1 cell development (89-91). Similar results with anti-IL-4 mAb treatment have recently been obtained with the experimental Candidiasis (92,93) and even infections that are primarily associated with cell mediated immunity such as Listeriasis exhibit enhanced resistance following elimination of IL-4 effects (94). The importance of IL-4 in the Th2 cell differentiation is supported by data from the *in-vitro* models as well. For example, in the TCR transgenic systems incubation of CD4⁺ cells with IL-4 during the primary stimulation leads to the development of Th2 cells, whereas addition of anti-IL-4 augmented the development of cells that produce IFN- γ (77,78).

Furthermore the differentiation of T-cell clones exhibiting a Th0 cytokine pattern towards a Th2 phenotype was shown to be dependent upon presence of IL-4 (95). In conclusion, these data prove the critical importance of cytokines in Th cell development.

Implicit in the studies described above is that IL-4 or IFN- γ production is induced soon after infection to direct the immune response. In fact, 72 hours after *L. major* infection enhanced IL-4 production is observed in the draining lymph nodes of susceptible (Th2) mice and IFN- γ is present in the lymph nodes of resistant (Th1) mice, suggesting that the production of these cytokines is, indeed, part of the innate immune response (85,96²¹⁴). NK cells appear to be a major source of IFN- γ in this system. Thus in resistant C3H/HeN mice, NK cells are activated within the lymph node while Balb/c mice fail to exhibit this response (97). Similarly, NK cells have been implicated as the source of IFN- γ that drives the differentiation of naive Th cells towards a Th1 phenotype when transferred to SCID mice infected with *L. major* (98). In contrast, the cells producing IL-4 during the initial stages of infection presumably are mast cells and/or basophils (99). Alternatively, IL-4 may be secreted by the T cells themselves. For example, it was suggested that recent thymic emigrants provide the IL-4 that drives Th2 cell differentiation (64). Furthermore, footpad injection of eggs from *Schistosoma mansoni* induces a rapid cytokine response in the lymph node characterized by elevated levels of IL-4 and IL-5 (100) and in this system CD44^{lo} T cells produce IL-4 (101). Another potential source of cytokines during the early stages of immunity are $\gamma\delta$ T cells. In *Listeria*, these cells have been reported to produce IFN- γ within 3 days of infection (102). Interestingly, $\gamma\delta$ T cells are also present in lesions from patients with *Leishmania* (103) and increased numbers are found in both resistant and susceptible strains of mice infected with *L. major* (104). However, whether they promote susceptibility or resistance remains unclear; $\gamma\delta$ T cells also appear at later stages of infection with murine influenza virus and have been thought to participate in resolution of the disease (105).

In contrast to *in-vivo* studies, a role for IFN- γ in Th cell subset differentiation *in-vitro* has been more difficult to demonstrate. Although not absolute, the inhibitory effect of IFN- γ on proliferation of murine Th2 cells is significant and seems to be sufficient to limit their clonal expansion. While IFN- γ does not inhibit lymphokine

secretion by stimulated Th2 cells, it does inhibit many of the agonist effects of those secreted lymphokines. For example, IFN- γ inhibits proliferation of murine bone marrow cells stimulated with IL-3, IL-4 or GM-CSF (106). In addition, IFN- γ can inhibit IL-4 dependent B cell differentiation (107,108). IFN- γ augments Th1 cell expansion *in-vitro* possibly through inhibition of Th2 cell proliferation (109,110). However when added to naive CD4⁺ T cells a strong bias towards Th1 cell development was not observed, suggesting that other cytokines might be required (77,78). The recently identified cytokine NK cell stimulatory factor (NKSF) or IL-12 is a likely candidate as it is produced by macrophages and B cells and stimulates the production of IFN- γ from T cells and NK cells (111,112,136). Originally described in the human (113,114), the murine equivalent has now been cloned (115). IL-12 inhibits Th2 cell expansion in cell lines taken from atopic patients (116,137). Most recently it has been found that the presence of IL-12 during primary stimulation of CD4⁺ T cells from TCR transgenic mice skews the response in the direction of Th1 cell differentiation (80,110,135). These are extremely important results which suggest that IL-12 may be the IL-4 counterpart for differentiation of Th1 cells. One of the appealing aspects of this hypothesis is that it directly links the induction of a Th1 response with the nature of the invading pathogen and the innate immune response. Those pathogens associated with Th1 cell development, including intracellular protozoa and bacteria, are the same pathogens shown to induce IL-12 production. For example, *S. aureus* induces IL-12 production by murine and human macrophages (111). Similarly, the ability of *Toxoplasma* to stimulate IFN- γ production by NK cells is dependent upon secretion of IL-12 by macrophages (117). In leishmanial system, IL-12 production correlates with Th1 cell development. Furthermore, IFN- γ production by cells from resistant mice was inhibited when IL-12 was neutralized with mAbs (118). It is likely that over the ensuing years a series of studies on IL-12 will further demonstrate a central role for this cytokine in directing CD4⁺ T cell development (119,154).

Several other cytokines may also participate in Th cell subset differentiation. For example, it was demonstrated that *in-vivo* depletion of TGF- β promotes resistance in mice normally susceptible to leishmania and that this resistance is associated with enhanced IFN- γ and decreased IL-4 production (120,121). While these results suggest

that TGF- β may augment Th2 cell development, conflicting data suggests that the presence of TGF- β during *in-vitro* stimulation of either human or murine T cells enhances IFN- γ production, and suppresses the development of IL-4 producing cells (122,123). This discrepancy may relate to the presence or absence of NK cells, as TGF- β has been shown to inhibit the NK cell activity (124).

IL-10, a lymphokine secreted by Th2 cells, B cells and LPS - stimulated macrophages, inhibits lymphokine production by Th1 cells but not by Th2 cells(125). It substantially inhibits secretion of γ -IFN and IL-3 but lymphokines such as GM-CSF and TNF as well as IL-2 are slightly inhibited (126-128). Although there appears to be reciprocal regulation of responses of Th1 and Th2 clones by lymphokines secreted uniquely by these subsets, the site of regulation differs. IFN- γ secreted by Th1 cells apparently can act directly to inhibit the proliferative ability of Th2 cells while IL-10 exerts its effect indirectly by acting on APC to reduce their ability to stimulate lymphokine production by Th1 clones (129-133). IL-10 may also play a modulatory role, as it inhibits the APC function of macrophage/dendritic cells and *in-vitro* neutralization of IL-10, when splenic cells were used as APCs, led to enhanced Th1 cell differentiation (131-134).

IL-13 is the most recently reported cytokine thought to regulate the selective differentiation of CD4⁺ Th cells. In human monocytes stimulated with LPS, the production of proinflammatory cytokines, chemokines and hematopoietic growth factors is inhibited by IL-13, particularly inhibiting the secretion of IL-1, IL-6, IL-8, TNF- α , IL-10, GM-CSF and G-CSF (138,139). Similar effects have been reported for IL-4 and IL-10 (140). Like IL-4 and IL-10, IL-13 also appears to inhibit NO production by activated macrophages (134). In addition to IL-4, IL-13 is another cytokine secreted by T cells that induces naive human B cells to switch to cells producing IgG4 and IgE (141-143).

IL-13 probably acts like IL-4, suppressing the development of Th1 cells through down regulation of IL-12 production by monocytes, thus favouring the generation of Th2 developmental pathways (144,149). The modulating effects of IL-13 on the functions of monocytes are observed in the presence of neutralizing anti-IL-4 mAbs, thus indicating that IL-13 acts independently of IL-4 (145).

IL-12 and IFN- γ induce development of Th1 pathways (146-149). The capacity of IL-13 to down regulate production of these cytokines by monocytes suggests that, like IL-4, it may favour the development of Th2 type of responses (150).

ii) Antigen presenting cells :

Studies in the TCR transgenic model indicate that the most efficient APCs for stimulating naive CD4⁺ T cells are dendritic cells, although other cell types may substitute for dendritic cells (77-79). It is now known that macrophages and B cells are also capable of inducing differentiation of Th1 or Th2 as they also express B7 (151-153). Several studies have demonstrated that B7 functions as the most important co-stimulatory molecule in the murine system (155-161). Particularly relevant to the stimulation of naive Th cells were studies showing that the presentation of the TCR ligand and the co-stimulatory molecules needed to be on the same cell for efficient T Cell activation (162). However, some studies report different outcomes following exposure to different APCs (179).

However, recent evidence suggests that co-stimulatory molecules on APCs may selectively influence T cell differentiation : antibodies against B7.1 or B7.2 selectively inhibit the development of Th1 and Th2 responses, respectively, both *in-vitro* and *in-vivo* (163). However, the contribution of B7.1 and B7.2 to T cell differentiation may be more complex than initially thought. Both B7.1 and B7.2 can induce Th1 or Th2 differentiation, and their relative contributions may be affected by antigen concentration. B7.2 appears to be the dominant co-stimulatory molecule during primary responses, whereas B7.1 which is upregulated later in immune responses, may be critical in maintaining primary and secondary immune responses (164).

iii) Antigenic concentration and epitopes :

The relationship between different pathogens and the induction of distinct Th cell subsets suggests that distinct epitopes might induce the development of a particular Th cell subset. However, most evidence indicates that differences at the epitope level are not a major factor in determining which Th cell subset predominates. For example, isolated T cell clones when stimulated with anti - CD3 mAb, could differentiate in either direction, depending upon culture conditions (165). Similarly, in the TCR transgenic

models either Th1 or Th2 cells can develop (77,78). In addition, in the leishmanial system Th1 and Th2 cell clones bearing the same TCR have been derived from infected mice (166).

Notwithstanding these observations, recent data obtained using a peptide derived from human Type IV collagen suggest that certain characteristics of the immunogenic peptide may influence subset selection (24,167). Thus, when H-2^s mice were immunized with collagen peptide a Th1 cell response develops, whereas H-2^b and H-2^d mice develop a Th2 cell response. The density of the ligand on the APC, which is a function of the affinity between the processed peptide and class II molecules, may be responsible for different outcomes in association with different MHC class II expressing APCs (24,167). This suggests that in certain events the epitope may indeed influence which subset predominates by dictating the affinity of the interaction between MHC and itself. In this regard, the continued presence in culture of an anti-V _{β} 8 mAb specifically enhanced IL-4 production in the V _{β} 8 T cell population, suggesting that continuous ligation of the TCR might influence subset development (168).

Finally, one of the oldest factors known to influence the development of Th cell subsets is antigen dose. This has been reinforced by the recent demonstration that low dose challenge in the murine leishmanial system induces a cell mediated immune response that leads to resistance to reinfection (169). This is more evident from the observed inhibitory effect of high concentrations of antigen on proliferation of Th1 clones but not Th2 clones. This may account for the observations that delayed hypersensitivity reactions are favoured by low daily doses of antigen while high antibody responses are favoured by high daily doses of antigen (170,212,213).

3. Relevance of T cell subsets in mycobacterial infections

3a. Polar dichotomy of immune responses in mycobacterial infections

The heterogeneity of CD4⁺ T cells is most evident in mycobacterial infections such as Tuberculosis and Leprosy. As stated earlier, both these diseases do not exist as single clinical entities but represent themselves as a wide spectrum of clinical and immunological manifestations exhibiting different patterns of immune responses to the same pathogenic agent in different individuals (1,2).

Leprosy is a polymorphic disease, manifesting itself through a wide clinical spectrum of severity, according to the immune status of the host. After gaining access to the host's tissues, if the leprosy bacilli are not destroyed by the phagocytes, they persist within the phagocytes resulting into a granuloma. Such a reaction is at first termed as indeterminate, as the lesion is too immature to be classified. This lesion can either persist for months or years or develop into one of the overt forms of clinical leprosy.

Ridley and Jopling (1) introduced a classification of clinical leprosy based upon the concept that it was a five point spectral disease. Polar tuberculoid individuals (TT) and borderline (BT) type have paucibacillary, localized self limiting disease whereas patients with polar lepromatous leprosy (LL) and borderline (BL) leprosy have disseminating, multibacillary disease which requires long term chemotherapy.

Both tuberculoid (TT) and lepromatous (LL) forms are relatively stable, whereas the borderline forms are unstable and without treatment deviate towards the more stable polar forms. The borderline forms are borderline tuberculoid (BT), borderline (BB) and borderline lepromatous (BL) going from tuberculoid to lepromatous pole of the spectrum (1).

At one pole, tuberculoid leprosy (TT) patients develop high levels of CMI which results in the killing and clearing of bacilli in the tissue and the localized signs are restricted to skin and nerves although there is concomitant tissue destruction.

At the other pole lepromatous leprosy (LL) patients exhibit a selective immunological unresponsiveness to *M. leprae* antigens leading to widespread lesions of skin, peripheral nerves, upper respiratory tract, eyes, testes and the reticuloendothelial system. This form of the disease is characterized by a massive bacillary infiltration of the tissues and continuous bacteremia, but tissue destruction is minimal and appears late.

Tuberculoid leprosy can be self-healing and typically has low levels of antibodies and good cell mediated immunity. In contrast to this, the lepromatous form of the disease is associated with high⁶ levels of relevant and irrelevant antibodies and poor cellular immune function (171,172).

Skinsnes (173) was amongst the first to suggest that tuberculosis like leprosy, is a disease characterized by an immune spectrum. He suggested that most patients

analogous to those with tuberculoid leprosy develop disease characterized by granuloma formation and cellular hypersensitivity whereas a few patients present a picture analogous to lepromatous leprosy. There have been a number of studies in which the immune status of anergic tuberculosis patients have been characterized further. Lenzinni as well as Rottoli and Rottoli (22) proposed that tuberculosis also presents an immune spectrum. They have produced a four group classification. The high resistant group (RR-reactive) is characterized by delayed hypersensitivity. The middle groups i.e. RI-reactive intermediate and UI unreactive intermediate are characterized by Jones-Mote form of hypersensitivity and the low resistant group i.e. UU-unreactive is characterized by lack of cellular response and a negative tuberculin reaction. The first form is self-healing and the last form, fatal. The number of acid-fast bacilli in the infected tissue increases towards the unreactive pole, accompanied by increase in tuberculin antibodies. The RR form is characterized by lymphocytes and epitheloid cells in their lesions. The UU form shows extensive spread of disease within the chest and other organs. The intermediate forms may shift towards one pole or the other, the course being affected by drug treatment. Thus the grouping is an arbitrary division along a continuous spectrum.

Thus polar dichotomy of cellular & humoral immune responses is strongly evident in both Tuberculosis and Leprosy, the two major mycobacterial infections.

3b. CD4+ T helper cell differentiation in mycobacterial infections

Since CD4+ T cells play the central role in immune responses to T-dependent antigens they form the basis for this heterogeneity. As stated earlier, Th1 and Th2 subsets of CD4+ Th cells are involved in cellular and humoral immunity respectively. Hence, the differential generation of Th1 and Th2 responses could explain the above immune deviation. In fact the existence of Th1 and Th2 type of responses has reportedly been proved not only in experimental animals but also in humans with selective cellular or humoral immune responses to allergic or parasitic antigens (174-178).

Most T cell lines and clones from normal donors have characteristics of Th0 cells, secreting a mixture of virtually all possible lymphokines and do not fit into the functional

Th1 or Th2 subsets of mice. However, recent studies from several laboratories studying individuals whose immune system is actively engaged by antigen suggest that functional subsets of CD4⁺ T cells do exist in man. Of particular relevance is the work of Romagnani *et al* (174), as they showed that T cell clones from normal and atopic individuals responsive to tuberculin purified protein derivative (PPD) or to parasite antigens, could be classified in Th1 and Th2 subsets of CD4⁺ T cells analogous to those in mouse. Similar results have been found by Salgame *et al* (176). CD4⁺ T cell clones from strongly lepromin positive contacts or patients produced predominantly γ -IFN while CD4⁺ tetanus-toxoid-specific clones produced IL-4 and IL-5 but little γ -IFN, thus indicating development of Th1 and Th2 subsets respectively.

Thus, the selective generation of cellular or humoral immune responses is entirely explainable by polar differentiation of CD4⁺ Th cell subsets.

However, the precise regulatory mechanisms for this selective expansion of Th cell subsets in mycobacterial infections are not known, which need to be investigated in an animal model.

In light of this knowledge, the following aims and objectives were set for the present study.

Aims and Objectives

- 1) To set up an experimental model in which there is selective expansion of Th cell subsets following immunisation with *Mycobacterium vaccae*.
- 2) To study the regulatory mechanisms for the selective expansion of T cell subsets.

MATERIALS & METHODS

1. Microorganisms :

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 (181). 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 the medium.

2. Animals :

a) Mice :

8-10 week old Balb/c and C3H/HeJ mice obtained from National Institute of Health, U.S.A. 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, U.S.A. and maintained in the animal colony of Haffkine Biopharmaceutical Corporation Limited, Bombay were used for studies in random bred animals. Prior to immunisation, all mice were tested for delayed type hypersensitivity (DTH) response to the sonicate of *M.vaccae*. Only those animals that showed no response were used in the experiments.

b) Rabbits :

Adult Albino rabbits weighing 1.5 to 2 Kg were purchased from Haffkine Biopharmaceutical Corporation Limited, Bombay were used for raising antisera.

3. Immunisation :

10 day old culture of *M. vaccae* in the Doub and Youman's medium was killed by γ -irradiation (2.4 Mrad). The bacterial cells were harvested and washed in phosphate buffered saline (PBS) (Append I). The washed bacterial cells were pelleted by centrifugation at 2000g max for 30 min. The supernatants were discarded and one ml of packed cell volume was made upto 50 ml with sterile PBS containing 0.05% Tween 80 to give a suspension containing 2×10^9 bacilli/ml (182). The required number of bacilli were suspended in 0.5 ml volume for intraperitoneal (i.p.) immunisation and in

0.05 ml for intradermal (i.d.) immunisation.

