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A NOVEL APPROACH TOWARDS INDUCTION AND EVALUATION OF ANTI-INFECTIOUS AND ANTI-INFLAMMATORY IMMUNE RESPONSE AGAINST SCHISTOSOMIASIS MANSONI

MEKURIA LAKEW

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A novel approach towards induction and evaluation of anti-infectious and anti-inflammatory immune response against schistosomiasis mansoni

by

Mekuria Lakew

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Department of Biology
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MEKURIA LAKEW

Department of Biology, Faculty of Science

Approval by:

Dr. Joseph Olobo
External examiner

Dr. Leyikun Jemaneh
Internal examiner

Dr. Beyene Petros
Research advisor

Dr. Masresha Fetene
Chairman, Dept. of Biology

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This dissertation is dedicated to our beloved late mother W/ro Hadas G/Hiwot, who sacrificed everything in her possession including her life to see us, her children, educated. This is an offer to her relentless and unfailing love.

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ABBREVIATIONS

ADCC	=	Antibody dependent cytotoxicity
APC	=	Antigen presenting cell
ASC	=	Antibody secreting cells
AWA	=	Adult worm antigen
BALT	=	Bronchus associated lymphoid tissues
BSA	=	Bovine serum albumin
CLA	=	Cutaneous lymphocyte associated
CT	=	Cholera toxin
CTB	=	Cholera toxin-B
CTB-GST	=	Cholera toxin B bound glutathione S-transferase
CTB-SEA	=	Cholera toxin B bound soluble egg antigen
DTH	=	Delayed type hypersensitivity reaction
DTT	=	Dithiotheitol
ELAM	=	Endothelial leucocyte adhesion molecules
ELISPOT	=	Enzyme linked immunospot
FCS	=	Fetal calf serum
FDC	=	Follicular dendritic cells
G-CSF	=	Granulocyte colony stimulating factor
GALT	=	Gut associated lymphoid tissue
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
GC	=	Germinal centre
GIT	=	Gastro-intestinal tract
GM-CSF	=	Granulocyte macrophage colony stimulating factor
GM1	=	Ganglyoside on all nuclear cells
ICAM	=	Intracellular activation marker
IEL	=	Intra-epithelial lymphocytes
INF	=	Interferon
LECAM	=	Leucocyte adhesion molecules (selectin)
LPL	=	Lamina propria lymphocytes
M-CSF	=	Macrophage colony stimulating factor
MadCAM	=	Mucosal associated cell adhesion molecule
MALT	=	Mucosal associated lymphoid tissue

MCP = Monocyte chemotactic protein

MHC = Major histocompatibility complex

MLN = Mesenteric lymph node

MNC = Mono-nuclear cell

NALT = Nasal associated lymphoid tissues

NK = Natural killer

PBL = Peripheral blood lymphocytes

PGE2 = Prostaglandin E-2

PHSC = Pluripotent haematopoietic stem cells

PP = Peyer's patches

RPM = Rounds per minute

SCF = Stem cell factor

SEA = Soluble egg antigen

Sm28GST = Schistosoma mansoni derived Glutathione-S-Transferase

SmPGK = Schistosoma mansoni phosphate glycerate kinase

SPDP = N- Succinimydyl (3[2-pyridyl] dithio) propionate

TCR = T-cell receptor

TdT = Terminal deoxy-nucleotidyl transferase

TGF = Transforming growth factor

TH = T-helper

TNF = Tumour necrotizing factor

TPI = Triose phosphate Isomerase

TT = Tetanus toxoid

VCAM = Vascular cell adhesion molecule

VLA = Very Late Antigen

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ABSTRACT

In humans, mucosal stimulation, like in systemic immunization, results in a transient appearance of B-cells capable of spontaneously producing antibodies. These cells are cells *en route* to their final destinations. They primarily go to the sites they were initially stimulated but also to other selected effector sites within the common mucosal immune system. During the migration, they express specific surface molecules that represent the sequence of developmental events taking place in the cells. Using these phenotypic cell markers B-cells have been divided into discrete populations that span the developmental stages. On the basis of these phenotypic markers, the migratory B-cells are sorted into functional groups. To achieve this, a novel approach that combines immunomagnetic beads with ELISPOT was developed. This method was applied to characterize the phenotypes, activation pattern and homing commitments of B-cells after oral (Cholera Toxin CT) and systemic (Tetanus Toxoid - TT) vaccination. Following its success in the known systems, CT and TT vaccines, it was used to describe immunity in chronically infected patients with diseases such as leishmaniasis, tuberculosis and schistosomiasis, whose pathology involves immunological hypersensitivity reaction (DTH). This method was shown to be capable of describing the immune status of an infected individual in terms of the phenotype and function of antibody secreting cells (ASC). The fact that the distinction could be made between mucosally and systemically activated B-cells by differential expression of homing receptors (L-selection, CD44 and $\alpha 4\beta 7$) is an additional tool to evaluate the contribution of Mucosal associate Lymphoid tissue (MALT) in disease causation and modulation of the immune system under natural infections. The comparison of mucosal immune responses following different routes of immunization was facilitated by the development of the cellular dispersion technique. The technique made the parallel analysis of cellular composition of lymphoid tissue such as the tonsil feasible. Induction from oral, intra-nasal, tonsillar and rectal routes of immunizations were compared. The intra-tonsillar and intra-nasal routes of immunizations were found to be capable of acting as induction sites for local and distant B-cell responses. The peroral and parenteral immunization of activated B-cells into the circulation, intra-tonsillar vaccination is highly localized. In the study of schistosomiasis, the CD19 surface

marker was used for functional characterization of the types of antigen specific immunoglobulin secreting cells. Accordingly, IgM > IgA > IgG secreting cells were found in the infected, while the reverse was characteristic of the control group. IgM dominance in acutely infected individuals was statistically significant ($P < 0.01$). Analysis of the result by age and intensity of infection revealed IgA secreting cells to be dominant in older age (> 15 years) and in those with light infections while in those that are under 15 years of age, IgM secreting cells to be dominant. Attempt to use the tonsils as induction sets to the common mucosal immune system resulted in highly localized response making it less interesting in this respect. Instead the intranasal route was found to be convenient to reach distantly related mucosal sites. In the immunological characterization of *S. mansoni* in the BALB/c mice, it was shown that following mucosal immunization with CTB-Sm28GST vaccine, parasite induced pathology, number of eggs and worms were reduced by more than 50%, 84% and 66%, respectively. This strongly suggested a strategy for induction of anti-infectious and anti-inflammatory immune response which could lead to development of a vaccine-mediated control measure. Such a strategy may be applicable not only to schistosomiasis but also to other diseases such as leishmaniasis, leprosy, and tuberculosis, that induce similar delayed type high resistivity (DTH) responses as an immunopathological mechanism. This work has improved the technique of assessing the effect of vaccines, and has evaluated the means of delivery of antigens that induce either stimulation and/or tolerance at systemic and mucosal levels. The technique is of a broader application to chronic disease with similar immunologic responses on mechanisms of pathogenesis.

I. INTRODUCTION

1.1 Epidemiology of schistosomiasis

Schistosomiasis is a debilitating water borne disease affecting nearly 10 % of the world population and threatening more than 600 million people who may contract it as they perform their daily duties in and around fresh water bodies. There are 3 major species of schistosome parasites: *Schistosoma mansoni, S. haematobium, S. japonicum and two minor species* confined in a few countries *S. intercalatum* and *S. mekongi* affecting man. On rare occasions other species that normally infect other hosts, *S. bovis, S. curassoni, S. margrobowiei, S. matthei and S. rodhani* can parasitize humans (Voge *et al.*, 1978; Majid, 1980).

The life cycle of schistosome parasites alternates between humans and snail hosts. The cercaria penetrate the skin and transforms into the next larval stage, the schistosomulum, that stays for a few days under the skin. Subsequently it migrates to the lung via the blood and the lymphatics. In the process of migration the larvae mature to adult male and female worms and descend to the liver. Young adults in copula move to the hepatic portal vein or attach to the wall of the mesenteric veins. Successful completion of the life cycle depends on release of embryonated eggs by the worm in copula, availability of susceptible intermediate hosts and a suitable environment. Most of the body mass and large portion of the energy consumed by the worm is devoted for reproduction of eggs. They convert nearly equivalent of their dry weight to eggs each day. During their 5-10 years of average life span in humans, each couple of *S. mansoni* can produce up to 40 million eggs (Clegg, 1965).

The initial pathology due to schistosomiasis are similar in all species. This includes cutaneous lesions at the site of cercarial entry due to tissue reaction to shistosomula under the skin and toxic and allergic reactions to immature and mature adults residing inside and outside the blood vessels. The early symptoms are fever, fatigue, urticaria and eosinophilia. The most serious pathology, however, arises from eggs deposited by mature worms residing in the liver and the mesenteric veins of the host. About 50% of the eggs produced by these worms (300 eggs/day) are passed out with the faeces while the rest are trapped in the Peyers Patches the major induction site of Gut Associated Lymphoid Tissues (GALT) or are carried through the portal system to the liver (Weinstock and Blum, 1987). The bloody mucoid diarrhoea and the granulomatous lesions causing hepatosplenomegaly, with abdominal tenderness, anorexia,

loss of weight, fever and weakness at 6-8 weeks after infection are the results of the eggs deposited in the liver and the mucosa. The eggs passing through the vascular epithelium apply pressure and secrete collagenase-like enzymes that cause endothelial and sub-endothelial inflammatory reactions. These reactions in some individuals cause polyps and pseudo-papilloma in mucosa and submucosa extending to the inside and resulting in chronic obstruction and blood loss (Demette and Sproat, 1955).

Chronically, schistosomiasis mansoni produces scaring (Symmer's pipestem fibrosis) in the liver and portal venules leading to portal hypertension that subsequently develop into splenomegaly, ascites and oesophageal and gastric varices. The portal hypertension and systemic shunting due to the granuloma at the hepato-intestinal level occasionally results in bleeding of the oesophageal varices or renal failure that could lead to death (Wilson, 1990). The splenomegaly could also give rise to hypertension with anaemia, leukopenia and thrombocytopenia.

The full course of the disease as characteristically described above presents only in less than 10% of infected individuals. In the rest 90 %, the granuloma builds up in a gradual manner for some time and spontaneously down-regulates without much symptoms. The severe manifestation of the disease both at the acute and chronic level is seen more in countries like Brazil, the Sudan and Egypt (Manson-Bahr and Bell, 1987; Cheever *et al.*, 1978).

The control of the disease at the moment depends on chemotherapy, snail control, health education, water engineering and sanitation. Although these measures are quite efficient in their short term effects, the need to use them repeatedly over a long period of time has made their utility more difficult both from the economic point of view and the emergence of drug resistant strains of the parasites and the snails.

As an alternative strategy, workers over the last decade have been engaged in an attempt at developing anti-inflammatory and or anti-infectious vaccines. A panel of antigens of all stages of the parasite have been screened and a number of candidate vaccines employing vast array of immunoregulatory mechanisms have been defined. Among these are radiation attenuated cercariae, Paramomysin, Glutathione-S-Transferase (Sm28GST), Triose Phosphate Isomerase (TPI), Phosphoglycerate kinase (SmPGK) and Glyceraldehyde 3-phosphate dehydrogenase

(GAPDH). All the antigens have been shown to provide partial protection against infections on experimental hosts (reviewed in Dunne *et al.*, 1995).

Epidemiological observations suggest that a similar protective immunity allowing rejection of the adult worm, preventing reinfections and immunomodulation of granulomatous reaction exists in humans (Butterworth *et al.*, 1988). Studies from Kenya, Gambia and Brazil (Wilkin *et al.*, 1984a&b; Butterworth *et al.*, 1988), have reported the association of IgA antibodies specific to Sm28GST and the fractions of major B-cell epitope (aa115-131). Association of high levels of age specific prevalence and intensity of infection and acquisition of resistance to reinfection have also been established. IgA specific to Sm28GST has been shown to reduce worm fecundity and egg viability in experimental infections and *in vitro* studies (Grzych *et al.*, 1993; Grezeel *et al.*, 1993). It has also been demonstrate that IgG and IgE antibodies to soluble egg antigen (SEA) in mice and *in vitro* contribute to protective immunity through the killing of schistosomula.

The basis for production of the protective and down regulating IgA antibody is known to be the human mucosal compartments specially the GALT on which the female worms from mesenteric vein deposit eggs to be excreted with the faeces. During this time some eggs are trapped in Peyer's Patches which are the major induction site for IgA response. These observations suggest the involvement of IgA and the mucosal immune response in the dynamics of vigorous granulomatous inflammation in acute phase of the disease and spontaneous down-sizing of it in chronic phase and its relationship to intensity of infection and age in humans. By taking these facts into account, recombinant Sm28GST, which is known to reduce worm fecundity and egg viability *in vitro*, could be delivered to a susceptible host with the view to developing an anti-infectious and an anti-inflammatory vaccine for schistosomiasis mansoni.

With regard to human response, it is clear that the mechanisms which regulate the evolution of immunopathology and the modulation processes vary significantly from species to species of schistosome parasites and results from animal studies cannot be directly applied or extrapolated to humans. For example, Sm28GST specific antibodies are not involved in protection in the mouse model whereas Sm28GST specific T-cells were shown to be protective in both humans and mice.

Due to lack of methods that allow detection of changes in the human immune system, most information on host immune responses against infectious agents are generated from animal studies. Thus it was thought that a proper understanding and appreciation of the mechanisms in mammalian immune system and developing a method that allows the detection of changes in human immune system during natural infection or following vaccination, are two steps necessary in order to set a reasonable vaccine strategy.

1.2 The mammalian immune system

1.2.1 The systemic immune response

The immune system is the means through which the body recognizes its own constituent as self and protects the body from non-self by clearing foreign particles. Its main role is to protect the organisms from infectious agents like, bacteria, fungi, parasites and from the consequences of infections. The foreign particles could be the products of the infecting organisms but also native cells that have undergone neoplastic transformation or fragmentation because of one or other reasons. To accomplish these functions, mammals through cell to cell interaction and release of cytokines and transfer factors from the stromal micro-environment give rise to two sets of genetically committed distinct cell linages. Both the linages arise from a common pluripotent haematopoietic stem cells (PHSC) located in the bone marrow (Fleming *et al.*, 1993) (Fig. 1).

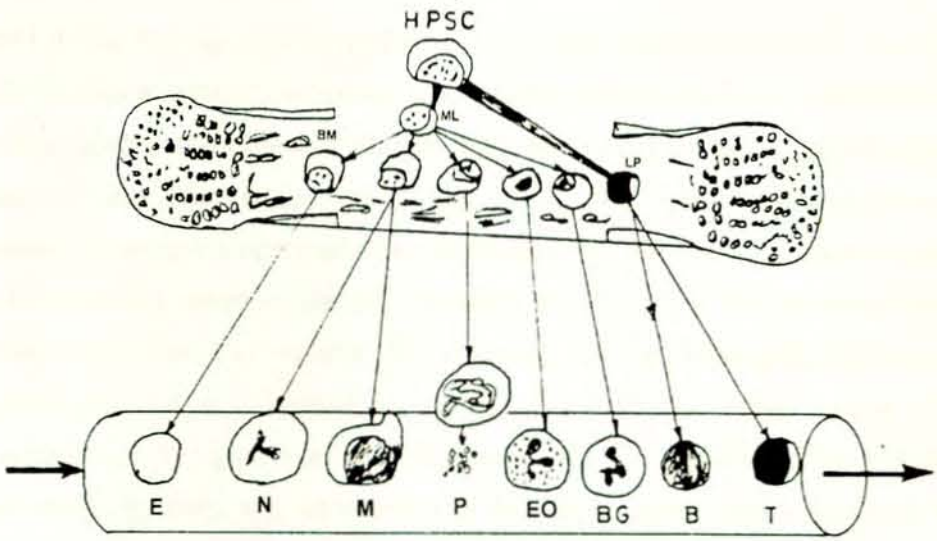


Fig. 1. **A scheme of Haemopoiesis:** E-Erythrocytes, M-monocytes, P-Platlets, N-Neutrophilic, EO-Eosinophil, BG-Basophil, LP-Lymphocyte Progenitor. HPSC-Haematopoietic stem cells, BM-Bone marrow, ML-Myelomonocytic linage.