4. Preparation of antigen :

i) Preparation of sonicate :

10 day old culture of *M. vaccae* in the medium of Doub and Youmans was harvested and killed by γ -irradiation. The killed cells were washed with PBS and subjected to sonic disintegration (B-30, Sonifier - Bransen Sonic Power Co., U.S.A.) at 20 kHz for 30 min with a 50% duty cycle, keeping the cell suspension constantly on ice. The sonicate was then centrifuged at 50,000g for 1h at 4°C (Sorvall Ultracentrifuge - OTD Combi) The supernatant was filtered through 0.22 μ m membrane filter (Millipore, U.S.A.) and its protein content was estimated by the method of Lowry *et al* (Append II) (183). After confirming sterility the solution was aliquoted and stored at -20°C and used as *M. vaccae* sonicate.

ii) Purification of 65 kDa antigen of *M.vaccae* from the sonicate

a) Lysozyme - papain treatment of sonicate :

The *M.vaccae* sonicate was prepared in 10 mM Tris - EDTA buffer (Append) as described earlier. The sonicate was centrifuged at 750 g for 10 min to remove cell debris. The supernatant was collected and treated with 2 mg/ml Lysozyme (Sigma) and 10 mM EDTA and incubated at 37°C for 2h (184). Thus the bacterial cell wall was disintegrated. The protein concentration of the sonicate was estimated. The lysozyme treated sonicate was then subjected to papain digestion. Papain (Sigma, Cat. No. P 3125) was added in the proportion of 1mg enzyme to 15 mg of protein. Also 0.1 M L-cysteine hydrochloride (Sigma) was added to the digestion mixture in the proportion of 0.5 ml of L-cysteine to 30 ml of mixture. The reaction was allowed to proceed at 37°C for 2h in a water bath with intermittent shaking. The reaction was then stopped by transfer of the flask to a chilled ice bath and addition of 0.13M iodoacetamide (Sigma) in the proportion of 0.2 ml for every 10 ml of the reaction mixture. The suspension was centrifuged at 50,000 g for 1h at 4°C and the supernatant was collected, aliquoted and stored at -20°C.

b) Purification of 65 kDa antigen from lysozyme papain digested *M. vaccae* sonicate :

The lysozyme papain digested sonicate was subjected to preparative agarose isoelectrofocussing (IEF) employing the technique described earlier (185). 20ml of 1% agarose (Sigma, IEF grade, type VIII, < 0.02 EEO) containing 12.5% (w/v) sucrose and 2% (w/v) Ampholine (Sigma) of the pH range 3.5 - 5.0, was layered on to a gel bond film of 7.5 cm X 7.5 cm and cooled to solidify. Filter paper (Whatman, Chromotography paper, 17 Chr.) strips dipped in anolyte (0.05M H₂SO₄ pH 1.5) and catholyte (0.4 M HEPES, pH 5.2) solutions were placed at either sides of the gel at the respective electrode ends. Platinum wire electrodes were placed directly in contact with filter paper strips and prefocussing was carried out at 12 mA current for 30 min. A trough of the size 0.5 cm X 6.5 cm was cut out 5mm from the cathodal end and 800 µl of sample containing 10-12 mg protein of the lysozyme papain digested sonicate was added in it. Electrofocussing was carried out at constant power 3.0 watts keeping maximum current at 12 mA and voltage at 1000 V for 2-2.5h, after which current was allowed to fall gradually to zero. At this point the electrophoresis was deemed complete. The entire electrofocussing was carried out at 4°C. Several precipitin bands, likely to be of proteins precipitating at their isoelectric points were observed. The pI of the precipitin bands was checked by a surface electrode (LKB, Pharmacia) and pH gradient formation was also confirmed. The gel portion containing 65 kDa antigen band at pI 4.3 (186,187) was cut and taken into a glass tube to which 1.0 ml of PBS - Tween 80 (PBS - T80) was added and the gel was crushed into it to fine particle size. This was kept at 4°C overnight for elution of 65 kDa antigen in PBS - T 80 and next day centrifuged at 10,000 g for 30 min at 4°C. The supernatant containing purified antigen was collected and used for various tests.

iii) Preparation of 65 kDa antigen bearing nitrocellulose membrane (NCM) particles :

The lysozyme-papain digested sonicate was subjected to preparative agarose IEF. The focussed agarose gel was then blotted onto a nitrocellulose membrane (NCM, Sigma) employing Midget Multiblot Electrophoresis transfer unit (LKB, Pharmacia, Sweden).

Sheets of thin (Whatman no.1) and thick (Whatman no. 17 chr.) filter paper of 7.5 cm X 7.5 cm were cut and soaked along with the synthetic sponges in the Tris-glycine transfer buffer (Append). The focussed 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 to expose the underside. A nitrocellulose membrane of the size same as the agarose gel (7.5 cm X 7.5 cm) was cut and placed over the inverted side of the gel slowly without entrapping air bubbles. A sheet each of thin and thick filter paper were placed on top of the NCM and gently pressed. Then on either side of this assembly, one each of thin and thick sheets of 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 3/4th 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 4.0 watts keeping the Vmax 215 for 2 -2.5 h. At the end of the run the NCM was washed 3 times with PBS - Tween 20 (Append I) for 5 min each time. A strip of 1 cm width was cut along the direction of the run and stained with India Ink to mark the position of 65 kDa antigen band. Then the 65 kDa antigen band blotted on NCM was solublized by the method of Abou Zeid *et al* (188) as described below. The portion of NCM containing 65 kDa antigen band was cut and its area measured. This strip was cut into small pieces and taken into a test tube to which DMSO (Dimethyl sulfoxide, Sigma) was added in the proportion of 250 μ l / 20 mm² area of NCM and allowed to dissolve for 1 h at room temperature. The suspension was mixed on a vortex mixer to ensure complete dissolution. Then the dissolved NCM particles were reprecipitated by addition of carbonate buffer (Append I) (250 μ l / 20 mm² area of NCM) and centrifuged at 10,000 g 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 *M. vaccae*. 100 μ l of the optimum dilution of the suspension was employed in all the assays.

5. Delayed type hypersensitivity (DTH) test :

DTH test was carried out by the method of Grey and Jennings (189). A 0.05 ml volume of *M. vaccae* sonicate containing 50 µg of protein was injected into the hind footpad and measurements of footpad thickness were taken before, 24 h and 48 h after injection. A set of non-immune naive mice of the same strain were also similarly tested. The results were expressed as corrected footpad enlargement (CPE), which is the difference between the footpad enlargements caused by the test sonicate in the immunised and control mice, simultaneously tested. The results were assayed by Student's 't' test, by comparing CPE values with zero.

Similarly the DTH response to purified 65 kDa antigen of *M. vaccae* was tested. The 65 kDa antigen was purified as described earlier. The precipitin bands containing purified antigen from three gels were pooled and the protein eluted in 1.0 ml of PBS-T20 (PBS Tween 20) as described earlier. 0.05 ml solution containing 50 µg of purified antigen protein was injected into hind footpad of the immunised mice showing significant DTH response to *M. vaccae* sonicate. The DTH response of these animals to purified 65 kDa antigen of *M. vaccae* was assayed.

6. Lymphoproliferation assay :

This assay was carried out employing standard microculture technique (190). 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). The cell suspension was spun at 400 g for 10 min to pellet the cells. The pellet was suspended in wash medium, approximately 3 ml for each spleen. 8 ml of this cell suspension containing around 5×10^7 cells was layered over 4 ml of Ficoll-Hypaque (Density - 1.083 ± 0.001 , Sigma) and then centrifuged at 400 g at room temperature (RT) for 20 min. The junctional buffy layer of mononuclear cells was gently aspirated with pasteur pipette and washed thrice with cold wash medium. Its viability was estimated by trypan blue exclusion test (191) and the viable cell count taken. The suspension was then centrifuged at 400 g for 10 min at 4°C and the pellet suspended at 2×10^6 cells/ml in culture medium, RPMI 1640 containing 10% foetal calf serum (FCS, Biological Laboratories, Israel), 2.0 mM glutamine (Sigma) and 1% Antibiotic Antimycotic (AA)

(Sigma). Each well of the microculture plate (Nunc, Denmark) received 2×10^5 cells and 20 $\mu\text{g/ml}$ of *M. vaccae* sonicate in the final volume of 0.2 ml/well. The culture medium of control wells did not contain antigen. All test and control cultures were set up in quadriplates. 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. 2 Ci/mM) for a period of 16 h. They were then harvested on 3 MM Whatman filter paper discs of 20 mm diameter and the latter were air dried. The dried discs were then transferred to 10% trichloroacetic acid (TCA) for 5 min followed by three washes for 2 min each with distilled water and finally a methanol wash for 5 min. The discs were then dried at 50°C . The dried discs were transferred into scintillation vials containing 5 ml scintillation fluid (Append I). Counts were taken in a scintillation counter (LKB) and results were expressed as counts per minute (cpm).

7. Separation of T cells :

A spleen cell suspension was made as described above. The mononuclear cells separated and the cell suspension was loaded onto a preactivated nylon wool column and non adherent cells eluted by the method of Julius *et al* (192) as described below. Nylon wool (Leukopak, Fenwel Lab, U.S.A.) was soaked in running tap water for 20 h. It was subsequently soaked in double distilled water for 2 days at room temperature with frequent changes. The washed wool was teased and soaked in RPMI 1640 with 5% FCS and 1% AA at 37°C for 16 h with three changes. It was then 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) nylon wool was packed. The syringe with the wool was sterilized by autoclaving and later held vertically under sterile conditions. It was then gently washed with ten volumes of medium containing 10% FCS and 1% AA and kept soaked in the same at 37°C for 30 min. 1.0 ml suspension of 5×10^7 lymphoid cells at 37°C were overlayed gently on the column and allowed to enter into the wool. The cell suspension was incubated at 37°C along with wool for 30 min. After incubation was over, culture medium at 37°C was delivered on top of the wool and the column eluted very slowly in 2-3 bed volumes of culture medium at the rate of 6-10 drops a minute. The non-adherent cells were collected, chilled, washed once, then suspended in the culture medium and viability estimated by trypan blue exclusion.

8. Preparation of peritoneal cell suspension :

The mice were given an intraperitoneal injection of 5.0 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, the cells were washed in culture medium once and viability estimated.

9. Irradiation of spleen and peritoneal cells for use as antigen presenting cells (APCs);

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

10. Antigen pulsing of peritoneal cells :

10^6 peritoneal cells were suspended in 2.0 ml of the culture medium containing 10% FCS and 200 μg of sonicate of *M. vaccae*. The cell suspension was delivered into 16 mm wells of a plastic culture plate (Nunc, Denmark) and incubated in a humidified incubator at 37°C with 5% CO_2 for different time periods as required. The cells were harvested and washed thrice and final cell pellet suspended in the culture medium.

11. Paraformaldehyde fixation of peritoneal cells :

This was done by the method described by Allen and Unanue (193). The antigen pulsed or nonpulsed 10^6 peritoneal cells were suspended in 2.0 ml of culture medium containing 1% (w/v) paraformaldehyde (analytical grade, Sigma) and incubated at 20°C for 15 min. At the end of the incubation period, they were washed three times in wash medium, incubated again at 37°C in the culture medium for 30 min and again washed three times.

12. Rabbit antibody against 65 kDa antigen of *M. vaccae* ;

The lysozyme-papain digested *M. vaccae* sonicate was subjected to preparative agarose IEF as described earlier and the visible precipitin band at pI 4.3 was cut. The bands from three such gels were pooled together and crushed in a glass tube with a

glass piston to fine particle size. To this mixture (approx. 2.0 ml), 2.0 ml of Freund's Incomplete Adjuvant (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 intradermally at multiple sites on the back. The immunisation was repeated twice at 10 days interval. 7 days after the last immunisation the rabbit was test bled from ear artery, blood collected and the serum was separated. The antiserum was tested against *M. vaccae* sonicate and 65 kDa antigen by agarose gel diffusion method of Ouchterlony (194) and the antibody content 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 1.0 ml aliquotes and stored at -20°C. IgG fraction was obtained from this antiserum by ammonium sulphate precipitation (Append II) (195).

13. Assay for 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 anti-mouse IgG specific peroxidase conjugate.

Reagents :

1. Coating antibody - IgG fraction of rabbit antiserum against 65 kDa antigen of *M. vaccae* in coating buffer (Append I).
2. Source of 65 kDa antigen - lysozyme papain digested *M. vaccae* sonicate in PBS.
3. Positive control serum - serum of mice immunised with 65 kDa antigen of *M. vaccae* in Freund's Incomplete Adjuvant given three times at one week interval by the intradermal route.

4. Anti- 65 kDa antibody containing known standard mouse antiserum - in our initial assays for standardisation, serum of mice given three 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.
5. Normal mouse serum.
6. Anti-mouse IgG specific HRP conjugate (Sigma) - affinity isolated goat anti-mouse IgG (heavy chain specific) antibody coupled to horse raddish peroxidase (Append II).
7. Substrate solution - TMB- 1 mg / 10 ml, H_2O_2 - 2 μ l / 10 ml (Append I).
8. Dilution buffer - PBS- T 20 (Append I).
9. Washing solution - saline- T 20 (Append I).
10. Coating buffer - Carbonate - bicarbonate buffer, pH 9.6 (Append I).

Procedure :

1. Wells of Elisa plates (12 X 8 plates - Nunc, Denmark) received 100 μ l of IgG fraction (100 μ g/ml) of rabbit antiserum against 65 kDa antigen. The plates were incubated overnight at 4°C.
2. Next day, the well contents were discarded and well surfaces were blocked with 2% gelatin in coating buffer at 37°C for 1-1.5 h.
3. The plates were washed 5-6 times with washing solution, Saline T 20 on an Elisa washer (Organon Teknika, Miniwasher) and 100 μ l of lysozyme papain digested *M. vaccae* sonicate (1 mg/ml) was added to the wells and incubated at 37°C for 2 h.
4. The wells were then washed 5-6 times and 100 μ l 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 1-1.5 h.
5. The plate was washed 5-6 times and 100 μ l of anti-mouse IgG HRP conjugate (1:10,000 dilution) was added to the wells and incubated for 1 h at 37°C.

6. The plate was then 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 2M H₂SO₄ and read at 450 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 standard mouse serum having reasonable amount of anti- 65 kDa antibody. Thus the results of antibody titres of serum samples done on different occasions were normalized with respect to the same standard.

$$\text{Antibody titre (Abs units/ml)} = \frac{A_{450} \text{ of test serum sample}}{A_{450} \text{ of standard mouse serum}} \times \text{dilution of test serum sample} \times 10.$$

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

14. Lymphokine Elisa :

Lymphokine levels in the culture supernatants were assayed by Elisa, employing commercial Elisa kits.

14a. IL-4 and IL-10 Elisa : These assays were performed employing kits purchased from M/s. Pharmingen, USA.

Design of the assay :

Wells of Elisa plate were coated with rat anti-mouse IL-4 / IL-10 mAb to which standard recombinant IL-4 / IL-10 (or test culture supernatants) were added. The levels of IL-4 / IL-10 were assayed by exposure to biotinylated rat (different specificity) anti-mouse IL-4 / IL-10 mAb followed by streptavidin HRP conjugate.

Reagents :

1. Capture antibody :

1a. Purified monoclonal rat anti-mouse IL-4 antibody (clone : BVD4 - 1D11)

1b. Purified monoclonal rat anti-mouse IL-10 antibody (clone : JES5 - 2A5)

10 µl of capture antibody solution containing 10 µg of antibody protein was diluted to 1.5 ml with coating buffer so as to get a concentration of 6.6 µg/ml.

2a. Lyophilised recombinant murine IL-4.

2b. Lyophilised recombinant murine IL-10.

The lyophilised powder was reconstituted with sterile distilled water to get 100 ng/µl concentration of the lymphokine. This was further diluted with PBS -T 20 containing 3% BSA to 20 ng/µl. This was again diluted with Conjugate Stabilizer solution (Pierce, USA) to give a range of concentrations from 1 to 100 ng/ml. Aliquotes of ready to use standards having the above ranges of concentration were stored at -20°C.

3a. Biotinylated rat anti-mouse IL-4 monoclonal antibody (clone : BVD6 - 24G2)

3b. Biotinylated rat anti-mouse IL-10 monoclonal antibody (Clone : SXC - 1).

10 µl containing 10 µg of antibody was diluted to 5 µg/ml with PBS -T 20 containing 3% BSA.

4. Streptavidin HRP conjugate :

2 mg of protein was reconstituted with 2.0 ml of PBS -T 20 which was further diluted to 10.0 ml with Conjugate Stabilizer (Pierce, USA). The final concentration of the solution was 200 µg/ml. This was aliquoted and stored at -20°C. When required this was diluted to 0.2 µg/ml.

5. Conjugate Stabilizer solution (for use with peroxidase label) (Pierce, USA) :

This was used for making final dilutions of conjugate and recombinant lymphokines so that the protein concentration did not fall on freeze-thawing of the aliquotes.

6. Superblock Blocking buffer in PBS (Pierce, USA) :

This reagent was used for blocking the surfaces of Elisa plate wells.

7. Substrate solution :

OPD - 4 mg / 10 ml, H₂O₂ - 2 µl / 10 ml (Append I).

Procedure :

50 µl of capture antibody (6.6 µg/ml) was delivered in each well of the Elisa plate (Maxisorp, Nunc, Denmark) and incubated overnight at 4°C. Next day, the plate was washed twice with PBS -T 20 and uncoated well surfaces were blocked with Superblock Blocking buffer in PBS for 1 h at room temperature. The plate was washed twice. 100 µl of various dilutions of standard recombinant IL-4 / IL-10 as well as culture supernates were added to respective wells and incubated at room temperature for 4 h. After the incubation period was over, the plate was washed 4 times and 100 µl of biotinylated anti-mouse IL-4 / IL-10 were added to each well and incubated at room temperature for 45 min. The plate was washed 6 times and 100 µl of streptavidin HRP conjugate was added to all the wells. The plate was incubated at room temperature for 30 min and then washed 8 times. 100 µl of the substrate solution was added to each well and incubated in the dark for 15 min at room temperature for colour development. The reaction was stopped with 2.5 M HCl and read at 492 nm.

Calculations :

Cut off absorbance value :

(mean absorbance of -ve control + 2 standard deviations) was employed as the cut off value.

$$\text{Amount of lymphokine} = \frac{A_{492} \text{ of sample}}{A_{492} \text{ of standard IL-4 / IL-10}} \times \text{Volume of culture well (ml)} \times \text{Concentration of lymphokine standard (pg/ml)} \times 2$$

The lymphokine levels were expressed as pg / 10⁶ T cells cultured.

14b. IL-2 Elisa :

This assay was performed employing kits purchased from M/s. Genzyme, USA.

Design of the assay :

Ready to use microtitre plates coated with anti-mouse IL-2 monoclonal antibody

were provided. Different dilutions of standard recombinant mouse IL-2 or culture supernatants were added to them. The IL-2 levels were detected by addition of biotinylated polyclonal anti-mouse IL-2 followed by streptavidin-peroxidase conjugate.

Procedure :

The assay was carried out as per the instructions given in the 'Instruction Booklet' provided along with the kit.

Calculations :

Same as described for IL-4 / IL-10 Elisa.

14c. γ -IFN Elisa :

This assay was performed employing kits purchased from M/s. Genzyme, USA.

Design of the assay :

The wells of Elisa plate were coated with hamster monoclonal antibody specific for mouse γ -IFN which captured the γ -IFN present in culture supernates or the standard recombinant mouse γ -IFN. The captured γ -IFN was detected by addition of polyclonal goat anti-mouse γ -IFN antibody followed by polyclonal donkey anti-goat Ig peroxidase conjugate.

Procedure :

The assay was carried out as per the instructions given in the 'Instruction Booklet' provided along with the kit.

Calculations :

Same as described for IL-4 / IL-10 Elisa.

15. Flow cytometry :

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, USA.

Stains used :

1) Phycoerythrin (PE) labelled anti-mouse B7 (PE-B7) :

This reagent was employed for assay of B7 expressing cells.

2) PE labelled anti-mouse IgM (PE-IgM) :

This reagent was employed for assay of IgM expressing B cells.

3) Fluorescein labelled anti-mouse kappa (Flu-kappa) :

This reagent was employed for assay of B cells.

4) Biotinylated anti-mouse IgD (Bio-IgD) :

This reagent was employed for assay of IgD expressing B cells. A second staining step with fluorochrome coupled avidin was required in this assay.

5) Fluorescein labelled avidin (Flu-avidin).

These reagents were purchased from M/s. Pharmingen, USA.

Staining Procedure :

The dilution of each antibody conjugate to be employed was standardised by using cells known to express the particular marker. Peritoneal cells harvested from treated or untreated mice were washed thrice in Hank's balanced salt solution (HBSS) and 200 μ l of 10^6 cells/ml cell suspension was delivered in each well of a microtitre plate. The cells were pelleted by centrifugation at 300 g for 10 min and the supernatant discarded. The cell pellet containing 10^6 cells was resuspended in 50 μ l of a mixture of two fluorochrome labelled antibodies, at appropriate dilutions for two colour analysis. The cells were incubated at 4°C for 1 h. After the incubation, the cells were washed once with PBS containing 1% FCS and 0.2% sodium azide and resuspended in 50 μ l of the same fluid. This was subjected to flow cytometric analysis. With each staining procedure control samples were incubated with washing buffer. When the analysis could not be done immediately the stained cells were fixed in 0.1% paraformaldehyde in PBS which was washed off before flow cytometry.

Data analysis :

Data were collected from 10,000 to 20,000 cells from each sample and stored in the list mode. The analysis was based on gated cell population varying from 1500-2000.

16. 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 by Ding *et al* (1996). 100 μl of culture supernatants were incubated in duplicates with equal amount of Griess reagent (1% sulphanylamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4) at room temperature for 10 min. For Calculation of nitrite concentration sodium nitrite was employed as a standard. The absorbance readings were taken at 550 nm with a microplate Elisa reader (Titretek Multiskan, Flow).

RESULTS

TABLE 1

Effector T helper responses of mice immunised with *M. vaccae* by i.d. or i.p. route

No.	Route of Immun.	DTH		Titre	
		Mean CPE $\pm$ S.E. (mm)		Anti-65-Kda antibody titre Mean AU / ml $\pm$ S.E.	IgG1 IgG2a Ratio
		Sonicate	65-kDa Ag		
1.	i.d.	0.36 $\pm$ 0.01	0.35 $\pm$ 0.02	501 $\pm$ 39	1.3
2.	i.p.	0.08 $\pm$ 0.03	0.04 $\pm$ 0.01	3339 $\pm$ 209	2.0

Balb/c mice, two groups of 50 each were immunised with 10^8 killed *M. vaccae* by i.d. or i.p. route. One week later DTH test was done on each of 10 randomly selected mice immunised by the two routes. The mice were bled from retro-orbital plexus, blood samples of 5 identically immunised mice were pooled into 10 pools, each of i.d. and i.p. immunised mice respectively. Elisa tests for anti-65 kDa antibody titre was performed with anti-mouse IgG conjugate on each pool and results expressed as AU / ml. Each pool was also tested for anti-65-kDa IgG1 and IgG2a antibodies, employing anti-mouse IgG1 and anti-mouse IgG2a conjugates. The relative amounts of IgG1 and IgG2a were expressed as ratio of AU / ml obtained with the two conjugates.

CPE = Corrected foot pad enlargement

AU = Absorbance Units

SE = Standard Error

1. DTH, lymphoproliferative and antibody responses against 65-kDa antigen of *M. vaccae* following i.d. and i.p. immunisation with *M. vaccae*.

Balb/c mice were immunised by i.d. or i.p. route with *M. vaccae* vaccine containing 10^8 killed bacterial cells. One week later, the DTH responses and serum antibody levels against the 65 kDa antigen of *M. vaccae* as well as IgG1: IgG2a ratio of the antibody were assayed in these animals.

The i.p. immunisation generated a poor DTH response whereas the i.d. immunisation generated an excellent DTH response. The anti-65 kDa antibody responses of the i.p. primed mice were almost 5-7 fold higher (mean titre - 3339 ± 229 AU/ml) than those of the responses of i.d. immunised mice (mean titre - 501 ± 39 AU/ml). Even though it was a primary immunisation, the amount of IgG1 antibody was higher than IgG2a antibody in both the i.d. and i.p. primed mice. However, the IgG1 component in the sera of i.p. primed mice was much greater than that of i.d. primed mice (Table 1).

This was considered as a preliminary evidence in support of different outcomes of T helper (Th) responses after i.p. and i.d. immunisation respectively.

It is difficult to visualise development of an immune response only in spleen following i.d. or i.p. immunisation. It is logical to expect development of primary responses in regional lymphnodes draining the site of immunisation. The responses observed in spleen could be due to spill over of antigen or APC with antigen into systemic circulation. Alternately, the responses observed in spleen could be due to antigen specific T and B cell clones, migrating to spleen, from the draining lymphnodes after they are primed by the antigen at that site.

Therefore CD4⁺ Th cell responses of - 1) dermal lymphnodes draining the site of i.d. immunisation. 2) mesenteric lymphnodes of i.p. immunised mice and 3) spleens of both i.d. and i.p. immunised mice were compared.

2. CD4 + Th cell responses of dermal lymphnodes, mesenteric lymphnodes and spleens of mice immunised with *M. vaccae* by i.d or i.p. route.

Balb/c mice were immunised with 10^8 killed *M. vaccae* by the i.p. or i.d. route. The i.d. immunisation was given in the lower lateral flanks drained by the inguinal

TABLE 2

Lymphoproliferative responses of DLN, Spleen and MLN cells of mice primed with *M. vaccae* by i.d. or i.p. routes

Route of Immun.	Mean CPM $\pm$ S.E.					
	Spleen		SI	DLN / MLN		
	- Ag	+ Ag		- Ag	+ Ag	SI
i.d.	2,205 $\pm$ 645	27,519 $\pm$ 4940	12.5	840 $\pm$ 133	16,868 $\pm$ 2667	20.1
i.d. ^{a)}	2,874 $\pm$ 415	21,273 $\pm$ 372	7.4	ND	ND	-
i.d. ^{b)}	2,874 $\pm$ 415	20,963 $\pm$ 1060	7.3	ND	ND	-
i.p.	1,703 $\pm$ 108	5,311 $\pm$ 584	3.1	6,351 $\pm$ 1858	22,342 $\pm$ 3783	3.5
i.p. ^{b)}	1,475 $\pm$ 119	3,910 $\pm$ 502	2.7	ND	ND	-

Balb/c mice (10 per group) were immunised with *M. vaccae* by i.p. or i.d. route with 10^8 killed *M. vaccae*. One week later, dermal inguinal lymph nodes (DLN) of i.d. immunised mice, mesenteric lymph nodes (MLN) of i.p. immunised mice and spleens of both were removed, single cell suspension made and cultured with *M. vaccae* sonicate/ 65-kDa antigen and a lymphoproliferation assay performed.

- a) As antigen a suspension of NCM adsorbed *M. vaccae* sonicate was employed
- b) As antigen a suspension of NCM adsorbed 65-kDa antigen of *M. vaccae* was employed.

lymphnodes. The mice were tested for DTH responses one week later, sacrificed and their spleens, inguinal and mesenteric lymphnodes removed by aseptic technique. Single cell suspensions of the dissected lymphoid tissues were made, loaded on Ficoll-Hypaque gradient to obtain lymphoid cells and passed over nylon wool column to obtain primed T cells from various tissues. A set of whole spleen and lymphnode, cells of the animals primed by different routes was also set aside.

2×10^5 dermal, mesenteric and spleen cells of i.d. or i.p. primed mice were cultured with *M. vaccae* sonicate / 65-kDa antigen for a 6 day standard antigen specific lymphoproliferation assay.

5×10^5 primed T lymphocytes obtained from different tissues were cultured with equal number of normal irradiated cells coming from the same tissue to serve as APCs and *M. vaccae* sonicate as antigen. The culture supernatants were harvested after 36h and assayed for IL-2, IL-4, IL-10 and γ -IFN by Elisa.

2a. CD4⁺ T Cell responses of i.d. primed mice :

Both the dermal lymphnode and spleen cells of i.d. immunised mice showed an excellent *in-vitro* proliferative response on stimulation with antigen (Table 2). When the lymphokine profiles of dermal lymphnode and splenic T cells were compared, it was observed that the IL-2 levels were comparable and quite low (Table 3). Dermal lymphnode T cells secreted marginally higher levels of IL-4. In contrast, IL-10 levels secreted by splenic T cells were considerably higher in comparison with those of dermal lymphnode T cells. Similarly γ -IFN levels of splenic T cells were also quite high (Table 2 and 3)

Since IL-2 is an autocrine growth factor for CD4⁺ T cells and since both splenic and dermal lymphnode T cells showed excellent proliferation on antigenic stimulation, it is logical to expect the proliferating cells to utilize the secreted IL-2 for growth. Therefore, levels of IL-2 seen in the culture supernatants were post-utilisation. Based on these arguments, it was concluded that both splenic and dermal lymphnode T cells of i.d. primed mice secreted significant amounts of IL-2 on antigenic stimulation.

The IL-4 levels of the two T cell sources of i.d. primed mice were marginally

TABLE 3

Lymphokines generated by *in-vivo* primed splenic T cells on antigenic stimulation

Source of T cells	Lymphokine (pg / 10 ⁶ cells) $\pm$ S.D							
	IL - 2		IL - 4		IL - 10		γ - IFN	
	- Ag	$\pm$ Ag	- Ag	+ Ag	- Ag	+ Ag	- Ag	+ Ag
Spleens of i.d. primed mice	- ve	22 $\pm$ 1.06	- ve	38 $\pm$ 0.7	- ve	766 $\pm$ 86	288 $\pm$ 5.0	> 690
Inguinal lymphnodes of i.d. primed mice	11 $\pm$ 0.5	21 $\pm$ 2.7	- ve	66 $\pm$ 5.3	- ve	189 $\pm$ 18.4	ND	ND
Spleens of i.p. primed mice	- ve	20 $\pm$ 0.5	- ve	35 $\pm$ 0.7	996 $\pm$ 196	1304 $\pm$ 384	226 $\pm$ 9.2	> 690
Mesenteric lymphnodes of i.p. primed mice	69 $\pm$ 11.3	28 $\pm$ 1.06	- ve	-ve	- ve	196 $\pm$ 10.0	ND	ND

Balb/c mice were immunised with 10⁸ killed *M. vaccae* by i.d. or i.p. route. One week later, the spleens and inguinal lymphnodes from i.d. primed mice as well as spleens and mesenteric lymphnodes of i.p. primed mice were removed, single cell suspensions made and T cells obtained. 5 x 10⁵ primed T cells of each lymphoid tissue were mixed with equal number of irradiated normal non-immune cells of the same tissue to serve as APCs and stimulated with *M. vaccae* sonicate. The cells were cultured for 36h, supernatants harvested and assayed for IL-2, IL-4, IL-10 and γ -IFN.

S.D. = Standard Deviation

different. However, IL-10 levels in splenic T cell secretion were considerably higher than those of dermal lymphnode T cells. Based on this one could hypothesize that the size of the effector T cell pool was much higher in the spleens than in the draining lymphnodes. Splenic effectors secreted IL-4, IL-10 and γ -IFN i.e. both Th1 and Th2 lymphokines were present in the secretions. Therefore, it was concluded that splenic effectors of i.d. primed mice were of Th0 type or the effector T cell pool consisted of a mixture of all the three types of T cell subsets namely Th0, Th1 and Th2.

Antigen induced proliferation is a classical feature of either precursor or memory CD4⁺ T cells, because of their ability to secrete large amount of IL-2. Therefore a good proliferative response of dermal lymphnode and splenic T cells of i.d. primed mice indicates generation of a good memory response after i.d. immunisation.

2b. CD4⁺ T cell responses of i.p. primed mice :

The mesenteric lymphnode and spleen cells of i.p. primed mice also showed an antigen specific proliferative response, though it was quite poor in comparison with that of dermal lymphnode and spleen cells of i.d. primed mice (Table 2).

The IL-2 levels in the supernatant of i.p. primed splenic T cells were quite low, and comparable to those of i.d. primed mice (Table 3). However, the spleen cells of i.d. primed mice showed an excellent antigen induced proliferation whereas the proliferative response of i.p. primed spleen cells was poor. Based on this, it was concluded that IL-2 secretion by the i.p. primed splenic T cells was poor. The mesenteric node T cells of i.p. primed mice, showed considerable IL-2 secretion, even without *in-vitro* restimulation with antigen (Table 3). Further, on antigenic stimulation, IL-2 levels showed a decline. Also, the lymphoproliferative responses of i.p. primed mesenteric lymphnode cells showed a comparable trend. The spontaneous basal proliferation of the cells (in the absence of antigenic stimulation) was quite high and antigenic stimulation did not bring about the same degree of enhancement in proliferation that was seen in the cultures of i.d. primed spleen and dermal lymphnode cells. Based on these, it was concluded that memory T cell response after i.p. immunisation was poor.

The mesenteric node T cells of i.p. primed mice did not show any IL-4 secretion, though their splenic T cells showed levels comparable to those of i.d. primed mice.

TABLE 4

Effect of i.p. primed spleen cells on DTH and anti-65-kDa antibody responses to i.d. immunisation with *M. vaccae*

No.	Nature of cells received before i.d. immunisation	DTH CPE $\pm$ S.E mm	Anti-65-kDa antibody titre AU/ml $\pm$ S.E.
1.	None	0.26 $\pm$ 0.02	501 $\pm$ 39
2.	i.p. primed cells	0.15 $\pm$ 0.02	80 $\pm$ 3

Balb/c mice were immunised by i.p. route. One week later, their splenic lymphocytes were harvested and transferred into syngeneic mice. 24h after the transfer the recipients were immunised by i.d. route. One week after the i.d. immunisation these animals were tested for DTH and anti-65-kDa antibody responses.

IL-10 levels of mesenteric node T cells were comparable to those of i.d. primed dermal lymphnode T cells. The splenic T cells of i.p. primed mice secreted much higher levels of IL-10 than the mesenteric node T cells. Thus again, the spleens appeared to have a much larger effector T cell pool as compared to mesenteric nodes (Table 3 and 4).