The first component belongs to the myeloid linage. The cells that belong to this group are equipped with receptors for a wide range of microbial antigens and are non-specifically protecting the body from harmful pathogens. They are found distributed in different tissues. The neutrophils, eosinophils, basophils, monocytes and thrombocytes in the blood; histocytes in connective tissues; meralgia in the brain; and the rest in the cells of the sinuses, the liver, spleen, adrenals, bone marrow, lymph nodes, tonsils, Peyer's patches, appendix, lumen and the thymus. The neutrophils provide the first line of defence against invading organisms in the circulatory system, in the bone marrow and at active infection sites. They use toxic form of oxygen to kill ingested or formed particles as bacteria and degenerating cells. Eosinophils are cells that increase in number during allergic response and parasitic infections. Mast cells are non-motile connective tissue cells found adjacent to capillaries throughout the body and basophils are motile white blood cells making up 1% of the total leucocytes. The latter two cell types when bound to antigen bound IgE antibody trigger the release of mediators like serotonin and histamine that are responsible for allergic reactions. Most phagocytic cells are the monocyte/macrophages. To these, are added the natural killer cells that display both myelomonocytic and lymphocytic characteristics and are not phagocytic. They can kill antibody coated nucleated cells, tumor cells, or virally infected cells in the absence of antibodies and activated macrophages. Cytokines produced by activated macrophages like IL-1, IL-6, tumor necrotizing factor-α (TNF-α) and the complement factors (C3 and C5) play important role by attracting these cells to the inflammatory sites.

The second group belongs to the lymphoid linage and constitutes a more specific line of defense. It consists of genetic, molecular and cellular components that are capable of exerting high and low effects according to the need of the body. The major cellular components are the B-cells, T-cells and antigen presenting cells. The B-cells undergo two types of developments: an antigen independent and dependent. The former takes place within the bone marrow. It involves a series of changes identified by (i) steps in the somatic rearrangement of 3 gene segments known as variable (V), Diversity (D), and Joining (J); (ii) changes in the surface molecules that are essential for various types of interactions among cells of the immune system such as signal transduction, interactions between cells of the immune system and blood tissue barriers and adhesion and homing receptor molecules; and (iii) their dependence in growth and regulatory factors (Mackay *et al.*, 1991). Based on these transformational changes B-cells are divided as pro B, Pre-B, immature and mature B-cells (Fig. 2).

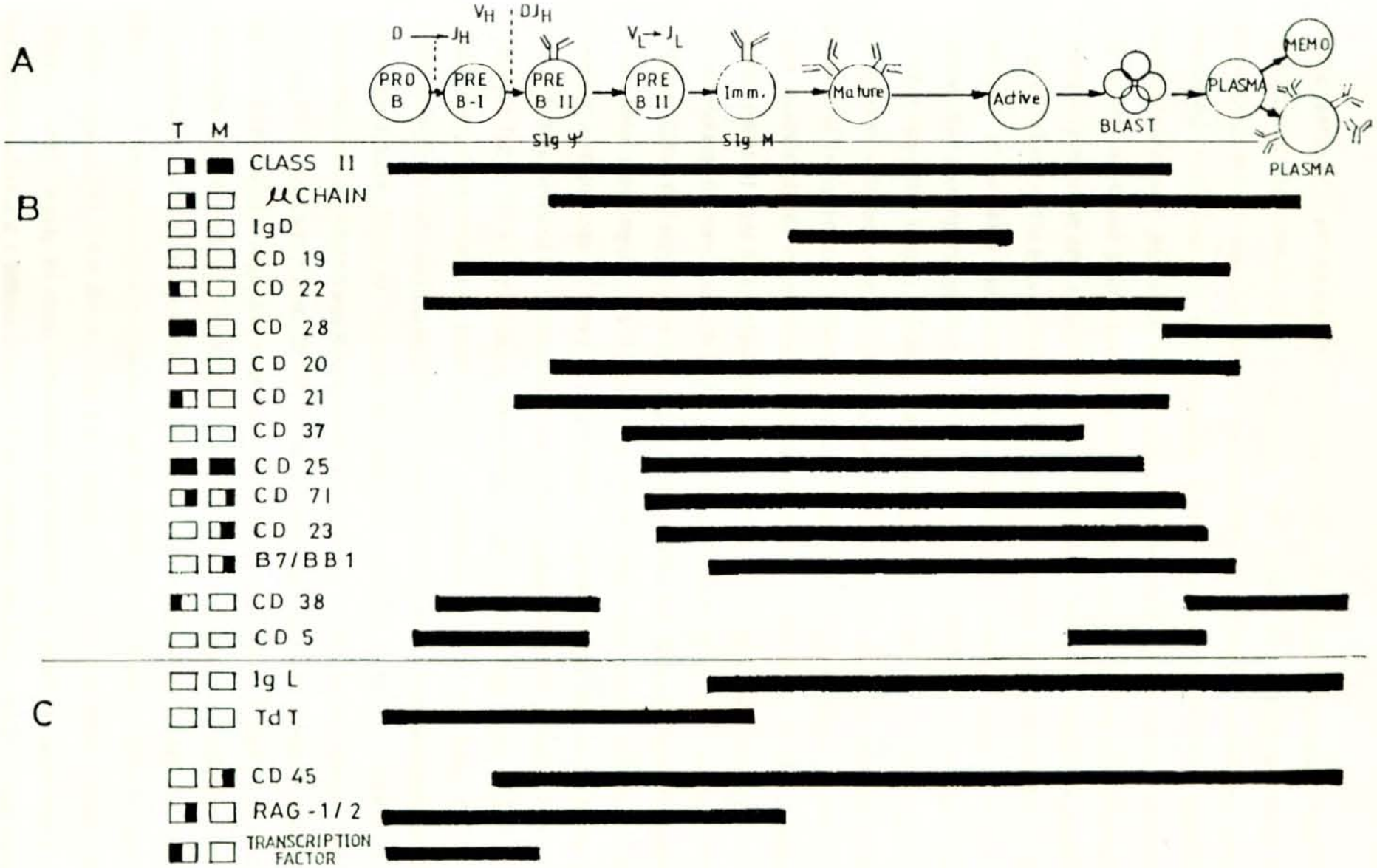


Fig. 2. Classification of human B cell and their differentiation (genetic and antigenic) markers.

At the genesis of pro-B cell stage the transcription factor binds to Ig enhancers and opens the chromatin at the immunoglobulin (Ig) gene. This makes the Ig-gene accessible for transcription and activation of responsible enzymes (RAG-1 and RAG-2 and terminal deoxy-neuclotidyl- transferase (TdT)) for encoding proteins required for the immunoglobulin rearrangement and expression of N-nucleotides (Malissen, 1995). Prior to the μ heavy chain expression, substantial proliferative expansion is initiated by the binding of transcription factors to Ig enhancer and giving chance for critical genetic events including rearrangement of genes to betide. The heavy chain gene that is opened first it is also transcribed first. The completion of the heavy chain gene rearrangement brings the promoter and enhancer genes together thus enabling the speeding up of the transcription rate of the next stage. The $\lambda 5$ and VpreB proteins that support the heavy chain together with accessory Ig like chains Igα and Igβ involved in transduction of signals that inhibit the synthesis of heavy chain and trigger the onset of the light chain. At this stage about 60% of the B-cells will have only one Ig light chain receptor alleles in germline configuration suggesting that there is little on going rearrangement of light chain genes (Rothenberg, 1995). The light chain as soon as it is formed replaces the surrogate light chains ($\lambda 5$ and VpreB), from heavy chain and in it's turn produces signals that suppress the light chain synthesis. This way each step in rearrangement of light chain genes regulate the production of the preceding steps ensuring that each B-cell expresses a unique type of heavy and light chains. The pro-B cells further make use of growth factors produced by stromal cells [macrophage colony stimulating factors (M-CSF), stem cell factor (SCF), granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), Interleukin 1 (IL-1), Interleukin 6 (IL-6), Interleukin (IL-7) and transforming growth factor-β (TGF-β)] and adhesion molecules like very late antigen (VLA-4), vascular cell adhesion molecule (VCAM), (VLA-5) and fibronectin, and β2-integrin and ICAM-1 that are produced by extracelluar matrix (ECM) (Bazan, 1990; Taga and Kishimoto, 1992). After these steps the B cell is transformed into the pre-B-cell, a cell stage that does not depend on stromal cells. It instead produces leucocytes surface markers (CD45), CD19, CD38, CD40 and more important, the major histocompatibility class antigens that are required for interactions with T-cell and signal induction of the mature B-cells (Janeway and Bottomly, 1994). It has a different growth requirements like IL-7 and stromal cells and is capable of long term expansion (Dittel and LeBien, 1995). During all these interactions the assembly of sIgM immunoglobulin is accomplished always in a defined sequence finally leading to the production of mature B-cells

bearing IgM and IgD of identical specificity (Nossal, 1994). The Igs are expressed with glycosylated and phosphorylated heterodimer molecules designated IgMα and IgMβ, respectively encoded by genes mb-1 and B-29 (Hardy, 1991).