3. Comparison of proliferative and lymphokine responses of spleen cells of i.d. and i.p. primed mice respectively :

Comparison of lymphokine profiles of i.d. and i.p. primed splenic T cells showed some interesting features. IL-2 levels were comparable, but as pointed out earlier i.d. primed spleen cells showed an excellent proliferative response whereas i.p. primed spleen cells did not. The IL-2 being an autocrine growth factor, the observed IL-2 levels in the supernatants of antigen stimulated i.d. primed spleen cells reflect the surplus IL-2, remaining after utilisation for proliferation. Comparable levels of IL-2 were observed in the antigen stimulated cultures of i.p. primed splenic T cells, even though the cells did not proliferate. Therefore it was concluded that the i.d. primed spleen cells generated much larger amounts of IL-2 than i.p. primed spleen cells.

The splenic T cells of both i.d. and i.p. primed mice secreted IL-4, IL-10 and γ -IFN. IL-4 levels were comparable and low, but IL-10 levels of i.p. primed spleen cell cultures were significantly higher. γ -IFN levels were not titrated till the end point; therefore it is not certain if the levels of this cytokine were truly comparable. Splenic CD4⁺ T effectors, of i.p. primed mice also were either Th0 type or they consisted of a mixture of three types of Th cell subsets namely Th0, Th1 and Th2. The i.p. immunisation seems to favour a larger Th2 component; because the splenic CD4⁺ T cells of i.p. primed mice secreted significantly higher levels of IL-10 in comparison with those of i.d. primed spleen cells.

Thus i.d. immunisation seems to induce memory and effector Th responses favouring a larger Th1 component; this is based upon the ability of the mice to mount a DTH response, low antibody titre and the lymphnode and spleen cells showing excellent lymphoproliferation on *in-vitro* stimulation with antigen. The i.p. immunisation in contrast, generated a poor memory response and an effector Th response favouring a larger Th2 component. This is based upon the observations that the mice failed to mount

a DTH response, their antibody titres against a major T epitope, i.e. 65 kDa antigen were significantly higher along with larger IgG1 : IgG2a ratio and their splenic effectors secreted significantly higher levels of IL-10. Both the splenic and mesenteric lymphnode cells showed a poor *in-vitro* proliferative response to antigen.

It has been reported earlier by other investigators of our group that i.p. immunisation expands CD8+ T cells, recognising the 65 kDa antigen of *M. vaccae*. The primed CD8+ T cells have been shown to suppress the generation of DTH and lymphoproliferative response of the animals (180). The primed CD8+ T cells were also shown to compete with CD4+ T cells for IL-2 (197). It can be hypothesized that absorption of IL-2 by the primed CD8+ T cells, may result into relative increase in the levels of IL-4 in the milieu of lymphoid tissue of the i.p. primed animals, driving the differentiation of CD4+ T cells to Th2 pathway. This issue was investigated.

4. Role of CD8+ T cells in Th cell subset differentiation after i.p. immunisation :

Balb/c mice were immunised by i.p. route. One week later their splenic lymphocytes were harvested and transferred into syngeneic mice. 24 h after the transfer, the recipients were immunised by i.d. route with *M. vaccae*. One week later, the DTH, lymphoproliferative response and anti 65- kDa antibody responses of the above animals were assayed. Similar responses of a control set of animals immunised as usual by i.d. route were also assayed.

The i.d. primed mice showed a good DTH response and low antibody response as before. In the contrast, the mice receiving i.p. primed cells prior to i.d. immunisation showed a poor DTH response and their antibody titres were still lower (Table 4). Hence, it was concluded that CD8 + T cells which were concomittently primed on i.p. immunisation were not responsible for driving the differentiation of Th cells to Th2 pathway.

Therefore, the differences in the outcomes of immunisation by i.d. and i.p. routes could most likely be due to differences in processing and presentation of antigen in the lymphoid tissues encountering the antigen delivered by the two routes. Lymphoid tissues where the primary immune responses would develop when the antigen is introduced as per the described protocols, would be the inguinal (dermal) and mesenteric lymphnodes.

TABLE 5

Comparison of antigen presentation by spleen, MLN, DLN and peritoneal cells in an *in-vitro* lymphoproliferation assay

No.	Antigen presenting cells	Mean CPM $\pm$ S.E.	S.I.
1.	Dermal lymph node cells	5,751 $\pm$ 715	15.7
2.	Mesenteric lymph node cells	8,162 $\pm$ 1985	19.1
3.	Spleen cells	7,508 $\pm$ 1161	11.4
4.	Peritoneal cells	1,523 $\pm$ 84	1.3

Single cell suspensions of the dermal inguinal (DLN) and mesenteric lymph nodes (MLN) and spleens from normal naive Balb/c mice were prepared. Peritoneal cells (PC) were also harvested from normal naive Balb/c mice. These cell suspensions were irradiated. A separate set of Balb/c mice were immunised by i.d. route with *M. vaccae*, sacrificed one week later, spleens harvested and splenic T cells obtained. 10^5 primed T cells were mixed with equal number of each of the above cells as APCs and *M. vaccae* sonicate as antigen and a lymphoproliferative assay performed.

There is always the question of spill over of antigen into circulation, in which case the response may also develop in the spleen. Therefore antigen processing and presentation by irradiated cells from dermal and mesenteric lymphnodes and spleens of normal animals was investigated. As a single source of primed T cells, T cells from spleens of mice immunised with *M. vaccae* by i.d. routes were employed. The i.d. primed splenic T cells were cultured with the above irradiated lymphoid cells and *M. vaccae* sonicate for 5 days to generate T effectors *in-vitro*. These were restimulated with the sonicate to induce cytokine secretion which was assayed 48 h later i.e. on day 7. A parallel set of cultures was processed for *in-vitro* antigen specific lymphoproliferation.

5. Comparison of lymphoproliferative and T effector responses generated *in-vitro* from i.d. primed splenic T cells induced by antigen presented by APCs from spleen, dermal or mesenteric lymphnodes of normal mice :

Balb/c mice were primed with *M. vaccae* by i.d. route; one week later the mice were sacrificed, their spleens harvested, single cell suspension made and passed over nylon wool column to obtain i.d. primed splenic T cells.

5×10^5 i.d. primed splenic T cells were mixed with equal number of irradiated normal spleen, dermal or mesenteric lymphnode cells as APCs and *M. vaccae* sonicate (20 $\mu\text{g/ml}$) as antigen and cultured for 5 days to generate effectors. These were restimulated with 20 $\mu\text{g/ml}$ of *M. vaccae* sonicate and 10^5 irradiated normal spleen cells were added to each culture. This was done to supplement the APCs, lost if any during the 5 days culture period. The culture supernatants were harvested 48 h later i.e. on day 7 and assayed for IL-2, IL-4 and IL-10.

All the three APC sources induced similar low amounts of IL-2 (Table 6). As pointed out earlier, the IL-2 being an autocrine growth factor, is utilised by the proliferating CD4⁺ T cells for their own growth. Therefore observed IL-2 levels would be post-utilisation for proliferation. As a consequence no conclusions can be drawn, without comparing these values of IL-2 with lymphoproliferation induced by the same source of APCs.

TABLE 6

Lymphokines secreted by T effectors generated *in-vitro* by different APCs when cultured with splenic T cells of i.d. primed mice.

Source of APCs	Lymphokine (pg / 10 ⁶ cells) $\pm$ S.D					
	IL - 2		IL - 4		IL - 10	
	- Ag	$\pm$ Ag	- Ag	+ Ag	- Ag	+ Ag
Spleen cells	10 $\pm$ 1.7	- ve	- ve	37 $\pm$ 3.5	- ve	628 $\pm$ 40
Peritoneal cells	10 $\pm$ 1.7	14 $\pm$ 1.2	- ve	- ve	- ve	755 $\pm$ 30
Inguinal lymphnode cells	10 $\pm$ 1.7	- ve	- ve	198 $\pm$ 0	- ve	175 $\pm$ 0.4
Mesenteric lymphnode Cells	10 $\pm$ 1.7	- ve	- ve	206 $\pm$ 3.9	- ve	168 $\pm$ 0

Balb/c mice were immunised with 108 killed *M. vaccae* by i.d. route. One week later the animals were sacrificed and the splenic T cells separated. 5 x 10⁵ i.d. primed splenic T cells were mixed with equal number of irradiated normal cells obtained from different tissues or peritoneal cells, to serve as APCs and cultured for 5 days with *M. vaccae* sonicate (20 μ g/ml) to generate effectors. These were stimulated with *M. vaccae* sonicate for 48h more. culture supernatants harvested on day 7 and assayed for IL-2, IL-4 and IL-10.

All the three lymphoid cell sources of APCs were capable of presenting antigen efficiently to induce excellent proliferative responses which did not differ from one another significantly (Table 5). Based on this it was concluded that all the three sources of APCs induced significant amount of IL-2 secretion. Proliferation and secretion of IL-2 being the property of memory CD4 + T cells, it was therefore concluded that all the three sources of APCs had the capacity to induce comparable memory CD4+ T cell responses.

Splenic APCs induced significantly higher levels of IL-10 than the APCs of dermal and mesenteric nodes. Based on this it was concluded that antigen presentation by spleen cells favours a greater effector differentiation than the APCs of mesenteric and dermal lymph nodes. Both dermal and mesenteric lymphnode APCs generated significantly higher levels of IL-4 than the splenic APCs. When IL-4 and IL-10 levels induced by splenic and lymphnode APCs are compared, one comes across a conflicting observation. Both IL-4 and IL-10 are supposed to be the Th2 or Th0 effector cytokines. Splenic APCs which induced significantly higher levels of IL-10 than lymphnode APCs, should have also generated higher levels of IL-4; however, findings were exactly the opposite (Table 6). In this context, it is interesting to note that i.p. primed mesenteric lymphnode effector cells (generated *in-vivo*) also did not show any IL-4 in the culture supernatant, whereas i.d. primed dermal lymphnode effectors showed significant amount of IL-4 (Table 3). This is despite the fact that i.p. priming generated significant Th2 component in the *in-vivo* response as judged by IL-10 levels of splenic T effectors of i.p. primed mice, and the DTH and anti-65 kDa antibody responses.

The data presented so far brings about following significant findings. Nature of CD4+ T cell response to a killed vaccine of *M. vaccae* depends upon route of immunisation. The i.d. immunisation generates a significant memory response whereas in the i.p. immunised mice memory response is poor. Effector responses to either route of immunisation, consisted of a pool of Th0, Th1 and Th2 type of cells. In i.d. immunised mice, the Th1 component was significantly larger whereas in the i.p. immunised mice, the Th2 component was significantly greater. These differences in the outcome of Th responses to immunisation by the i.d. and i.p. route could not be explained by differences in the antigen presenting function of the APCs of the draining lymphnodes namely the inguinal and mesenteric nodes. Therefore one has to look elsewhere for explanation.

Peritoneum contains a mixture of lymphoid cells, significant amongst them being the cells of monocyte / macrophage lineage and the B cells, which can present antigen. It is possible that peritoneal macrophages might process and present the antigen, when immunisation is done by the i.p. route. Therefore antigen presentation by the peritoneal cells (PC) could be the obscure factor, responsible for poor memory Th response and for driving the differentiation to Th2 pathway on i.p. immunisation. Therefore antigen presentation by the PC was investigated.

6. Lymphoproliferative and T effector responses generated *in-vitro* from i.d. primed splenic T cells induced by antigen presented by the APCs in peritoneal cells (PC) :

T cells from spleens of i.d primed Balb/c mice were prepared as described earlier. PC were obtained from normal non-immune Balb/c mice and given 800 rad gamma radiations.

5×10^5 i.d. primed splenic T cells were mixed with equal number of PC to serve as APCs and *M. vaccae* sonicate (20 $\mu\text{g/ml}$) and cultured for a period of 5 days to generate effectors. These were restimulated with 20 $\mu\text{g/ml}$ of *M. vaccae* sonicate and 10^5 irradiated normal spleen cells were added to each culture, to supplement the APCs, lost if any during the 5 day culture period. The culture supernatants were harvested 48h later on day 7 and IL-2, IL-4 and IL-10 were assayed.

A parallel set of cultures containing 10^5 i.d. primed splenic T cells and equal number of irradiated PC and *M. vaccae* sonicate (20 $\mu\text{g/ml}$) was also established for a standard 6 day lymphoproliferation assay.

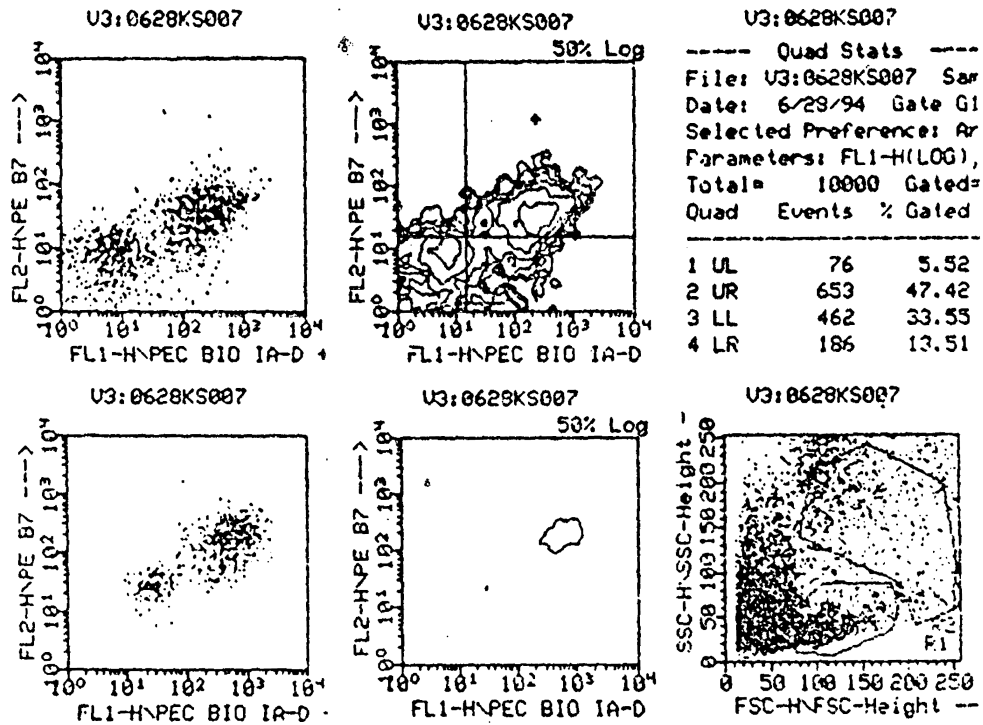
The supernatants of cultures revealed very low amounts of IL-2 (Table 6). Proliferative response of i.d. primed T cells induced by PC as APCs were also very poor (Table 5). Based on these findings it was concluded that, the PCs did not induce significant amount of IL-2 secretion nor memory CD4⁺ T cell responses.

The culture supernatants revealed no IL-4, IL-10 levels were very high and comparable to those induced by spleen cell APCs.

Thus based on *in-vitro* experiments of antigen presentation by the PCs, it was

FIGURE 1

MHC class II expression by peritoneal exudate cells



concluded that whereas PC functioned as APCs, they did not induce a significant memory response. Further, based on IL-10 levels induced by the PCs, it was concluded that the PCs favoured effector differentiation of the primed T cells and significant Th0/Th2 type of differentiation took place in the cultures. In these experiments, γ -IFN levels were not assayed. Therefore, it was not possible to establish unequivocally if the antigen presented by PCs favoured Th2 type of differentiation. Poor IL-2 secretion induced by PCs appears to support a Th2 differentiation.

Thus the above experiments, appear to suggest that when antigen is presented by PCs, one can expect to see the type of CD4⁺ Th responses encountered in i.p. primed mice, i.e. poor memory response and significant Th2 component in the effector responses. Therefore antigen presentation by the peritoneal cells was investigated in greater details.

Antigen presentation to CD4⁺ T cells is dependent upon expression of MHC class II and co-stimulatory molecules, particularly the CD28 ligand, B7/BB1 (198,199). Peritoneal cells contain a mixture of T cells, B cells and cells of monocyte / macrophage lineage. In order to investigate the reasons for the unique behaviour by the peritoneal cells, it was necessary to examine the expression of MHC Class II and B7/BB1 by the latter two cell populations in the peritoneal aspirates.

7. MHC class II expression by peritoneal exudate cells :

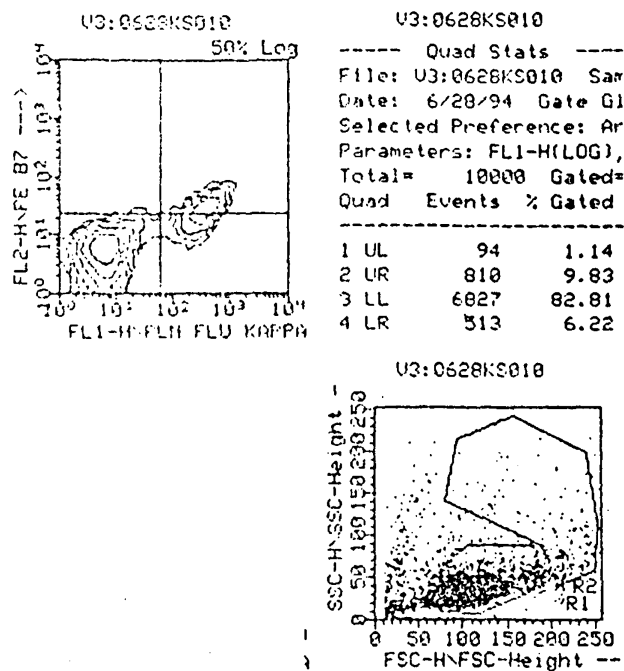
Peritoneal cells of Balb/c mice (H-2^d) were harvested and stained with Bio-anti-I-A^d to assess the MHC class II expression. When these cell preparations were subjected to flow cytometric analysis, it was observed that 52% of the total cells were I-A^d positive (Fig 1).