Parallel to the synthesis of IgM, the genes for enzymes RAG-1 and RAG-2 genes and TdT are mobilized to encode proteins required for the immunoglobulin rearrangement and expression of N-nucleotide. The range of the operation times for each enzyme varies. TdT functions from the start of the synthesis until the heavy chain is formed. The RAG-1 and RAG-2 genes turn on with the expression of the Ig rearrangement and decay when the signals from the μ heavy chain and surrogate light chain that are shut off. The λ5 and Vpre-B molecules regulate and support the heavy chain to stay stable until the light chain rearrangement is completed. Transcription factors like E32 and OCt-2 continue to be produced until the light chain rearrangement is completed.

Matured B-cells from bone marrow and T-cells from the thymus equipped with their respective antigen receptors are released in to the circulation. They continue to circulate through out the body in primary and secondary lymphoid organs via the high endothelial venules (HEV), lymphatic drainage through the thoracic duct until they are selected by antigen to proliferate and transform into effector cells. When the B-cells encounter an antigen they transduce signals that initiate tyrosine kinases and phosphatases and enhance expression of MHC-class II antigens, ICAM-1 and B1/BB7 and alter inositol phosphate metabolism. If they fail to get appropriate antigen they continue to patrol the body for foreign antigens from blood through tissues, into lymph and back to blood. Wherever they come in contact with foreign antigens they home or recirculate more at those sites they first encountered the antigens (Picker and Butcher, 1992).

Systemically activated B-cells preferentially end up in bone marrow, the spleen and peripheral lymph nodes, which contribute the bulk of immunogloblins found in serum. The antibodies produced from these mature plasma cells are capable of neutralizing toxins, viruses and bacterial effects as well as facilitate the engulfment of microorganisms by phagocytes or their direct killing by cytolytic cells. Some immunoglobulin classes can activate the complement system. When the antigen is administered for the first time it initiates a primary immune response that is of a low level and does not persist for long

period. In the second response the antibodies appear early and remain at high level for a long period. IgM appears first in response but soon is switched to other classes depending upon the type of antigen. There are 5 classes of antibodies: IgG, IgA, IgM, IgD and IgE. Each functional Ig consists of a single unit of two short light and two long chains, structurally similar but functionally different to each other. The IgG and IgA on the basis of antigenic and structural differences are further classified into four IgG subclasses (IgG1-IgG2, IgG3 and IgG4), and two IgA subclasses (IgA1 and IgA2) in humans (Delacroix *et al.*, 1985; Mesteky and McGhee, 1987).

IgG antibodies are the most common form of antibody in circulation and are passed from mother to fetus before birth. It is present in high concentration in the blood only after prolonged exposure to antigen and thus represents the bulk of normal immunoglobulin in human blood. It activates complement and thus plays active role in induction of lysis of foreign cells. There are two forms of IgA: monomeric occurring in circulation and dimmeric form in secretions. The later is resistant to proteolytic enzymes. IgE is responsible for the causation of severe acute allergic reactions and is associated with acute parasitic infections.

In humans, $3x10^{10}$ lymphocytes come into the blood each day via the thoracic duct. By contrast $4.5x10^{11}$ lymphocytes migrate each day into organs such as lung, spleen, liver and bone marrow via flat venules. There, they initiate secondary immune responses and perform an effector function precisely where they are needed. In doing that they follow selective routes to get to lymphoid tissue like lymph node, tonsils and Peyer's patches where antigens and antibody producing B-cells, cytotoxic, helper and suppressor T-cells are concentrated in order to reach the site at the shortest possible time. This process of surveillance, their activation and transmission of the activating immune reactions are directed by pro-migratory factors and specialized 'adhesion and homing' molecules. These molecules facilitate direct cell-cell, cell to matrix or soluble mediators like cytokines or antigens which are prerequisites to effect a well-ordered immune response. The non Ig cell surface markers (differentiation markers, adhesion molecules and homing receptors) are divided into four groups on the basis of whether they are single or multiple hydrophobic stretches and the position of the amino-terminus in reference to the membrane. Type I and II are single stretches with respectively extracelluar and intracellular demarcations. The type III are multiple stretches whose polypeptides are imbedded in the membrane. And the IVth type are channel forming types

with homologous units. Among those known to have a clear influence on B-cell interactions with bone marrow stromal cells, T-cells, interdigitating dendritic cells, follicular and endothelial cells are illustrated in Fig. 2 and 3.

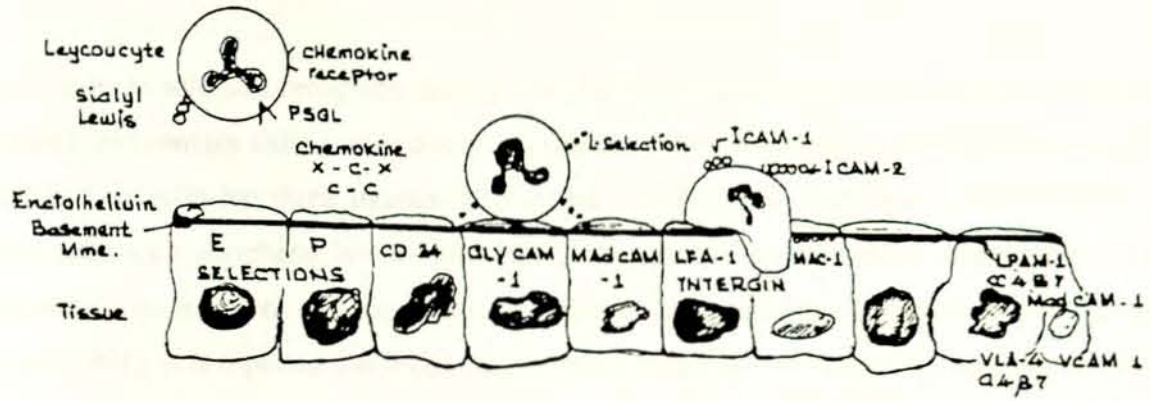


Fig. 3. Adhesion molecules and their role at inflammatory sites.

The adhesion molecules are classified according to their structure into four families of cell surface proteins known to facilitate such activities: Integrin family, Ig-related family, the selectins and chemokines that are widely distributed and acting via ligand-receptor binding. They affect the migration and adhesion of lymphocytes in general and inflammation in particular. Several of these (CD19, CD20, CD22, CD37, HLA-class II) molecules appear during early activation and stay all the way through to maturation while a few either appear for a short period after activation (CD25, CD71 and B7) or are even lost upon interaction with antigen. The antibody secreting cells, as has been shown from surface markers analysis of spontaneous IgG secreting cells in tonsil, bone marrow and peripheral blood at the different effector sites exhibit different phenotypes and functional levels of maturation (Brieva *et al.*, 1994).

Selectins or LECAMs are a small family of adhesion molecules represented by three members, L-selectin (CD62L), E-selectin (CD62E) and P-selectin (CD62P) (McEver, 1991). They are made up of an N-terminal extra cellular ligand, a trans-membrane region and a short cytoplasmic tail. Mel-14 is a selectin on mice lymphocytes and its homologue in man is Leu 8 (Tedder *et al.*, 1990; Laskey *et al.*, 1992). Other members include endothelial

leucocyte adhesion molecule-1 (ELAM-1) and GMP-140 that is transiently expressed in endothelial cells. They are important in lymphocytes, monocytes and neutrophils interactions in vascular endothelium (Dustin and Springer, 1991). During the early phase of inflammation, selectins that mediate weak and unstable interaction between lymphocytes and endothelial cells forcing the migratory cells to roll along vessel wall. Triggering factors like IL-8, MIP-1β activate lymphocyte adhesion molecules.

All the three selectins recognize ligands that are fucosylated, sialyl Lewisx tetrasaccharide (sLeX) and contain sialic acid and sulphate (Rosen, 1993; Anjun *et al.*, 1995; Adams *et al.*, 1993). L-selectin has three ligands: GlyCAM-1, CD34 and MadCAM-1. GlyCAM-1 is associated with peripheral lymph node HEVs. In serum it is present at concentration 1-2 μg/ml thus its function as a tissue specific adhesion molecule is rather controversial (Knol *et al.*, 1994). It is reported that it can trigger specifically Ca++ influxes in lymphocytes and can induce adhesion to integrin ligands as well as expression of activation epitopes on β1 and β2 integrins suggesting that it could provide the integrin activating signals. Contrary to that, the fact that its expression in lymph nodes decreases during antigenic challenge is implicated as an inhibitor of L-selectin dependent migration. It is believed that it is inactive in serum presumably due to bound selectin. CD34, is a molecule expressed on vascular endothelium and haematopoietic stem cells acting as L-selectin ligand in HEV of peripheral lymph nodes (Arbones *et al.*, 1994). The third, MadCAM-1, support L-selectin by its mucin like domain binding via its N-terminal ICAM/VCAM-like domains mainly on HEVs in peyers patches and mesenteric lymph nodes. Although L-selectin is important in peyers patches, mesenteric and peripheral lymph nodes, it is shed during activation and is absent from most mucosal cells. The absence of its binding carbohydrate from MadCAM-1 expressed at the lamina propria also makes it less effective as a homing molecule to the mucosa. The majority of memory and α4β7 lymphocytes lack expression of selectin. α4 integrin associate with β1 and β7 forming LPAM-1 a receptor for the mucosal vascular addresin MadCAM-1, whereas only a minority of mucosally induced blood antibody secreting cell (ASC) expressed L-selectin (Wagner *et al.*, 1996).

P-selectin and E-selectin each has glycoprotein ligands (PSGL-1 and ESGL-1 respectively) that are presumed to serve as a ligand for each of them. The former is mucin while the later is fucosylated but like fibroblast growth factor receptor. The mucins are serine and threonine-

rich proteins that are heavily O-glycosylated and have an extended structure. A subset of 10-15% of peripheral blood L-selectin positive T-cells that express the cutaneous lymphocyte associated (CLA) carbohydrate antigen can bind to E-selectin.

The three adhesion molecules selectins, chemoattractants and integrins with some overlap act in sequence. Leucocytes and endothelial cells express selectins which mediates a weak leucocyte endothelial interaction. The L-selectin molecules guide leucocytes to sites of inflammation. At the end they are cleaved by unusual proteolytic activity at membrane proximal sites resulting in rapid shedding from the cell surface. Early phase of inflammatory response are dominated by P-selectin followed by either L-selectin or E-selectin. Lymphocytes use $\alpha 4\beta 7$ integrin to mediate rumbling like behaviour and firm adhesion under flow condition. Depending on the carbohydrate sequence each of the selectin-recognised circulating cells are forced to role along the vessel wall.

The integrins are trans-membrane glyco-proteins consisting of 15-α and 8 -β units that non-covalently associated to form a family of more than 21 heterodimer members classified in three subfamilies: $\beta 1$, $\beta 2$ and $\beta 3$ families (Schweighoffer *et al.*, 1993; Yang *et al.*, 1995). They are activated by Chemo-attractants, induced confirmatory changes to produce activation epitopes (Hogg and Landis, 1993; Diamond and Springer, 1994). Integrins have a diverse role in the immune response maintenance, development, tissue integrity and homing. They are the main mediators of cell-extra-cellular matrix and cell-cell adhesion. Triggering factors like IL-8 and MIP-1 activates them. This step is important since integrins on lymphocytes bind well to their counter receptors when activated.

The Beta 1 subfamily, CD49/CD29 molecule, also known as very late antigen (VLA) appears a few days after mitogenic stimulation. It is formed by association of $\alpha 1$ with any of the 6 subunits, (CD49 a-f). Most tissues express the $\alpha 1\beta 1$, $\alpha 1\beta 3$, $\alpha 3\beta 3$, and $\alpha 6\beta 4$, either late after activation or constitutively. Most peripheral blood lymphocytes (PBL) express VLA-1 and VLA-2 late after stimulation and VLA-4,-5 and-6 constitutively (Dustin and Springer, 1991). They are required for adhesion to fibronectin, collagen and laminin. When an antigen-receptor complex is triggered, receptors responsible for cell-cell (LFA-1) or cell-matrix ($\beta 1$ integrins) dramatically and transiently increase their avidity for the respective ligands (Schimizu *et al.*, 1990; Dustin and Springer, 1991). VLA-4 ($\alpha 4\beta 1$), is specific to VCAM-1

that is induced on endothelial cells during inflammation. Freshly isolated monocytes express considerable amount of VLA-4,-5 and -6. VLA-4 synergies with LFA-1 in signal transduction following successful antigen-receptor complex. High rate immunoglobulin secretion by B-cells in bone marrow is stimulated by VLA-4 (Roldan *et al.*, 1992). The instructions seem to convert cells from non-adhesive to an adhesive mode, promoting confirmatory changes involving cytoskeletal rearrangement, which ultimately increase the area of contact, strengthen adhesion and reorientation of the lymphocytes protein secretory apparatus to words bound target cell (Haung *et al.*, 1992; Dustin and Springer, 1991). Cells whose stickiness can be regulated by such mechanism include mature T and B-lymphocytes, NK and Neutrophils that bear FMCβ receptors (Haung *et al.*, 1992; Sung and Goldfine, 1992) and also on monocytes and muscle cells.

The β2 family is also called Leu-CAMs or CD11/CD18 molecules. They are composed of three members exclusively expressed by leucocytes: CD11a/CD18 (Leu-CAMa, LFA-1,$\alpha L\beta$2 CD11b/cd18 (Leu-CAMb, Mac-1, CR3, $\alpha M\beta$2 and CD11c/CD18 (LeuCAMc, p150, 95-,$\alpha X\beta$2). They are important in the interaction of leucocytes with endothelial cells. They, increase in quantity during activation and collaborate on adhesion between themselves (Mac.1 and LFA-1) and with other-β2 families in the regulation of T-cell proliferation (LFA-1, VLA-4, VLA-5 and VLA-6) cytotoxicity (LFA-1 and VLA-4) and adherence of lymphocytes to endothelial cells, epithelial cells and fibroblasts (VLA-4, VLA-5). LFA-1 is promiscuously involved in a number of functions: T and B-cells and antigen presenting cells (APC) interactions, interactions of killer cells with their target natural killer (NK), antibody dependent cytotoxicity (ADCC), adherence to endothelial cells, fibroblasts and epithelial cells (Springer *et al.*, 1987; Kishimoto *et al.*, 1990). It facilitates lymphocytes adhesion to endothelial tissues and diapedesis of lymphocytes, monocytes and polymorphonuclear cells (Springer, 1994). The ligands for LFA are ICAM-1 and ICAM-3. It primarily interacts with ICAM-1 but during activation ICAM-3 on T-cells also binds to LFA-1 on APCs and activates T-cell $p56^{lck}$ and $P59^{fyn}$ tyrosine kinases. It is expressed on all lamina propria monocytes but not on intra-epithelial lymphocytes for they do not produce its ligands.

The β7 integrins, $\alpha 4\beta 7$ and $\alpha E\beta 7$ are found on all lymph node B and T-cells and very little in thymus and bone marrow (Kilshaw and Murant, 1990). An $\alpha 4\beta 7$ is a homing receptor for mesenteric lymph node lymphocytes to gut associated tissues and binds to

MAdCAM-1 in an L-selectin dependent manner. It also binds to VCAM-1 and fibronectin suggesting that it also act at the sites of inflammation. It is found in abundance in memory T-cells while L-selectin is dominant in naive T-cells. It is suggested that L-selectin/MAdCAM-1 interaction may occur between naive lymphocytes and Peyer's patch HEV, and gut endothelium and $\alpha 4\beta 7$/MAdCAM-1 in memory T-cells and gut endothelium (Mackay *et al.*, 1992a&b; Hamann *et al.*, 1994). It cooperates with CD44 and LFA-1 in binding gut lymphocytes to mucosal venules. Another important integrin expressed predominantly on intestinal epithelium and subpopulation of lamina propria lymphocytes (40%), but also found on respiratory epithelia in mesenteric lymph nodes and tonsils (5%) is $\alpha E\beta 7$ molecule. It is inducible on peripheral blood T-cells but not B-cells. Its ligand, E-cadhrin located on intraepithelial tissue helps trapping the T-cells. TGF-β produced by both epithelial cells and activated lymphocytes maintains the expression of $\alpha E\beta 7$ on PBL. In contrast lamina propria lymphocytes without a need for any stimulation express high $\alpha E\beta 7$. (Heusser *et al.*, 1991).