8. Comparison of B7 expressing population of B and non B cells in spleen, lymphnode and peritoneal aspirate :

Single cell suspensions of inguinal lymphnodes, spleens and peritoneal aspirates of Balb/c mice were prepared as described earlier. Spleen and peritoneal cells (PC) were stained with PE anti B7.1 (CD80) / PE-anti-B7.2 and FITC-anti-kappa antibody conjugates. Inguinal lymphnode cells were stained with only PE-anti-B7.1 and FITC-anti-

FIGURE 2

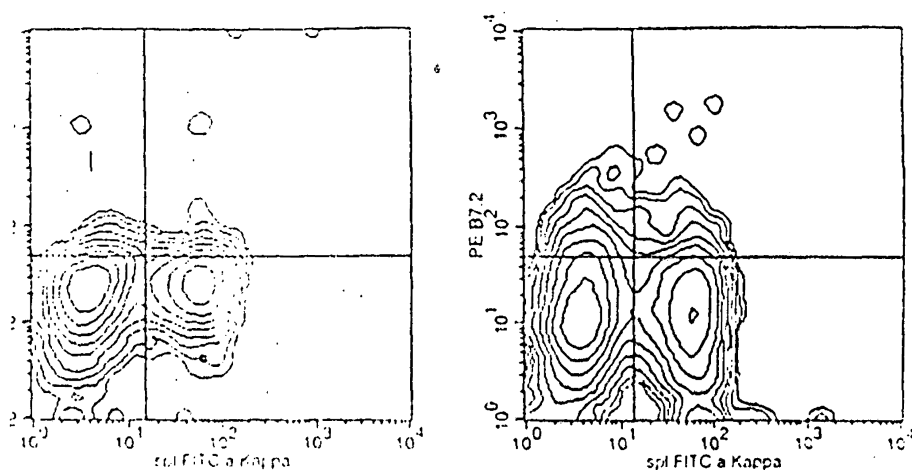
B7 expression by dermal lymphnode B and non-B-cells



The suspension of dermal lymphnode cells was stained with PE-anti-B7.1/B7.2 and Flu-anti-kappa antibodies. The gated (R1) cells were analysed.

FIGURE 3

B7 expression by splenic B and non-B-cells



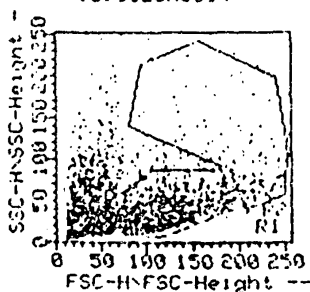
Acquisition Date: 9-Jul-95
Total Events: 10000

Quad	Events	% Gated	% Total
UL	136	2.66	1.36
UR	83	1.26	0.83
LL	4539	58.04	45.39
LR	1836	27.04	18.36

Acquisition Date: 9-Jul-95
Total Events: 10000

Quad	Events	% Gated	% Total
UL	303	4.65	3.03
UR	113	1.73	1.13
LL	3679	56.48	36.79
LR	2419	37.14	24.19

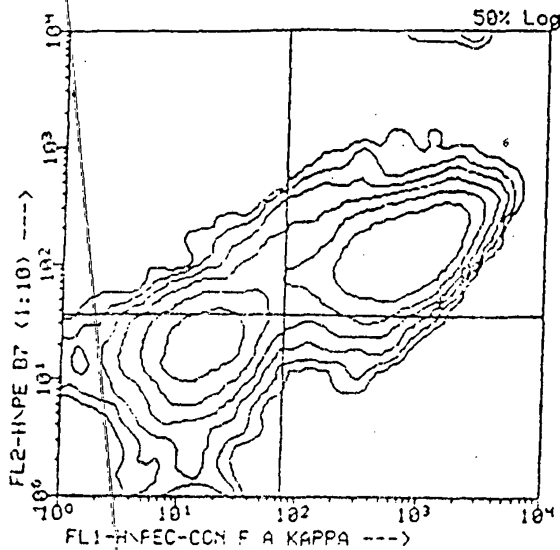
U310628KS014



The suspension of splenic cells was stained with PE-anti-B7.1/B7.2 and Flu-anti-kappa antibodies. The gated (R1) cells were analysed.

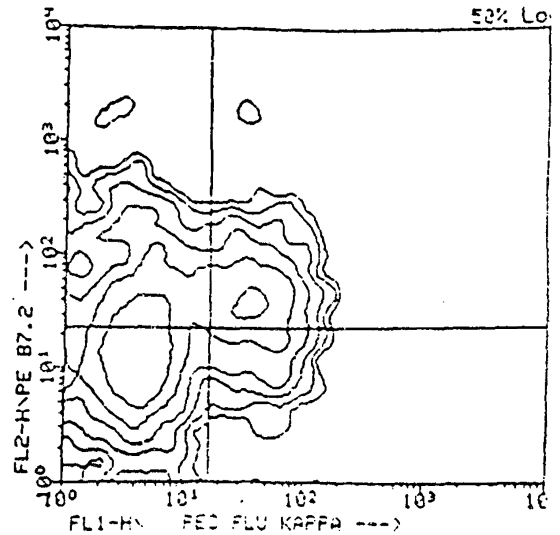
FIGURE 4

B7 expression by peritoneal B and non-B-cells



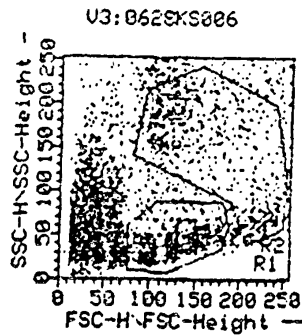
----- Quad Stats -----
 File: U3:090794PP24 Sam
 Date: 9/7/94 Gate G1:
 Selected Preference: An
 Parameters: FL1-H(LOG),I
 Total= 30220 Gated=
 Quad Events % Gated

1 UL	643	10.03
2 UR	3548	55.33
3 LL	2819	31.49
4 LR	220	0.15



----- Quad Stats -----
 File: U3:0316KS204 Sam
 Date: 3/16/95 Gate G1:
 Selected Preference: An
 Parameters: FL1-H(LOG),I
 Total= 10220 Gated=
 Quad Events % Gated

1 UL	832	30.11
2 UR	581	21.03
3 LL	1122	40.61



The suspension of peritoneal cells was stained with PE-anti-B7.1/B7.2 and Flu-anti-kappa antibodies. The gated (R1) cells were analysed.

TABLE 7

B7 expression by splenic, dermal lymphnode and peritoneal B and non - B Cells :

No.	Cell Suspension	% Frequency (Gated cells)			
		B Cells		Non - B Cells	
		B 7.1 + ve	B 7.2 + ve	B 7.1 + ve	B 7.2 + ve
1.	DLN cells	9.83	ND	1.14	ND
2.	Spleen cells	0.83	1.13	1.36	3.03
3.	Peritoneal cells	55.33	21.03	10.03	30.11

Normal naive Balb/c mice were sacrificed and peritoneal cells, DLN and spleens were harvested and single cell suspensions made. These were stained with Flu-anti-kappa and PE-anti-B7.1 / B7.2 and flow cytometric analysis done.

kappa antibody conjugates. The stained preparations were subjected to flow cytometric analysis.

B7.1 or B7.2 expressing cells formed a minor component of cells in the suspensions of spleen and lymphnode cells Kappa-positive i.e. B cells, expressing B7.1 and / or B7.2 constituted 1-3% of gated cells in the spleen and 9-10% of gated cells in the lymphnode cell suspensions. Kappa-negative i.e. non - B cells expressing B7.1 and / or B7.2 constituted 2-5% of cells in the spleen, and upto 1% of cells in the lymphnode cell suspensions. In contrast, B cells expressing B7.1 and/or B7.2 constituted at least 55% of cells in the PC suspensions. B7 expressing non B cell number was also quite high in the PC suspensions. Non B cells expressing B7.1 or B7.2 constituted at least 30% of the cells of the PC suspensions (Table 7) (Fig 2-4). Thus it appears, practically close to 80-90% of cells in the PC suspension express one or the other isotype of B7 in contrast to spleen and lymphnodes where B7 expressing cell fraction is 5-10%. Interestingly, the predominant B7 molecule expressed by non-B fraction in the PC suspension appeared to be B7.2, relative frequency of B7.1 and B7.2 expressing non-B cells being 10% and 30% respectively.

One has to take into account, this surprising finding of B7 expression by non-B and B cells in the peritoneal aspirates at such a high-frequency, while interpreting results of in-vivo and in-vitro antigen presentation by the PC.

It is known that a significant component of non-B cells in the spleen and lymphnode suspensions are the professional APCs, namely the dendritic cells. Whether dendritic cells constitute a significant component of PC suspension is not known. We know that effective CD4+ T cell responses can develop in the lymphnodes and spleens with dendritic cell number as low as 1% in the former and 2-5% in the latter. In the light of this, large size of B7 expressing non-B cell fraction (30-40%) in the PC suspensions should be adequate to induce CD4+ Th response in the peritoneum. However, if large majority of these B7 expressing non-B cells in the peritoneum turn out to be macrophages and not dendritic cell, then the antigen processing parameters in these cells would be different from those in the dendritic cells. The major function of professional macrophages is the effector function characterized by degradation of

TABLE 8

Duration of antigen processing by the peritoneal cells can influence proliferative response : Antigen pulsed, fixed peritoneal cells are superior to live functionally active peritoneal cells.

No.	Nature of APCs	CPM $\pm$ S.E.	S.I.
1.	Pulsed for 4h and fixed	672 $\pm$ 111	2.1
2.	Pulsed for 8h and fixed	4,428 $\pm$ 508	13.8
3.	Pulsed for 24h and fixed	3,018 $\pm$ 899	9.4
4.	Irradiated PC + Ag	2,608 $\pm$ 411	2.7
5.	Irradiated spleen cells + Ag	7,698 $\pm$ 486	20.1

Pooled PC of normal naive Balb/c mice (10^6 cells/ml) were pulsed with *M. vaccae* sonicate (200 μ g/ml) for 4, 8 and 24h, washed and fixed in 1% paraformaldehyde. A separate set of Balb/c mice were immunised with *M. vaccae* by the i.d. route and one week later, the mice were sacrificed, the spleens removed and T cells separated. 10^5 i.d. primed T cells were mixed with equal number of antigen pulsed peritoneal cells and cultured for a lymphoproliferation assay. Cultures of i.d. primed T cells with non-fixed irradiated peritoneal cells and spleen cells and *M. vaccae* sonicate (20 μ g/ml) were also set up for comparison.

phagocytosed particles and macromolecules, secretion of biologically active agents such as reactive oxygen and reactive nitric oxide intermediates (ROIs and RNOIs), prostaglandins and TNF- α . Each of these biological activities can influence the CD4⁺ Th cell activation and differentiation, if antigen is processed and presented by such cells. This kind of influence is expected when macrophages function as direct APCs. In addition, the B7⁺ macrophages can function as "bystander accessory" cells also, which is likely if the frequency of such cells is very high. The above issues, need to be investigated in the context of *in-vivo* and *in-vitro* responses of CD4⁺ Th cells, on recognition of antigen presented by PC. Therefore influence of degradation of peptide antigen and the products of macrophage secretion on the direct antigen presentation and the indirect "bystander accessory" function was investigated.

9a. Duration of antigen processing can influence the proliferative response: Antigen pulsed fixed PC are superior in APC function than live functionally active PC :

Normal peritoneal cells were pulsed with *M.vaccae* sonicate for different durations - 4 to 24h and then fixed with 1% (w/v) paraformaldehyde. These antigen pulsed and fixed PC were used as APCs and cultured with i.d. primed splenic T cells in a standard 6d lymphoproliferation assay.

In three different experiments peritoneal cells pulsed for upto 4h did not support lymphoproliferation at all. The PCs pulsed for 6-24h showed increasing proliferation, maximum being obtained from 6h-12h pulsing. Longer pulsing i.e. greater than 24h or continuous processing did not support lymphoproliferation (Table 8). Therefore, excessive processing and degradation could be one of the factors contributing to the peculiar nature of the CD4⁺ T cell responses of i.p. primed mice.

It is important to note that in this experiment two different issues were investigated. After permitting the cells to process for varying durations, the cells were also fixed. This eliminates their ability to secrete bioactive molecules such as ROI, RNOI, prostaglandins by the macrophages and lymphokines by B cells. Role of these agents in the direct APC function and "bystander accessory" function is thus stressed by this finding. However it was essential to validate these findings by actual evidence of

TABLE 9

DTH response of indomethacin treated, i.p. immunised mice :

No.	Pre-treatment	Route of Immunisation	DTH CPE $\pm$ S.E.
1.	None	i.p	0.08 $\pm$ 0.02
2.	Indomethacin	i.p	0.18 $\pm$ 0.02
3	None	i.d.	0.28 $\pm$ 0.02

Balb/c mice were treated intraperitoneally with 6 doses of 50 μ g each of indomethacin at 12 h interval. 12 h after the last dose the mice were immunised by i.p. route with 10^8 *M. vaccae* and one week later their DTH response was tested against the sonicate.

TABLE 10

Antigen presentation by indomethacin treated peritoneal cells.

No.	Source of APCs	Mean CPM $\pm$ S.E.	S.I.
1.	Spleen cells	5,787 $\pm$ 837	8.5
2.	Naive peritoneal cells	1,523 $\pm$ 84	1.3
3.	Indomethacin treated peritoneal cells	4,833 $\pm$ 419	3.9

Balb/c mice were given 6 doses of indomethacin, 50 μ g each by the i.p. route at 12 h interval and 12 h after the last dose the peritoneal cells harvested. Peritoneal cells and spleens were harvested from normal mice and single cell suspensions made. All these were irradiated for use as APCs. A separate set of Balb/c mice were given an i.d. immunisation with *M. vaccae*, their spleens harvested one week later, single cell suspensions made and T cells separated. 10^5 i.d. primed T cells were mixed with equal number of spleen or peritoneal cells to serve as APCs and *M. vaccae* sonicate as antigen and a standard lymphoproliferation assay performed.

secretion of these biologically active agents by the PC and evidence of influence on the function of these cells as APCs and as the "bystander accessory" cells.

Prostaglandins play a major role in the inflammatory and immune response. It is well known that macrophages produce prostaglandins which down regulate the lymphoproliferation (196). The possibility that prostaglandin secretion by PC contributes significantly towards the APC function or bystander function of PC could be tested by studying the effect of prostaglandin blockers such as indomethacin on antigen presentation by PC as well on the effector responses to i.p. immunisation.

10. Effect of prostaglandin blocker (indomethacin) on *in-vivo* response to i.p. immunisation : antigen presentation by the indomethacin treated peritoneal cells :

Balb/c mice were treated intraperitoneally with 6 doses of 50 µg each of indomethacin at 12h interval, given in 0.5 ml PBS (200). 12h after the last dose the mice were given an i.p. immunisation with 10^8 *M. vaccae* and their DTH response was tested against the sonicate one week later. As before the normal, non treated mice failed to mount the DTH response whereas the indomethacin treated mice showed a definite DTH response (Table 9).

In-vitro antigen presentation by the indomethacin treated peritoneal cells was also investigated. Balb/c mice were treated with indomethacin as before. 12h after the last dose of indomethacin the PC were harvested and 10^5 PC were cultured with equal number of splenic T lymphocytes from i.d. primed Balb/c mice alongwith *M. vaccae* sonicate in a lymphoproliferation assay.

As before the PC of non treated animals did not support antigen specific lymphoproliferation; however the PC of indomethacin treated mice did provide adequate antigen specific stimulation to induce T cell proliferation response even though it was less than that induced by spleen cell APCs (Table 10). These results clearly indicate that prostaglandins adversely affect the T cell proliferative responses when antigen is presented by the peritoneal macrophages.

Having examined the role of prostaglandins in the APC function of PC, it was then

TABLE 11

Peritoneal cells suppress an antigen specific *in-vitro* T cell proliferation induced by splenic APCs : Role of prostaglandins

No.	Composition of APCs	Mean CPM $\pm$ S.E.	S.I.
1.	Spleen cells	5,787 $\pm$ 837	8.5
2.	Spleen cells + PC	726 $\pm$ 98	0.9
3.	Spleen cells + indomethacin treated PC	3,230 $\pm$ 326	3.9

Peritoneal cells were harvested from normal Balb/c mice and those treated with 6 doses of indomethacin. Spleens were also removed from normal Balb/c mice and single cell suspensions made. All these suspensions were irradiated to serve as APCs. A separate set of Balb/c mice was immunised i.d. with *M. vaccae*, sacrificed 1 week later, spleens removed and T cells obtained. 10^5 i.d. primed T cells were mixed with equal number of irradiated normal spleen cells to serve as APCs and 5×10^4 PC to serve as "bystander" cells, cultured with *M. vaccae* sonicate as antigen and a lymphoproliferation assay performed.

decided to investigate the next issue, the role of peritoneal macrophages as "bystander accessory" cells in influencing the outcome of immune response.

11. Effect of peritoneal cells on antigen specific T cell proliferative responses induced by splenic APCs :

10^5 splenic T lymphocytes from i.d. primed Balb/c mice were cultured with equal number of normal irradiated spleen cells as APCs and *M. vaccae* sonicate as antigen. 10^4 normal irradiated peritoneal cells were added and lymphoproliferation was assayed.