CD44 also called Hermes is a family known in variant isoforms formed of glycoprotein with varying glycosylation and insertion of variant exons into the standard or haematopoietic CD44s. It is expressed on many types of tissues and is supposed to play a key role in tissue formation and patterning. It is expressed on stromal cells of central lymphoid organs as well as on haematopoietic progenitor cells. High levels of expression were described in particular for myeloid and erythroid progenitors. Lymphoid progenitors also express it but loses it during the final stage of maturation. It interacts with protein kinase C with its cytoplasmic tail and increases its expression during lymphocyte activation (Kelleher *et al.*, 1995; Koopman *et al.*, 1993). The major ligand for it is hyaluronic acid, secreted by stromal cells, but others like fibronectin, collagen and sulphated proteoglycan have been described. Many functions are labelled to CD44 among which are: the production of lymphoid and myeloid progenitors, the homing and seeding of bone marrow and specific enhancement of mucosal homing. Lymphocytes binding to MadCAM-1 mucosal addressin is blocked by Hermes 3, anti-CD44 monoclonal antibody via a domain distinct from the hyaluronate-binding sites (Salami and Jalkanen, 1991).

Once the cells traverse the endothelial tissue it is attracted chemotactically to the inflammatory loci. At the site of inflammation chemotactic factors: C-X-C (IL-8, PAF, C5a

fMLP); C-chemokines (MIP-1α MIP-1β, Monocyte chemotactic protein-1, (MCP-1), and RANTES activate both the expression and function of adhesion molecules in finely regulated manner (Springer, 1994). They assist the trans-endothelial migration but also act on local tissue like the mast cells and the basophils that release histamine and TNF (Huber *et al.*, 1991; Kuna *et al.*, 1993). The histamine induces P-selectin and TNF-α induces E-selectin expression. Stimulation of vascular endothelial cells with inflammatory cytokines such as INF-γTNF-α or IL-1 largely increase ICAM-I expression while ICAM-2 remains constant. On the other hand stimuli from chemokines and fMLP are able to activate pre-existing cell surface integrins within seconds by changing their conformation (Walsh *et al.*, 1990). The involvement of β2 integrins and ICAMs (VLA-4 and VCAM-1) may also participate (Butcher, 1991), and cause the cells to stick firmly to the endothelium (Andrew *et al.*, 1996). At that time the L-selectin is shed by proteolytic enzyme. The fact that these adhesion molecules are inter-convertible makes them important in the generation of immune and inflammatory responses and to patrol the body and more important for cytotoxic cells to identify and kill one and move to other cells.

The immunoglobulin super family comprises more than 70 members, including the T-cell receptor, Immunoglobulins, MHC antigens, CD2, CD3, CD4, CD8, NCAM and ICAM 1-3. Members of this family have Ig domains composed of 90-100 amino acid residues which are organized into two parallel -β sheets which are stabilized by disulphide bonds. The tertiary structures may represent a conformation, which is resistant to protease attack enabling the molecule to survive in the hostile extracelluar environment and thus being maintained by evolutionary pressures (Altomonte *et al.*, 1992). Their complementary receptors are, the integrins, on the surface of endothelial cells or APCs.

Members of the Ig family like CD3, CD28, CD4 and CD8 mediating adhesion to major histocompatibility complex (MHC) or T-cell receptor (TCR) with MHC and other molecules essentially derive T and B-cell activation, through signal transduction but hardly participate in lymphocyte adhesion. The blocking of CD4 and CD8 molecules cause as high as 100 times decrease in the sensitivity of TCR interaction (Bierer and Barakoff, 1989). Members like ICAM-1 bind with integrins LFA-1 and Mac-1 and to diverse range of counter receptors as is between ICAM-1 and CD43. The interaction of VLA-4, a β-1 integrin ($\alpha 4\beta 1$), with VCAM-1, expressed on lamina propria lymphocytes, can thus support all the types of

adhesive steps that are thought to be required for lymphocyte arrest on vascular endothelia at sites of inflammation, i.e., tethering and arrest of lymphocytes, perhaps in co-operation with other endothelial adhesion molecules. VCAM-1 is inducible by cytokines on endothelial cells and some non-vascular cells. VCAM-1 has many isoforms that have different binding sites. Chemoattractants might be more important in providing directional cues during trans-endothelial migration than regulating integrin adhesiveness and it is unclear whether up regulation of adhesiveness of other integrins such as LFA-1 would occur concomitantly with adhesion strengthening through VLA-4 or would require separate stimuli. Experimental observations however suggest its role in T-cell activation, killing and lymphocytes homing to chronic sites of infections (Schall *et al.*, 1990; Johnson, 1994).

The most probable thing is that naive B-cell from bone marrow, and T-cells from the thymus, express receptor antigens for both peripheral and mucosal high endothelial venules. However, the memory or effector lymphocytes, produce organ specific migratory pattern, through production of receptors, specific for the endothelial characteristics at the site of their initial stimulation. The adhesion of B-cells to follicular dendritic cells in early activation for example, require VLA-4 in addition to LFA-1. Ligation of either one prevents apoptosis among germinal B-cells. TCR binding activates LFA-1 expression and increases the avidity of VLA-4 and VLA-5 for fibronectin and VLA-6 for laminin. Proliferation is inhibited completely by anti-CD29 MoAb (β1 chain), while antibodies to VLA-4 and VLA-5 is partial, unless both antibodies are used (Sato *et al.*, 1995). During inflammation the influx of migrating lymphocytes through the vascular tissue is increased. Mediators like the cytokines and chemoattractants induce expression of endothelial adhesion molecules for lymphocytes and exert chemotactic effects. First the migration through the skin is dominated by a population of CD4 cells with a memory-cell phenotype. In cytotoxic T-cell activities, both LFA-1 and VLA-4 seem to be involved. Antibodies to any one LFA-1, ICAM-1 or VLA-4 inhibits cytotoxicity activity of T-cell. LFA-1 adhere human lymphocytes to activated endothelial cells via VLA/VCAM1 interactions (Neish *et al.*, 1992). LFA-1 on T-cells as co-stimulator receiving signal from ICAM-1 on APC and makes B-cells more efficient antigen presenters. Among the cytokines, INF-γ through induction of ICAM-I is known to have a delayed onset that peaks in 24 hours with the accumulation of CD4+ lymphocytes. TNF-α and IL-1α preferentially collect neutrophils to lymphocytes. They are inducers of ELAM-1 and IL-8. Activated complement are also mediators of lymphocytes.

TNF-α and INF-γ are potent inducers of lymphocyte migration into the skin whereas IL-8 and activated complements are likely to take part in induction of lymphocytes into dermal inflammatory lesions and T lymphocyte migration to the skin (Laskey *et al.*, 1992).

T cells just like the B-cells are derived from precursors in haematopoietic tissue undergo differentiation within the thymus and are then seeded to the peripheral lymphoid tissue and to the recirculating pool of lymphocytes. Developing thymocytes differ from one another with respect to the level of expression of the α and β chain of the TCR, the associated non-polymorphic CD3 complex proteins and the co-receptor CD4 and CD8. In the thymus single positive cells comprise 5%and are derived from much larger pool (80%) of double positive (CD4+CD8+) intermediates approximately 50% of which express the CD4-TCR$\alpha\beta$complex at low levels. The precursors to the double positive population and CD4-CD8- double negative immature cells represent only 5% of the adult thymocytes.

The CD3 $\gamma\delta\epsilon\xi\xi\eta$ and TCR $\alpha\beta$ transcripts are independently regulated during thymocyte ontogeny and T-cell activation, CD3 subunit transcripts appear at day 14 fetal thymocytes. They are important for signal transduction events that control thymic selection and T cell activation. The CD3 $\gamma\delta\epsilon$ subunits are derived by alternately splicing from a single gene. For efficient expression of this complex on the cell surface the CD3 proteins must be co-expressed with the T-cell receptors heterodimer.