As described earlier, cultures receiving only splenic APCs showed good lymphoproliferation whereas those which received peritoneal cells alongwith spleen cells as APCs showed inhibition of lymphoproliferation (Table 11). It is possible that the peritoneal cells not only bring suppression of T cell proliferation but also induce Th2 differentiation by secretion of cytokines or agents such as RNOs and prostaglandins.

In order to investigate the role of prostaglandins in the bystander behaviour of PC, the above experiment was repeated with indomethacin treated PC. Indeed distinct reduction in suppression was observed, when indomethacin treated PC were used as "bystander" cells (Table 11).

It was therefore concluded that PC did suppress an *in-vitro* antigen specific T cell proliferative response, induced by professional APCs and a major factor in the suppression was prostaglandin secretion by the peritoneal macrophages.

Evidence of prostaglandins favouring a Th2 type of cytokine secretion by inhibiting production of Th1 cytokines particularly IL-2 and γ -IFN has also been reported (201,202). The γ -IFN in turn has been shown to favour Th1 type of response and IL-2 secretion (17). In view of this the prostaglandin secreted by peritoneal macrophages possibly could bring about both *in-vivo* and *in-vitro* effects at least partly through reduction of γ -IFN secretion by T cells getting activated or by bystander cells such as NK cells. It is therefore logical to expect enhanced IFN secretion to nullify some of the effects of prostaglandins. Therefore, it was decided to investigate effect of poly I : poly C, an IFN inducer on the responses of mice to i.p. immunisation and on the *in-vitro* antigen presentation by PC in a proliferative assay.

TABLE 12

DTH, lymphoproliferative and anti - 65 kDa antibody responses of poly I : poly C treated mice

No.	Pre-treatment	Route of Immunisation	DTH CPE $\pm$ S.E.	Mean CPM $\pm$ S.E. (S.I.)	Anti-65kDa Ab titre mean AU/ml $\pm$ S.E.
1.	None	i.p.	0.08 $\pm$ 0.02	5,701 $\pm$ 439 (3.44)	3,339 $\pm$ 229
2.	Poly I : poly C	i.p.	0.24 $\pm$ 0.02	11,570 $\pm$ 506 (6.06)	765 $\pm$ 149
3	None	i.d.	0.28 $\pm$ 0.02	16,772 $\pm$ 7491 (7.4)	501 $\pm$ 39

Balb/c mice were given two doses of 100 μ g each of poly I : poly C by the intravenous (i.v.) route at 24 h interval and 3 h after the last dose they were immunised with *M. vaccae* by i.p route. One week later, their DTH response was tested. anti-65-kDa antibody titres assayed and lymphoproliferative assay performed on their spleen cells against *M. vaccae* sonicate.



TABLE 13

Antigen presentation by poly I : poly C treated peritoneal cells

No.	Antigen presenting cells	Mean CPM $\pm$ S.E.	S.I.
1.	Spleen cells	7,508 $\pm$ 1161	11.4
2	Peritoneal cells	1,523 $\pm$ 84	1.3
3.	Poly I : poly C treated peritoneal cells	6,225 $\pm$ 987	8.8

Balb/c mice were treated with two doses of 100 μ g each of poly I : poly C 24 h apart. 3 h after the second dose peritoneal cells were harvested and irradiated. 10^5 cells were cultured as APCs with equal number of i.d. primed splenic T cells and *M. vaccae* sonicate as antigen and lymphoproliferative assay performed. As controls 10^5 i.d. primed splenic T cells were also cultured with 10^5 irradiated normal spleen cells or peritoneal cells.

12. Effect of poly I : poly C treatment on i.p. immunisation :

12a. Effector T helper responses of poly I : poly C treated; i.p. immunised mice :

Balb/c mice were given two doses of 100 µg each of poly I : poly C by the intravenous (i.v) route at 24h interval (203) and 3h after the last dose they were immunised with *M. vaccae* by i.p. route. One week later, their DTH response was tested, anti-65-kDa antibody titres assayed and a lymphoproliferative assay performed on their spleen cells against the sonicate.

Poly I : poly C treatment of animals induced them to mount a DTH and a lymphoproliferative responses on i.p. immunisation which were comparable to those after i.d. immunisation. Also the antibody response was lowered to the level comparable to that of i.d. immunised mice (Table 12).

12b. Antigen presentation by poly I : poly C treated peritoneal cells to primed splenic T cells :

Antigen presentation ability of peritoneal cells of poly I : poly C treated animals was also studied.

Balb/c mice were treated with two doses of poly I : poly C 24h apart as before. 3h after the second dose peritoneal cells were harvested and irradiated for use as APCs. These (10^5) were cultured with equal number of i.d. primed splenic T cells and *M. vaccae* sonicate as antigen and lymphoproliferative assay performed.

The poly I : poly C treated peritoneal cells supported good lymphoproliferation unlike the normal peritoneal cells (Table 13).

These results clearly indicate that poly I : poly C treatment alters the response to i.p. immunisation. Whereas in the untreated mice the antigen specific proliferative response of CD4⁺ Th was poor and effector response showed a major Th2 component, the treated mice revealed a significant proliferative response and a major shift to Th1 effector response. This change was accompanied by improved *in-vitro* antigen presentation by the PC. Thus poly I : poly C treatment seemed to neutralise the effects of prostaglandin secretion by peritoneal macrophages.

TABLE 14

Nitrite levels in the culture supernatants of normal and indomethacin treated peritoneal cells :

No.	Composition of culture medium	Nitrite levels (μ moles / 10^6 cells)	
		Normal PC	Indomethacin treated Pc
1.	Culture medium	47.5	37.0
2.	Culture medium with 50 IU/ml of γ -IFN	60.0	94.0
3.	Culture medium with 100 IU/ml γ -IFN	72.5	101.0

Balb/c mice were given 6 doses of indomethacin and PC harvested 12h after the last dose. PC were harvested from non-treated Balb/c mice also. These were cultured in the medium containing either no γ -IFN or 50 and 100 IU/ml γ -IFN (see methods) for 20h, the supernatants harvested and nitrite levels estimated.

13. Production of reactive nitric oxide intermediates by peritoneal cells :

PC of normal Balb/c mice were cultured as 10^6 cells/ml in RPMI 1640 containing 10% FCS and γ -IFN (50-100 IU/ml). After 20 h culture supernatants were harvested and nitrite levels were estimated as an index of RNOI synthesis by the method of Ding et al (196).

Significant nitrite levels were observed even without γ -IFN in the medium. There was a significant increase in nitrite levels on addition of γ -IFN to the culture medium. This indicated that RNOIs were generated by the peritoneal macrophages.

One may argue that prostaglandins favour NO synthesis which in turn is responsible for poor APC and bystander accessory function of PC. Therefore it was essential to study nitric oxide synthesis by PC of indomethacin treated mice.

When nitrite levels were assayed in the culture supernatants of indomethacin treated PC significant increase in nitrite levels was observed.

6

DISCUSSION

An experimental murine model was set up to study the Th cell subset differentiation in mycobacterial infections, as a function of route of immunisation. Immunisation of animals with killed *M.vaccae* by i.d. and i.p. routes resulted into different Th responses. The i.d. immunised mice showed an excellent DTH response, a low serum antibody titre and an excellent *in-vitro* antigen specific lymphoproliferative response of spleen cells and dermal lymph nodes draining the site of immunisation. All these responses were directed against the immunodominant 65-kDa antigen of *M.vaccae*. Even though it was a primary immunisation, predominant isotype of anti-65 kDa antibody was IgG1. However, the IgG1 : IgG2a ratio of the antibody was much lower than that of i.p. immunised mice. The antigen specific *in-vitro* proliferation of primed cells is an indication of their ability to secrete IL-2 on activation by antigen. This is a characteristic of precursor and memory T cells. Hence it was concluded that i.d. priming generated a significant memory component of Th cells. The i.d. primed splenic and lymph node effector cells, when restimulated by antigen *in-vitro*, produced both Th1 and Th2 type of lymphokines. This is possible only if the effector population is of Th0 type or a mixture of all three subsets namely Th0, Th1 and Th2. In contrast, the i.p. immunised mice showed poor DTH and *in-vitro* antigen specific lymphoproliferative responses. The serum anti-65kDa antibody titre was high and IgG1 : IgG2a ratio of the antibody was higher than the corresponding ratio of the i.d. primed mice. The i.p. primed splenic effectors also produced both Th1 and Th2 type of lymphokines on restimulation with antigen *in-vitro*. However levels of IL-10 produced by i.p.primed effectors were significantly higher than the levels generated by i.d. primed effectors.

It was thus concluded that antigen specific T cell proliferative responses (memory?) of i.d. primed mice were excellent whereas they were poor in the i.p. primed mice. The Th effector responses of both i.d. and i.p. primed mice were mixed, but the former showed a major Th1 component and the latter a major Th2 component.

The above differences in the outcomes of Th responses to i.d. and i.p. immunisation could be due to differences in the functional state of various APCs in the nodes draining the immunisation site. Hence, presentation of *M.vaccae* sonicate by irradiated normal DLN and MLN cells to i.d. primed splenic T cells, was studied employing *in-vitro* proliferative assay. Irradiated normal spleen cells as APCs were also

included for comparison. All the three sources of APCs induced excellent and comparable proliferation. Despite differences in the outcomes of immunisation, by i.d. and i.p. routes, the APCs in the spleen, DLN and MLN behaved identically in the proliferation assays.

It is generally believed that when antigen is introduced in tissues, it is internalised by the APCs at the site and then transported to the draining nodes for presentation to T and B cell clones. Therefore, logically the nature of APCs available at the site of immunisation, would constitute an important factor deciding the outcome of immunisation. In i.d. immunisation the dendritic cells of dermis and Langerhan's cells of epidermis, both professional APCs, would present the antigen (204-206). In case of i.p. immunisation, peritoneal cells can act as APCs. The peritoneum definitely contains macrophages and B cells; however whether it contains dendritic cells is not known. Therefore the ability of the PC to present antigen was compared with that of DLN, MLN and spleen cells known to contain dendritic cells. The DLN, MLN and splenic APCs induced excellent proliferation in an *in-vitro* T cell proliferative assay with a single source of i.d. primed T cells, whereas PC were found to be poor in antigen presentation. Also using a single source of i.d. primed splenic T cells, effectors were generated *in-vitro* employing irradiated normal DLN, MLN and spleen cells and PC as APCs and their lymphokine secretion studied. DLN, MLN and spleen cells generated effectors produced IL-2, IL-4 and IL-10. However, PC generated effectors produced only IL-10 and in significantly higher amounts. Based on this it was concluded that antigen presented by PC favoured Th2 differentiation. Thus the *in-vitro* antigen presentation by PC in proliferation and effector generation assays correlated with the *in-vivo* proliferative and effector responses of i.p. immunised mice.

It could be argued that i.p. immunisation results into abortive Th response with predominant outcome being T cell tolerization. However our data do not support this argument. Though the DTH and lymphoproliferative responses of the i.p. primed mice were poor, their antibody responses were good. The spleen cells of i.p. primed mice did show evidence of generation of Th effectors, capable of cytokine secretion on restimulation *in-vitro*. Thus the conclusion that i.p. immunisation activated Th cells was quite rational. Therefore, it was essential to look for a logical explanation in the antigen presenting cells of the peritoneum for generating the observed type of Th response.

For inducing tolerisation, there would be two requisites. Firstly, the PC would be required to express MHC Class II so that they can present antigen, secondly the PC would have to be deficient in B7 expression, so that antigen would be presented without a costimulatory signal. Therefore PC were assayed for MHC Class II and B7 expression, the two important requisites for a cell to function as APC. Surprisingly, both the molecules were expressed at a very high frequency by both B and non-B cells in the PC preparation. MHC class II was expressed by 60% of total cells while B7.1 and/or B7.2 was expressed by at least 55% of B cells and at least 30% non-B cells. Further, the possibility of presence of dendritic cells in the peritoneum can not be ruled out. Thus a simple logical explanation for poor *in-vitro* antigen presentation by PC could not be offered.

Also, the finding of poor antigen presentation by the PC in the *in-vitro* proliferative assay could not account for the *in-vivo* responses of the i.p. immunised mice because they did show evidence of Th cell activation, the outcome of which is qualitatively different from that observed in i.d. immunised mice. The likelihood that the PC induced differentiation instead of proliferation had to be considered.

It was therefore decided to investigate if this type of response i.e. poor memory (T cell proliferation) response and predominant Th2 effector component generated in the peritoneum could be a consequence of high frequency B7 expression by the peritoneal macrophages and B cells.

As discussed earlier, the dendritic cells, the professional APCs, form a major component of splenic and lymphnode APCs in a primary response. With dendritic cell frequency as low as 1% and 2.5% respectively, both spleen and lymphnodes can develop efficient CD4⁺ Th response. Whether, dendritic cells constitute a significant component of total 30-40% of B7 expressing non-B cell fraction of peritoneum is not known. Even if it is assumed that the dendritic cells are present in the peritoneum at a frequency comparable to that of spleen and lymphnodes, the large majority of this B7 expressing non-B cell fraction would be macrophages. In this case the macrophages would outnumber the dendritic cells in the peritoneum and therefore compete with them for antigen uptake and presentation.

It is possible that the peritoneal macrophages would be active in their effector functions such as degradation of phagocytosed material and secretion of biologically active agents namely prostaglandins, reactive oxygen and nitric oxide intermediates (ROI and RNOI) and TNF. Each of these can influence CD4 + T cell activation if antigen is presented by macrophages actively secreting them. Hence, the possibility of each of the above effector functions of peritoneal macrophages adversely influencing their antigen presentation function were investigated.

When PCs were used as sole APCs and allowed to process antigen for different durations in an *in-vitro* proliferative assay, prolonged periods of processing (24h or more) lead to poor response, whereas after a short duration of processing of 6-12 h the PC could present antigen efficiently. It is likely that peritoneal macrophages degrade the antigen to peptides of very small size when allowed to process it over long duration. Evidence supporting small size of the processed peptide, its MHC affinity and consequent effect on Th cell cytokine secretion, particularly IL-2 has been reported (170).

The experiments of pulsing the peritoneal cells with antigen for different durations followed by fixation bring out another feature. The PC that were pulsed for short duration with antigen and then fixed, showed the capacity to support lymphoproliferation that was comparable to that of splenic or lymphnode APCs, whereas live PCs were poor in antigen presentation. This brings into focus the role of the (secretory) products of the PC in down regulating the APC function. Hence, the effect of secretion of prostaglandins by the PC on the *in-vitro* proliferative response and *in-vivo* response to i.p. immunisation were studied using prostaglandin inhibitors.

Our findings indicated that prostaglandin secretion indeed has negative influences on T cell activation by the PC. PC of indomethacin treated mice were quite efficient in antigen presentation whereas the PCs of untreated mice were not. Even *in-vivo* responses to i.p. immunisation of treated mice were different from non-treated mice. The indomethacin treated mice mounted a DTH response whereas the untreated mice did not. Evidence of prostaglandins favouring a Th2 type of cytokine secretion by inhibiting production of Th1 cytokines, particularly IL-2 and γ -IFN has also been reported (201,202). The γ -IFN in turn has been shown to favour Th1 type of response and IL-2 secretion (17).

In this context, it was logical to study the effects of increased interferon levels on i.p. immunisation. Mice were treated with poly I:poly C, an IFN inducer generating about 900 IU/ml of serum IFN following single injection of 100 µg dose. As expected, poly I : poly C altered the Th responses of mice to i.p. immunisation. The treatment induced the mice to mount a significant proliferative (memory) response and an effector response consisting of DTH and an antibody response with lower titres and higher levels of IgG2a. Based on this data, it was concluded that poly I : poly C treatment enabled the mice to generate a significant Th1 component. In contrast, the non-treated mice generated no DTH, a poor proliferative response and high titres of antibody with low levels of IgG2a, on i.p. immunisation, implying a major Th2 effector component. Thus the effects of prostaglandins of the peritoneal macrophages seem to be neutralized by poly I : poly C treatment.

Peritoneal macrophages can also generate reactive oxygen and reactive nitric oxide intermediates (ROI and RNOI). These in turn can interfere with antigen presentation. RNOI produced by peritoneal macrophages have been shown to inhibit antigen induced proliferation of T cells (196). RNOI have also been shown to favour Th2 differentiation (207). In our study the PC were shown to generate nitrite (an index of NO production) when cultured *in-vitro*. Nitrite levels significantly increased when the PC cultures were stimulated with γ -IFN. It is felt that these results reflect *in-vivo* events, as outlined below. I.p. immunisation probably induces effector generation of Th0 type in the first instance. Th γ -IFN generated induces the antigen presenting macrophages to secrete ROI and RNOI, which drive the differentiation of Th clone to Th2 pathway at the same time inhibiting their proliferative response. In fact inhibition of NO production by N^G monomethyl arginine has been shown to increase *in-vitro* antigen specific proliferative response induced by peritoneal macrophages (196).

So far as primary immunisation is concerned, antigen presentation by B cells is not likely to play a major role, because naive B cells do not express costimulatory B7 molecules and because of low frequency of antigen specific B cells. In our model the first reason has no relevance.

However, all B7 expressing cells and macrophages, can behave as so called

"bystander accessory" cells. A "bystander accessory" cell can provide the costimulatory signal without actually presenting the antigen, the antigen being presented by another APC lacking B7 (208). Bystander accessory cell has even been shown to rescue a cell from tolerance due to lack of CD28 modulation. Hence the bystander accessory function of the peritoneal cells was investigated.