Before the T-cells get to the periphery they undergo through the process of positive selection, clearing of the self-reactive cells. By the time they get to periphery they are divided in two exclusive cell groups based on their surface markers, CD4 and CD8. The CD8 are functionally grouped into T-cytotoxic and T-suppressor cells. The CD4 similarly based on the phenotypic markers and the cytokine profiles they produce are put into two groups: TH1 and TH2. TH1 cells produce IL-2, INF-γ and lymphotoxin whereas TH2 secretes IL-4, IL-5, IL-6, and IL-10. They have antagonistic effects. INF-γ increases the development of TH1 cells by suppressing TH2 differentiation and proliferation. Conversely the TH2 inhibits production of cytokines by TH1 cells (Mossmann and Coffman, 1989). Functionally also TH1 plays a part in destruction of infected cells and mediates delayed type hypersensitivity reaction (DTH). TH1 is important in the deal with intracellular pathogens and parasites. TH1 clones specific for mycobacterial, leishmanial, Trypanosoma and malaria antigens producing

IL-2 and interferon-γ (IFN-γ) have been associated with a protective role. TH2 on the other hand are identified with antibody production by B-cells and killing of target organisms or tissue by antibody dependent cytotoxicity or class I restricted cytotoxicity and induction of immunosuppression by CD8 cells (Wang *et al.*, 1994).

The antigen presenting cells (APCs) carry their functions by interacting with the T-cells. There are also evidences that they also represent susceptibility to several diseases. For example susceptibility to the rheumatoid like disease, ankylosing spondylitis (stiffening of vertebrae) is observed in 90 % of individuals expressing the HLA-B27 determinant.There are two classes of MHC in man. The class I expressed on all nucleated cells whereas class II is found on immunocompetent APCs like B-cells, macrophages/monocytes, dendritic cells and on activated T-cells. They influence T-cell function firstly by limiting the TCR repertoire by participating in negative and positive selection of T-cells in the thymus and secondly by determining the repertoire of peptide that can be presented to the TCR. The antigen specific receptors on T cells, the Fc region of the Ig, the gene system associated with histocompatibility complex. The H2 complex located in small site of mouse chromosome 17 consists of at least 9 regions and subregions. Within the complex the I-region gene-molecules have been shown to be most versatile with respect to the immune response. In mouse 5-I sub-gene each with different functions are defined I-A, I-B. I-J. I-E and I-C. For example I-A gene determines susceptibility to auto-immunity and allergy. Immune response gene (Ir) determines individual susceptibility to diseases by delimiting the capacity to respond to certain antigen and viruses (Merino *et al.*, 1994). Other important regions of H-2 are K, L and D regions attracted by cytotoxic T-cells. The S-region is responsible for synthesis of molecule of the complement component. Different MHC phenotypes have also been shown to present different peptide determinants to T-cells (Brown *et al.*, 1989). Both are heterodimeric cell surface glycoproteins. MHC class I antigens are composed of a 44 kilo dalton (KD) transmembrane protein associate non-covalently with the 12 KD protein β2 microglobulin encoded on human chromosome 15 (Germain, 1994).

The class II protein is composed of two transmembrane glycoproteins of 27-29 KD α-light chain and 33-35 KD heavy chain and two extra cellular domains of 90-1000 amino acids each. The N-terminal domain with no homology to immunoglobulins α1 and β1 and a C-terminal domain with sequence homology to immunoglobulin domains α2 and β2 and are

therefore considered to share a common evolutionary origin with immunoglobulin family receptors (Bonnerot *et al.*, 1995).

Human class II genes are encoded within the MHC on the short arm of chromosome 6 and the total size of the MHC class II region is about 1000 kilo base (KB). The genes of the MHC that encode the human leucocytes antigens (HLA) are the most polymorphic genes known in man. There are three subregions that contain an α-chain and at least one β chain; The DP, DQ and DR genes. The structure of HLA-antigen has a grove like formation located on the top surface of the molecules and on the side of the groove are two parallel α helices of the α 1 and α 2 domains which are critical for the T-cell recognition of MHC class I by the TCR and the α 1 and β1 domains on the MHC class II. Mutagenesis of amino acids in the grove has previously been reported to influence the immunological response to antigenic fragment causing auto-immune disease such as diabetes and also positive and negative selection in the thymus (Jacob *et al.*, 1992).

The class II $\alpha\beta$ dimers as soon as formed bind to the invariant chains (Ir) in the endoplasmic reticulum (ER) that protect it from binding peptides all the way through endosomal and lysosomal compartments. From the ER they transit through the golgi apparatus and enter a post golgi compartment where they appear to be delayed and interact with the endocytic pathway. In the process the invariant chains are cleaved and the processed peptide loaded on to the class II binding sites (Cavani *et al.*, 1995). The T-cells then would be able to recognize the complexes that are specific for the foreign peptide and the self-MHC molecules. A critical event in T-cell activation is the subsequent production of IL-2 resulting in proliferation of T-cells through autocrine and paracrine loops. It causes CD8+ cells differentiation to mature cytotoxic lymphocytes (CTL) and CD4+ and CD8+ cells to proliferation. However the way phosphorylated substrates induce nuclear events leading to IL-2 gene transcription is yet to be cleared.

There is the third type of T-cell stimulation that does not require accessory cells. That is using super-antigens. Both CD4+ and CD8+ cells MHC II expressing cells sensitize super-antigen coated to lysis. The co-expression of ICAM-I allowed lysis of target cells at 1000 fold lower concentration (Parra *et al.*, 1993). Such mechanism is to the advantage of the infectious agents. When T-cells are activated in mass by super-antigen causes

immunosuppression in mice and antigen specific non responsiveness in human T-cells occurs. The bacteria use super-antigens to destroy class II antigens or by activating the inflammatory cytokines such as IL-1, TNF-$\alpha\beta$ and INF-γ increases blood flow and nutrient supply thus benefiting bacterial growth (Bahadoran *et al.*, 1993).

The other form of pathway of antigen processing is employed for proteins, such as viral antigens that are synthesized within the cell and appears to induce protein fragments in the cytoplasm. Peptides produced in this manner associated with MHC-I are recognized by T-cells expressing CD8+. Since virtually all nucleated cells express class I MHC molecules all can serve as targets for CD8+ CTLs. This is an important defense mechanism against viruses, which infect many types of nucleated cells and tumor specific antigens. The class I restricted cytotoxic cells have been shown to be induced by lipid bound exogenous peptides, non confirmatory viral antigens from dead and UV treated virus or mycobacterial antigens suggesting that unbound class Iβ2m dimers intersect the endocytic route and transport processed peptides to the cell surface (Rock *et al.*, 1990).

1.2.2 Mucosal immune response

The mucosal membrane which represent a vast area, (400 m^2), is the most frequent portals of entry of common viral, bacterial and parasitic infectious agents and potentially harmful antigenic substances from the environment. In mammalian immune system distinct from the blood borne immune system that is described above is made up of lymphoid cells in the mucosa-associated lymphoid tissue (MALT), of the respiratory, gastrointestinal and genitourinary tracts, the eye conjunctiva and the ducts of exocrine glands (McGhee, 1992; Spencer *et al.*, 1986). It protects not only the mucous membranes but also the draining lymph nodes, spleen and the bone marrow. This system covers more than 200 times greater area than the surface area of the skin and harbour the largest organ specific immune cell population in humans. Of these the gut associated lymphoid tissue (GALT) is a prominent part. It comprises of Peyer's Patches, (PP) the appendix, mesenteric lymph nodes (MLN), and the diffusely spread lymph nodes in lamina propria and intraepithelial compartments of the intestinal mucosa and the tonsils in the oropharyngeal cavity. They possess more than half of all peripheral lymphocytes (10^{10} lymphocytes/m-2) (Mowat, 1987). They produce the best known protector of the gut and other mucosal tissues, the secretary immunoglobulin A (s-IgA) in quantities more than the total amount of IgG in the body (Shalaby, 1995). Unlike