An *in-vitro* lymphoproliferation assay was performed with i.d. primed splenic T cells and irradiated normal spleen cells as APCs. The PC were added to serve as "bystander accessory" cells. The PC suppressed the proliferative response. This was contradictory to the expected behaviour of "bystander accessory" cells. A review of a report on bystander accessory function by the PC may throw greater light on this complexity.

Isobe and Nakashima (196) have investigated presentation of the superantigen, staphylococcal enterotoxin B (SEB) by irradiated spleen cells, depleted of adherent cells (dendritic cell deficient) and studied the effect of addition of PC. In the absence of PC, SEB induced proliferation was poor, because the only available APC, i.e. the MHC Class II positive naive B cells do not express B7. Addition of PC resulted into responses that were dose dependent. Smaller numbers of PC, when added, improved the proliferative response and made it comparable to that induced by whole spleen cells as APCs. When large numbers of PC were added (number comparable to those in our experiments), the proliferative responses were suppressed. Thus from the above reported data of Isobe and Nakashima, it seemed that the nature of effect of bystander peritoneal cells, on *in-vitro* proliferation of T cells appeared to be dose dependent. It is possible that PC when added in small numbers do have a positive bystander accessory influence, enhancing the T cell response induced by B7 deficient APCs. However addition of larger numbers of PC induced suppression of proliferation, possibly due to production of prostaglandins and RNOI by the macrophages and IL-10 by B cells. Isobe and Nakashima confirmed this by blocking the suppressive effects of PC by N^G monomethyl arginine, which inhibits RNOI production.

In our experimental design, the antigen was presented by the dendritic cells in the spleen cells employed as the APC source. Therefore there was no deficiency in the

costimulatory signal delivered to the T cells. Hence, the question of enhancement of the T cell proliferative response by the bystander PC does not arise. However, as in the reported experiment of Isobe and Nakashima, addition of PC in large numbers resulted into suppression possibly due to products of macrophages and B cells. In order to verify this, the effect of indomethacin treatment on bystander suppressive function of PC was studied.

Indeed it was observed that the suppressive effects were inhibited considerably by indomethacin treatment, confirming the role of prostaglandins.

The peritoneal B cells, which express B7 at a high frequency, could also be responsible for suppression of *in-vitro* proliferation by the bystander PC. They can do it by IL-10 secretion (129). The B1 B cells which form about 45% of total PC population (209) are known to secrete IL-10 constitutively (210,211). These agents, i.e. prostaglandins, RNOI and IL-10 are not only known to inhibit IL-2 induced proliferation but also favour a Th2 differentiation (129,201,207).

These findings have direct *in-vivo* relevance if peritoneum does contain dendritic cells and there is no *a priori* reason to consider otherwise. Since B7 expressing macrophages and B cells would outnumber the dendritic cells (if present at the frequency comparable to that in other lymphoid tissues) by a factor greater than 10, the *in-vivo* response of T cells to antigen presented in the peritoneum would be comparable to that generated *in-vitro* by the splenic dendritic cells in presence of large numbers of PC (10^5 irradiated spleen cells as source of APCs and 5×10^4 PC).

In-vivo relevance of the above data i.e. bystander inhibition by PC may be questioned, as B7 positive macrophages and B cells would be present in lymphnodes and spleen, the sites where the primary response usually develops and such an influence by these cells has not been observed here. Low frequency of B7 expressing cells and compartmentalization of T cells, B cells and effector macrophages in these tissues i.e. spleen and lymphnodes could be the two reasons why similar effects of B7 positive macrophages and B cells do not significantly affect the responses in these tissues. The situation in the peritoneum is quite different. Firstly, the frequency of B7 positive macrophages and B cells in the peritoneum is so high that they would outnumber the

dendritic cells even if present. Second important difference between the former lymphoid tissues and peritoneum is that there is no compartmentalization of the cells in the peritoneum. For these reasons, the reported *in-vitro* findings are likely to have an *in-vivo* relevance.

Reasons for high frequency expression of B7 by the PC are not clear. It is possible that agents such as bacterial LPS which can stimulate both macrophages and B cells are brought to the peritoneum by the lymphatics from the intestine. Alternatively, the B7 positive,

macrophages and B cells may selectively migrate to the peritoneum from the sites of activation such as gut mucosa. The third possibility is that the B1 B cells and serosal cells differentiating into macrophages, probably express B7 constitutively.

Thus, the two major reasons could be cited for a poor proliferative and a major Th2 type of immune response following i.p. immunisation. Firstly peritoneal macrophages, actively secreting prostaglandins and RNOI and very efficient in degrading phagocytosed protein/peptide antigen, function as predominant APCs, because of high frequency B7 expression. Secondly, both macrophages and the peritoneal B cells can behave as bystander accessory cells and drive the immune response to the unique pathway because of high frequency B7 expression.



SUMMARY AND CONCLUSIONS

Distinct differences were observed in the Th responses of mice immunised with killed *M.vaccae* by i.d. and i.p. routes. The i.d. primed animals mounted a good DTH response and an excellent *in-vitro* antigen specific lymphoproliferative response whereas in the i.p. primed animals both the responses were poor. All these responses were directed against the immunodominant 65-kDa antigen of *M.vaccae*. The anti-65kDa antibody levels were low in i.d. primed mice with lower IgG1 : IgG2a ratio while i.p. primed mice showed high titres of anti-65kDa antibody with higher IgG1 : IgG2a ratio. The Th effectors of both i.d. and i.p. primed animals generated mixed effector response inducing secretion of both the types of lymphokines viz. Th1 and Th2; however the i.d. primed effectors showed a major Th1 component while i.p. primed effectors generated a major Th2 component. Despite these differences, the APCs in the spleen, DLN and MLN behaved identically in an *in-vitro* proliferative assay with a single source of i.d. primed splenic T cells and induced an excellent proliferation. We considered the probability that in i.p. immunised mice the antigen is presented by peritoneal cells which consist of macrophages and B cells. Whether they contain dendritic cells or not is not known. Therefore their ability to present antigen was compared with that of DLN, MLN and spleen cells known to contain dendritic cells. Indeed it was found to be poor. Also, the effectors generated by DLN, MLN and spleen cells as APCs produced IL-2, IL-4 and IL-10 whereas peritoneal cells (PC) generated effectors producing only IL-10 in significantly higher amounts. Based on this, it was concluded that antigen presented by PC favoured Th2 differentiation. Thus the *in-vitro* antigen presentation by PC in proliferation and effector generation assays correlated with the *in-vivo* proliferative and effector responses of i.p. immunised mice.

Our data do not support the argument that i.p. immunisation results into abortive Th response with predominant outcome being T cell tolerization. Though the DTH and lymphoproliferative responses of i.p. primed animals were poor, their antibody responses were good and their spleen cells contained Th effectors capable of cytokine secretion on restimulation *in-vitro*. Thus it was concluded that i.p. immunization does result in Th cell activation.

In order to find out a logical explanation for poor antigen presentation by the PC in the *in-vitro* proliferative assay, they were screened for MHC class II and B7

expression, the two important requisites for a cell to function as APC. Surprisingly both of these were expressed at a very high frequency by both B and non-B cells in the PC. Further, there is no *a priori* reason to believe that the peritoneum does not contain dendritic cells. Thus a simple logical explanation for poor *in-vitro* antigen presentation by PC could not be offered. Also the possibility that the PC induced differentiation instead of proliferation following Th activation had to be considered.

Hence, it was decided to investigate if this type of response i.e. poor memory (indicated by T cell proliferation) response and predominant Th2 effector component generated in the peritoneum is a consequence of high frequency B7 expression by the peritoneal macrophages and B cells.

Even if it is assumed that the dendritic cells are present in the peritoneum at a frequency comparable to that of spleen and lymph nodes, the B7 expressing macrophages would outnumber and therefore compete with them for antigen uptake and presentation. Also the effector functions of these macrophages such as degradation of phagocytosed peptide and secretion of biomolecules like prostaglandins, ROI and RNOIs may influence T cell activation if antigen is presented by these macrophages. This possibility was investigated.

Indeed when PC were used as the sole APCs, excessive degradation and prostaglandin secretion by them did downregulate the T cell proliferative responses. *In-vivo* studies did seem to suggest a major role for prostaglandins for Th2 differentiation of CD4⁺T cells, activated on i.p. priming of the mice. Also the effect of increased IFN levels on i.p. immunisation was studied by treating the animals with Poly I : Poly C, an IFN inducer. Poly I : poly C treatment of animals before i.p. immunisation induced them to mount good DTH and a significant proliferative response and an effector response with a major Th1 component. Thus the effects of prostaglandins of the peritoneal macrophages seemed to be neutralised by poly I : poly C treatment. RNOI production by PC has been reported to inhibit T cell proliferation and favour Th2 differentiation. Our data showed generation of RNOIs by PC when cultured *in-vitro*. Thus it was concluded that excessive degradation of peptides and secretion of prostaglandins and RNOIs by peritoneal macrophages may contribute towards the poor *in-vitro* antigen presentation by PC.

The "bystander accessory" function of B7 expressing PC was also studied since the peritoneal macrophages actively secreting prostaglandins and RNOIs and B cells capable of secreting IL-10 would outnumber the dendritic cells. Indeed the bystander PC were shown to inhibit *in-vitro* T cell proliferation by secreting prostaglandins. Similar bystander inhibition by RNOI production by PC has been reported.

The B1 B cells which form a major component of PC could contribute to bystander inhibition by PC as they constitutively secrete IL-10. These three agents, i.e. prostaglandins, RNOIs and IL-10 are not only known to inhibit IL-2 induced proliferation but also favour a Th2 differentiation. Low frequency of B7 expressing cells and compartmentalization of T cells, B cells and effector macrophages in tissues like spleen and lymph nodes could be the two reasons why similar effects of B7 positive macrophages and B cells do not significantly affect the responses in these tissues. As both these reasons do not stand valid in case of peritoneum, the reported *in-vitro* findings are likely to have an *in-vivo* relevance.

There could be different possibilities to explain the high frequency B7 expression by PC. Agents such as bacterial LPS which can stimulate both macrophages and B cells are probably brought to the peritoneum by the lymphatics from the intestine. Alternatively, the B7 positive cells may selectively migrate to the peritoneum from the sites of activation such as the gut mucosa. The third possibility is that the B1 B cells and serosal cells differentiating into macrophages, constitutively express B7.

APPENDICES

APPENDIX - I

1. Doub and Youman's medium :

Ingredients :

Asparagine	5.0 g
KH_2PO_4	5.0 g
K_2SO_4	0.5 g
Magnesium citrate	1.5 g
Glycerol	20.0 ml
Distilled water	980 ml

All the ingredients except magnesium citrate were dissolved in 980 ml distilled water. 20 ml of glycerol was added. After thorough mixing the pH was adjusted to 6.8 to 7.0 by adding appropriate amount of 1N 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 sterilized by autoclaving at 15 lbs and 121°C for 20 min.

2. Phosphate buffered saline (PBS) (pH 7.2, 0.5 M)

Sodium Chloride	8.0 g
Di-potassium hydrogen phosphate (K_2HPO_4)	1.21 g
Potassium di-hydrogen phosphate (KH_2PO_4)	0.34 g
Distilled water	1000 ml.

3. Tris buffer with EDTA (0.01 M Tris, pH 7.8; 0.01 M EDTA)

Tris	0.121 g
EDTA	0.292 g
Distilled water	100 ml.

Tris was dissolved in 100 ml distilled water. The pH was adjusted to 7.8 with 0.1N

HCl. To this EDTA was added and dissolved.

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

Tris	3.03 g
Glycine	14.4 g
Distilled water	1000 ml.

The ingredients were dissolved in 1000 ml distilled water and pH adjusted to 8.3 with 0.1N HCl, if required.

5. Saline-Tween (S-T20) :

Sodium chloride	9.0 g
Tween-20	0.5 ml.
Distilled water	1000 ml.

Sodium chloride was dissolved in 1000 ml distilled water to which 0.5 ml Tween 20 was added and mixed thoroughly.

6. Phosphate buffered Saline-Tween (PBS-T)

Tween-20	0.5 ml.
Phosphate buffered saline (PBS)	1000 ml.
(0.15 M, pH 7.2)	

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

Na_2CO_3	0.159 g (0.015 m)
NaHCO_3	0.293 g (0.035 m)
Distilled water	100 ml.

Both the salts were dissolved in 100 ml. distilled water and pH adjusted by addition of monobasic or dibasic salt solution at same molarity. Prepare fresh buffer every few days.

8. Substrate buffer-Sodium citrate buffer (0.15 M, pH 6.0)

Citric acid	2.1 g
Sodium citrate	2.4 g
Distilled water	100 ml.

Both the salts were dissolved in 100 ml. distilled water and pH adjusted by addition of either salt or acid solution of same molarity. Prepare fresh buffer every few days.

9. Scintillation Fluid :

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

PPO and POPOP were dissolved in 800 ml. toluene in a volumetric flask mixed thoroughly.

After complete dissolution the volume was made to 1.0 litre with toluene.

10a. Substrate solution (for TMB substrate) :

1 mg TMB (3,3', 5,5' - Tetra methylbenzidine) (Sigma) was dissolved in 10 ml. of sodium citrate buffer (0.15 M, pH 6.0). After complete dissolution of TMB, just before use H_2O_2 was added to this solution to final concentration of 2 μl . of 30% H_2O_2 /10 ml. The solution was mixed gently and immediately used.

10b. Substrate solution (for OPD substrate)

4 mg OPD (O-phenylenediamine dihydro-chloride) (Sigma) was dissolved in 10 ml. of sodium citrate buffer (0.15 M, pH 6.0). After complete dissolution of OPD, 2 μl . of 30% H_2O_2 solution was added to above 10 ml solution and mixed gently. The substrate solution is immediately used.

Both substrate solutions (10a and 10b) must be made up freshly each time (just before use) as they are rapidly inactivated in aqueous form. They are also lightactivated, so wrap the container completely in aluminium foil as soon as prepared. Use disposable gloves to handle OPD in powder form.

APPENDIX - II

1. Estimation of protein by Lowry's method :

Reagents :

Reagent A-1

Sodium potassium tartarate (hydrated)	2.0 g
Distilled water	100 ml.

The tartarate was dissolved in 40 ml distilled water in a volumetric flask and the volume was made upto 100 ml.

Reagent A-2

Cupric sulphate (hydrated)	1.0 g
Distilled water	100 ml.

The copper salt was dissolved in 40 ml distilled water in a volumetric flask and the volume was made up to 1000 ml. with the same.

Reagent A-3

Sodium carbonate	20.0 g
Sodium hydroxide (0.1N)	1000 ml

The salt was dissolved in 400 ml of the alkali in a 1000 ml volumetric flask and the volume was made up to 1000 ml with the same.

Reagent A - 0.5 ml. A-1 + 0.5 ml A-2 + 50 ml A-3.

Reagent A was prepared just before use and the remainder, if any, discarded after the assay.

Reagent B :

Phenol reagent (Folin-ciocalteu's reagent) was purchased from Sisco Research Laboratories. It was diluted 1 : 2 in distilled water just before use.

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 was taken to which 0.1, 0.2, 0.3, 0.4 and 0.5 ml. of standard BSA was added in duplicate. The volume of each tube was made upto 0.5 ml. with distilled water. Blanks of 0.5 ml. distilled water were also set up. 0.5 ml. of appropriate dilution of test sample was added in duplicate.

2.5 ml 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 (OD) readings were taken at 660 nm. adjusted against the distilled water blank. A graph of OD against protein concentration was plotted from which the protein concentration of the test sample was calculated.

2. Siliconisation of glassware

The glassware to be siliconised was washed thoroughly and dried. The glassware was rinsed with Sigmacote (silicone 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.

3. Preparation of IgG fraction from serum :

The IgG fraction was separated from 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, keeping the mixture being gently stirred at 4°C. After 30 min. the solution was centrifuged at 10,000 g for 30 min. The supernatant discarded and the precipitate was resuspended in the buffer of choice. This solution was dialysed against the same buffer extensively to remove SO_4^{2-} ions. The absence of

SO₄⁻² ions was tested by BaCl₂ precipitation test. The protein content of the IgG fraction was estimated both by spectrophotometry (extinction coefficient for 1.0 mg/ml solution at 280nm taken as 1.4) and by Lowry's method.

4. Standardisation of Conjugate dilution :

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

1. Solution of the required antigen (for example, mouse IgG for anti-mouse IgG HRP conjugate) was prepared in the coating buffer at concentrations 1 µg/ml, 5µg/ml. And 10 µg/ml.
2. 100 µg/ml BSA solution in coating buffer was also prepared.
3. 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 to test the various dilutions of the conjugate.
4. Plate was covered and incubated in a moist storage box overnight at 4°C.
5. Next day, the well contents were discarded and the unbound excess coating solution was removed by banging the inverted plate several times onto a pad of paper towelling.
6. All the wells were filled with 2% Gelatin, the blocking solution and incubated at 37°C for 1½ h.
7. The plates were washed 5-6 times with saline-tween 20.
8. 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.
9. 100 µl of each conjugate dilution were added to the wells. PBS-Tween 20 was added to a set of wells as conjugate blank and incubated at 37°C for 1 h.
10. The plate was then washed 5-6 times and 100 µl of the substrate solution (TMB + H₂O₂) was added. The plate was kept in dark at room temperature for 10-12 min.

11. When optimal colour developed, the reaction was stopped with 50 μ l. of 2M H_2SO_4 and absorbance was read at 450 nm in Elisa plate reader.

12. A_{450} values were plotted against the dilution of conjugate.



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SYNOPSIS

A SYNOPSIS OF THE THESIS
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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN MICROBIOLOGY

TITLE OF THE THESIS : STUDIES ON T CELL SUBSET
DIFFERENTIATION FOLLOWING
IMMUNISATION BY DIFFERENT
ROUTES WITH *M. vaccae*.

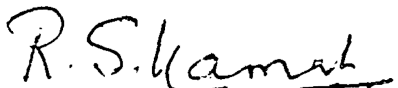
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INTRODUCTION

Immune response to pathogens consists of two major effector mechanisms namely humoral and cell mediated immunity. However , both the effector mechanisms may not have a protective role in a given infection simultaneously. It is also possible that a given infection or a given mode of immunisation selectively generates either cellular or humoral immunity^{1,2}. Leprosy and tuberculosis represent classical examples of such distinct patterns of immune responses to the same pathogenic agent in different individuals. The polar forms of lepromatous and tuberculoid leprosy and similar cases of tuberculosis show differential generation of humoral and cellular responses respectively^{3,4}. In tuberculoid leprosy, eventhough antibody levels are low, the cellular immunity is good, while in lepromatous leprosy in spite of high antibody levels the cellular response is poor.

As the CD4 + T cells are the principal controlling elements for immune response to T-dependent antigens, they form the basis for heterogeneity of humoral and cellular immunity. Recent studies of murine CD4 + T cell clones by Mosmann et al have shown that CD4 + T cells can be divided into two subsets - TH1 and TH2 - with distinct, and non overlapping patterns of lymphokine secretion ^{5,6}. TH1 cells produce IL-2, γ -IFN and TNF whereas , TH2 cells secrete IL-4, IL-5, IL-6 and IL-10. It has been reported that TH1 cells

mediate delayed type hypersensitivity (DTH)⁷. γ -IFN produced by TH1 activates macrophages^{8,9,10} and induces B cell proliferation^{11,12}, enhances IgG1 and IgE production¹³ and induces increased expression of MHC antigens on B cells¹⁴. These functional differences between TH1 and TH2 cells suggest that they are involved in cellular and humoral immune responses respectively.

It follows therefore that the heterogeneity of immune responses may result from the induction of TH1- and TH2-like effector CD4 + T cells by different antigens or immunisation conditions leading to the production of cytokines with distinct effector functions. In polar forms of leprosy, selective expansion of TH1 and TH2 subsets has been reported^{15,16}. However, the underlying mechanisms for the selective T subset expansion in these conditions are not understood. Hence, to understand the underlying mechanisms for the selective generation of cellular or humoral response in mycobacterial infections, following aims and objectives were set-up.

Aims and Objectives :

1) To set up an experimental model in which there is selective expansion of TH cell subsets following immunisation with *Mycobacterium vaccae*.

2) To study the regulatory mechanisms for the selective expansion of T cell subsets.

MATERIALS AND METHODS

1. Microorganisms:

Mycobacterium vaccae was kindly supplied by Dr. J. L. Stanford of Middlesex Hospital, London. The strain was maintained on L. J. slants and cultivated on the medium of Doub and Youmans ¹⁷.

2. Animals :

8-10 week old Balb/c and C3H/HeJ mice obtained from National Institute for Health, U.S.A. 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, U.S.A. and maintained in the animal colony of Haffkine Biopharmaceutical Corporation Limited, Bombay were used for studies in random bred animals. Prior to immunisation, all mice were tested for delayed type hypersensitivity (DTH) response to the sonicate of *M. vaccae*. Only those animals that showed no response were used in the experiments.

3. Immunisation :

A vaccine of one week old culture of *M. vaccae* was prepared, so as to contain 10^8 Γ -irradiated bacilli in 0.05 ml or 0.5 ml volume of phosphate buffered saline (PBS) containing 0.05% Tween-80, employing the method described

earlier ¹⁸. The former was used for intradermal (id) and the latter for intraperitoneal (ip) immunisation respectively.

4. Preparation of antigen:

i) Preparation of sonicate:

A one week old culture of *M.vaccae* in the medium of Doub and Youmans was killed by Γ -irradiation as before. The killed cells were washed with PBS and disrupted in a sonicator (Branson Sonifier, B-30) and spun at 50,000 g for 1 h. The supernatant was collected, filtered through 0.22 μ m membrane filter (Millipore, USA) and its protein content estimated by the method of Lowery et al ¹⁹. After confirming sterility the solution was aliquoted and stored at -40° C and used as "test antigen".

ii) Preparation of 65 kDa antigen bearing particles :

65 kDa antigen of *M.vaccae* was purified from the sonicate and nitrocellulose membrane particles bearing adsorbed 65 kDa antigen were prepared as described earlier²⁰. The suspension of particles in RPMI was titrated for use in lymphoproliferation assay employing spleen cells of mice primed i.d. with *M.vaccae* and optimum particle concentration was determined with each batch of 65 kDa antigen.

5. Delayed type hypersensitivity (DTH) test :

DTH test was done by the method of Grey and Jennings²¹.

6. Lymphoproliferation assay :

Lymphoproliferation assay was carried out employing standard microculture technique²².

7. Separation of T cells :

This was done by the method of Julius et al²³.

8. Peritoneal cells :

These cells were collected from normal Balb/c mice and those treated with an intravenous dose of 100 µg of poly I : poly C, 3 h earlier. The mice were given an i.p. injection of 8.0 ml Hank's balanced salt solution (HBSS) and then killed. Their abdomen were massaged gently and within 5 minutes of killing the contents were aspirated by a sterile pasteur pipette. Aspirates of several mice were pooled, the cells washed thrice and viable cell number estimated.

9. Irradiation of spleen and peritoneal cells for use as antigen presenting cells :

Spleen and peritoneal cells of mice were subjected to 800 rad gamma radiation in a ⁶⁰Co source. The irradiated cells were washed in RPMI 1640 and used as APCs.

10. Antigen pulsing of peritoneal cells :

4×10^5 peritoneal cells were suspended in 2.0 ml of the culture medium RPMI 1640 with 10% Foetal Calf Serum,

containing 200 μ g of sonicate of *M.vaccae* and incubated for 2 h. They were then washed three times and the final pellet suspended in the culture medium.

11. Paraformaldehyde fixation of peritoneal cells :

This was done by the method described by Allen and Unanue ²⁴. Briefly, antigen pulsed or non-pulsed 4×10^5 peritoneal cells were suspended in 2.0 ml RPMI containing 1% paraformaldehyde (Analytical grade, SIGMA) and incubated at 20°C for 15 min. At the end of incubation period they were washed three times, incubated again at 37°C in the culture medium for 30 min. and washed again three times.

12. Assay for anti-65 kDa antibody levels :

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 100 μ l/well of the antibody solution (IgG concentration 250 μ g/ml) overnight at 4°C. Next day the well contents were discarded and wells were blocked with 2% Gelatin for 1 h at 37°C. Then they were washed 5-6 times and 100 μ l of lysozyme - papain digested *M. vaccae* sonicate (1 mg/ml) was added to the wells and incubated for 1 h at 37°C. The wells were then washed 5-6 times and 100 μ l of different dilutions of test mouse serum samples along with appropriate controls (positive control serum and PBS-Tween 20 and normal mouse serum as negative controls) were added and

incubated for 1 h at 37°C. These were washed 5-6 times. 100 μ l of anti-mouse HRP conjugate (dilution 1:10,000) was added to the wells and incubated for 1 h at 37°C. The plate was then washed 5-6 times and 100 μ l of substrate solution (TMB - 1mg/10ml, H_2O_2 - 2 μ l/10 ml) was added to the wells and kept at room temperature in the dark for 10-15 min. The reaction was stopped with 2M H_2SO_4 and read at 450 nm.

13. Flow cytometry :

Peritoneal exudate cells of normal, poly I : poly C or LPS treated Balb/c mice were studied for expression of the co-stimulatory molecule B7 by flow cytometry. The cells were stained with phycoerythrin (PE) anti-B7 commercially obtained from Pharmingen. In order to identify the cell type, the cells were also stained with Fluorescein labelled anti-kappa 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

1. DTH, lymphoproliferation and antibody responses against 65 kDa antigen of *M. vaccae* following i.d. and i.p. immunization with *M. vaccae* :

Two sets of mice (outbred Swiss white and inbred Balb/C) were immunised by i.d. or i.p. route with 10^8 killed *M. vaccae* cells. One week later the DTH response and anti-65 kDa antibody levels were estimated in the outbred mice. Simultaneously lymphoproliferative response was assayed in the Balb/c mice using *M. vaccae* sonicate as antigen.

Single i.p. immunisation generated a poor DTH and a poor lymphoproliferative response and a high anti-65 kDa antibody response; while single i.d. immunisation generated good DTH and lymphoproliferative responses but low anti-65 kDa antibody levels.

Three i.p. immunisations generated high antibody levels as before; however, three i.d. immunisations also generated high antibody levels, thus making the difference in the antibody levels between two modes of immunisation non-significant. The pattern of DTH and lymphoproliferative responses after three immunisations by i.p. or i.d. route remained the same as in single immunisation.

This was considered as a preliminary evidence showing differential generation of humoral and cellular responses after i.p. and i.d. immunisation respectively.

2. Lymphokine profiles of i.d. and i.p. primed effectors :

To obtain the confirmatory proof for selective expansion of TH1 or TH2 subsets after priming , the lymphokine profile generated by i.d. and i.p. primed effectors was studied by using commercial Elisa kits.

Balb/c mice were primed by i.d. or i.p. route. One week later 5×10^5 splenic T lymphocytes were cultured with equal number of normal irradiated splenic lymphocytes as APCs and *M. vaccae* sonicate as antigen. Culture supernates were harvested after 36 h and assayed for IL-2, IL-4, IL-10 and Γ -IFN by Elisa, employing commercial kits (Genzyme and Pharmingen).

Both i.d. and i.p. primed effector T cells secreted all four lymphokines IL-2, IL-4, IL-10 and Γ -IFN thus indicating TH0 type of differentiation.

However, i.p. primed T effectors secreted considerably higher amounts of IL-10 compared to i.d. primed T effectors.

3. DTH, lymphoproliferation and antibody responses of poly I : poly C treated i.p. primed mice :

Balb/c mice were treated with 100 μ g/mouse of poly I : poly C i.v. and 3h later they were immunised by i.p. route with *M. vaccae*. One week later their DTH, lymphoproliferative responses and the anti-65 kDa antibody levels were assayed.

Poly I : poly C treatment of animals induced them to mount a DTH and a lymphoproliferative response on i.p. immunisation and the antibody response was lowered .

Lymphokine profile of T effectors generated in poly I : poly C treated i.p. primed mice were also studied as before.

5×10^5 splenic T lymphocytes of poly I :poly C treated , i.p. primed mice were harvested, cultured with equal number of normal irradiated spleen cells as APCs and M. vaccae sonicate as antigen for 36 h. The culture supernates were harvested and assayed for different lymphokines using commercial Elisa kits.

The IL-2 levels secreted by effector T cells of poly I : poly C treated animals were same as those of untreated animals, though the primed T cells of treated animals showed excellent antigen induced proliferation; whereas, those of untreated animals did not. The IL-2 levels in the cultures of primed T cells of poly I : poly C treated animals were post-utilisation for proliferation, implying that poly I : poly C treatment induced higher levels of IL-2 secretion by T cells. The other lymphokines showed the same levels as those seen with effectors of untreated animals.

4. Role of CD8 + T cells in T helper cell subset differentiation after i.p. immunisation :

It has been reported earlier²⁰ that i.p. immunisation expands CD8 + T cells, recognising the

65 kDa antigen of *M. vaccae*. The primed CD8 + cells have been shown to suppress generation of DTH and lymphoproliferative response of the animals. The primed CD8 + T cells were also shown to compete with CD4 + T cells for IL-2²⁰. One may hypothesise that this possibility leads to higher concentration of IL-4 in the lymphoid milieu which may drive differentiation of CD4 + T cells along TH2 pathway.

To test this possibility Balb/c mice were immunised by i.p. route. One week later their spleen cells were transferred into syngeneic mice. The recipients were later immunised by i.d. route with *M. vaccae*. One week later the DTH response and anti-65 kDa antibody titre of the above animals were assayed.

The i.d. primed mice showed a good DTH response and a low antibody response as before. In contrast, the mice receiving i.p. primed cells before i.d. immunisation showed poor DTH and the antibody responses were suppressed. Hence it was concluded that CD8 + T cells could not drive the CD4 + T cell response to TH2 pathway in this experimental model.

These data indicate that both i.p. and i.d. immunisation generate TH0 type of response; however, TH2 component of the response of i.p. immunised animals is greater. Poly I : poly C treatment which induces α -interferon secretion appears to enhance TH1 component.

5. Antigen presentation by splenic and peritoneal cells to primed splenic T cells :

10^5 splenic T lymphocytes from i.d. primed Balb/c mice

were cultured with equal number of normal irradiated spleen cells or peritoneal cells as APCs and *M. vaccae* sonicate as antigen for 6 days and proliferative response was observed.

Irradiated spleen cells supported good lymphoproliferation; whereas, peritoneal cells did not support lymphoproliferation. This indicated that splenic cells function as efficient APCs; whereas peritoneal cells do not.

Antigen presentation by poly I : poly C treated peritoneal cells was also studied.

Balb/c mice were treated with two doses of poly I : poly C 24 h apart. 3 h after the second dose peritoneal cells were harvested, pulsed for 2 h with *M. vaccae* sonicate as antigen and fixed with paraformaldehyde (This was done to avoid the effect of lymphokines secreted by peritoneal cells on stimulation with poly I : poly C). The lymphoproliferative response of i.d. primed T cells cultured with these APCs was studied.

It was observed that poly I : poly C treated peritoneal cells supported good lymphoproliferation unlike the normal peritoneal cells.

The above data gives rise to the question whether antigen presentation by peritoneal cells is responsible for TH2 differentiation.

6. Lymphokine profile of T effectors generated *in vitro* in presence of different APCs and primed T cells :

Balb/c mice were primed i.d. with *M. vaccae*.

One week later splenic T cells were harvested and cultured with equal number of different cell suspensions to serve as APCs for 7 days with *M. vaccae* sonicate as antigen. The APCs used were -

- 1) Normal irradiated spleen cells.
- 2) Normal irradiated peritoneal cells.
- 3) Normal irradiated dermal lymph node cells.
- 4) Normal irradiated mesenteric lymph node cells.
- 5) Poly I : poly C treated fixed peritoneal cell.

On 7th day culture supernates were harvested and different lymphokines assayed by commercial Elisa kits. Simultaneously the *in vitro* antigen induced lymphoproliferative response of primed T cells with same APCs was also assayed.

IL-2 levels induced by different APCs were similar. However, spleen cells and poly I : poly C treated peritoneal cells induced excellent proliferation; whereas, the non-treated peritoneal cells did not. IL-2 levels induced by spleen cells and poly I : poly C treated peritoneal cells were post-utilisation for proliferation. This implies that poly I : poly C treatment of peritoneal cells improves their antigen presentation efficiency which enables T cells to secrete adequate IL-2.

Eventhough effectors generated by peritoneal cell APCs secreted very little IL-2 and IL-4, they secreted considerable amount of IL-10. This is highly significant, favouring the hypothesis of TH2 differentiation when antigen is presented by peritoneal cells.

7. B7/BB1 expression⁶ by different cells in the peritoneal exudate :

Peritoneal cells of Balb/c mice were studied for B7 expression by Flow cytometry. 42.63% cells were B7 positive, kappa positive (B cells) and 5.78% cells were B7 positive, kappa negative (non-B cells). Also when these cells were analysed for expression of IgM and IgD, 27.19% cells were IgM positive, IgD negative; while 20.77% cells were IgM positive, IgD positive.

From this data it was concluded that the majority of cells expressing B7 in the peritoneal exudate of normal animals are B cells and only about 5% of non-B peritoneal cells express B7.

In the peritoneal exudate of poly I : poly C treated animals, 63.98% cells were kappa positive, B7 positive and 12.49% cells were kappa negative, B7 positive. In the peritoneal exudate of LPS treated animals, kappa positive, B7 positive cells were 74.58% and kappa negative, B7 positive cells were 1.28%.

From these results, it was concluded that poly I : poly C treatment increased B7 expression by non-B cells,

whereas, LPS treatment increased B7 expression by B cells.

Based on these results the following hypothesis has been proposed.

Immunisation with killed *M. vaccae* vaccine generated predominantly TH0 type of response, i.p. priming favoured a significant TH2 component. This effect was a consequence of antigen presentation by the peritoneal cells. The B cells and the macrophages of the peritoneal exudate of normal mice expressed B7, implying that both were capable of presenting antigen. Poly I : poly C₁ almost doubled the number of B7 expressing macrophages. This could explain the *in vivo* effect of poly I :poly C treatment, enabling the mice to respond to i.p. immunisation by mounting a DTH response. Almost half the B cells in the peritoneal exudate were IgM positive and IgD negative, implying that they were B1 B cells. These were B7 positive, indicating that they were capable of presenting antigen. B1 B cells have been reported to secrete IL-10 constitutively ^{25,26}. This would explain, TH2 type of response when effectors were generated *in vitro* with peritoneal cells as APCs. The LPS treatment increased the B cell fraction expressing B7 in the peritoneal exudate.

Based on this, one may conclude that the LPS of the normal gut flora may activate B1 B cells in the gut associated lymphoid tissue. A gut to peritonium traffic of B1 B cells has been reported ²⁷.

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