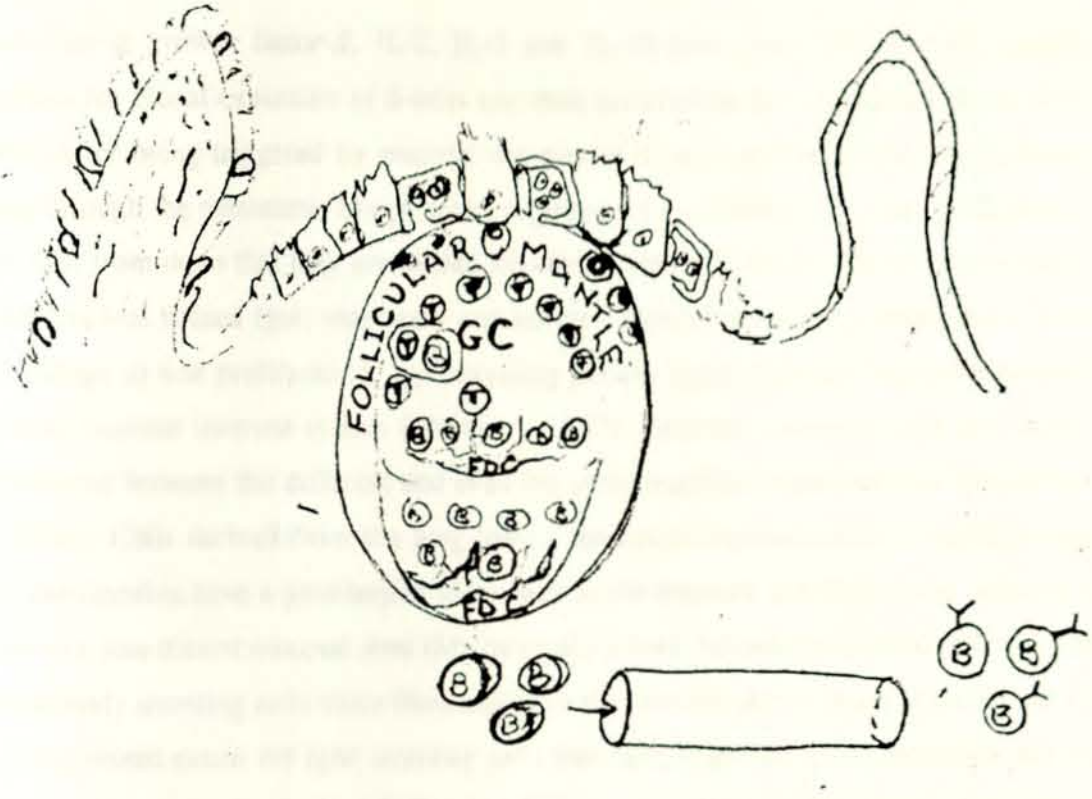
the systemic compartment the mucosal immune system consists of distinct lymphoid sites where antigens are encountered and processed and initial B-and T-cell triggering occurs (inductive sites), and separate areas where immune cells actually function (effector sites) (Clark and Ledbetter, 1994).

The mucosal surface of the upper aero-digestive tracts being located at the portal of entry of ingested and inhaled antigens, play a pivotal role in both GALT and the bronchus associated lymphoid tissues (BALT). The tonsil strategically placed at the entrance of this tract plays a leading role in antibody mediated protection of the whole upper aero-digestive tracts. It occurs in 3 pairs all over the oral cavity: palatine, lingual, and pharyngeal tonsils. It functions like the PP of the gut in the upper aero-digestive and respiratory organs but because of lack of secretory components and the J chain produce mainly monomeric-IgA (m-IgA).

The other components are the perioral lymphoids: Salivary gland lymph nodes, salivary gland lymphoid tissue, nasal associated lymphoid tissues (NALT), The oral mucosa associated gingival and diffusely scattered lymph node tissues. The lymph nodes and other lymphoid tissues situated in major salivary glands include parotid, submandibular, sublingual and minor salivary glands. The NALT is situated on both sides of naso-pharyngeal ducts. It consists of B-follicles and inter-follicular T-cell areas covered by specialized epithelium bearing the M-cells. The T-cell areas contain many lymph and blood vessels while the B-cell areas have a network of capillaries. Most of the B-cells express IgM or IgG.

The oral mucosa associated lymphoid tissues located below the oral epithelium comprise mostly of T-cells and macrophages. Occasional aggregations of lymphoid cells containing a central crypt are found in the soft palate, the ventral surface of the tongue and the cheek mucosa. the gingival lymphoid tissue contains predominantly T-cells with few phagocytic cells and B-cells. Upon stimulation by dental plaque antigens, the T- and B-cells with few shifts in favour of the B-cells until numerous plasma secreting IgA appear. Gingival tissue does not produce secretory component, hence IgA produced at gingival tissue cannot be secreted by the epithelial cells IgG producing cells constitute the majority of gingival plasma cells. They kill by IgG opsonization and complement mediated killing. The rectal and genital tract mucosa and their draining lymphoid tissues are also equipped with follicular construction and are IgA producers upon repeated local challenges.

All these mucosal compartments leaving a room for a few exceptions have similar structural and cellular composition organised into inductive and effector tissues. For example PP, solitary lymphoid follicles and the appendix of GALT have B-cell follicle and follicle associated epithelium-bearing M-cells (Fig. 4).



Key: B, B-cells; T, T-cells; GC, Germinal centre; M, M-cell; FDC, Follicular dendritic cells; Y, Antibody.

Fig. 4. General structure of the mucosal wall.

Luminal proteins, bacteria, viruses, particulate and soluble antigens are endocytosed through the M-cells and transported to the underlying APCs in the follicle. There B-cells bearing Ig specific for the antigen are held to come in contact with the antigens by accessory cells like dendritic cells and macrophages, at the extra-follicular sites in the presence of T-cells that is itself activated thus capable of helping B-cells (Kroese *et al.*, 1991). The T-cells at the para-follicular regions are > 95% $\alpha\beta$ TCR+ of which about 40 % are CD8+ cytotoxic/suppressor while the rest are T-helper (TH)-cells. $\gamma\delta$-T-cells are found in small quantities. Depending on the nature of the antigen the B-cells can also be directly activated. Then the activated B-cells start the germinal centre at the B-cell zone. Follicular dendritic cells (FDC) colonizing the follicular mantle there transform to B-blast and eventually to

centroblasts that literarily express no Ig but undergo rapid cell cycle (Zhang and Michael 1990). The germinal centre (GC) at first gives rise to a dark zone relatively devoid of T-cells by rapid proliferation. Upon maturation move to light zone where undergo expansion and point mutation in the variable region of the immunoglobulin genes.

Transforming growth factor-β, IL-2, IL-5 and IL-10 have been identified as important cytokines for clonal expansion of B-cells and their preferential IgA expression (Rizzo *et al.*, 1995). After being triggered by mucosal antigens it is believed that B and T-lymphocytes transit through the mesenteric lymph nodes migrate to the circulatory system via the thoracic duct. It is from there that they are seeded into the lamina propria of different compartments of the mucosal tissues (gut, mammary and salivary glands, respiratory tracts, etc.), where they change to non proliferating, IgA secreting plasma cells. This has led to the notion of common mucosal immune system (Mestecky, 1987). However it is also clear that there is a difference between the different and even the same mucosal organ in terms of degree of specificity. Cells derived from the lung have a tendency to go to the lung where as those from the intestine have a predilection to go back to the intestine and those from colon to the colon but also distant mucosal sites (Maloy *et al.*, 1994). Mucosally derived IgA, IgM and IgG antibody secreting cells move from inductive site into circulation but it is mainly the IgA and to a limited extent the IgM secreting cells that have migratory potential to the mucosa. But even the mucosally derived IgG antibody has selectivity to the mucosal sites than to other peripheral lymph nodes (Leung *et al.*, 1995).

The factors that are responsible for this cellular localization in the mucosal immunological network are not well described. Blood flow rate, surface characteristics of the IgA secreting cells, the organ derivation of these cells and the possible local presence of TH-cells specific for IgA are implicated (Xu *et al.*, 1994; Marinaro *et al.*, 1995). Cytokine production at the mucosa is biased towards TH2 responses. IL-4 and IL-5 predominate in Peyer's patches. IL-6 is expressed abundantly in lamina propria at the sites of IgA production. IL-4 in conjunction with TGF-β is an essential factor for the development of sIgA. IL-5 come at the terminal differentiation of eosinophils to be followed by IL-6 that enhances IgA production in those already committed ones. IL-4 and IL-5 deficient animals lose their ability to secrete IL-5 and IL-10 and to mount eosinophilia in response to helminth infection (Kopf *et al.*, 1996; Foster *et al.*, 1996). IL-6 deficient mice mount sustainable mucosal antibodies except to virus

infection. These deficiencies in terms of IgA response have no deleterious effect. The adhesion molecules specific for corresponding determinants on endothelial cells in mucosal and glandular tissues provide traffic signal. The $\alpha 4\beta 7$ (Picker *et al.*, 1991) and $\alpha E\beta 7$ integrins tend to migrate further into intestinal epithelium where they appear to be retained mainly by the binding of this integrin to E-cadherin expressed on epithelial cells (Cepek *et al.*, 1993). Moreover those that produce abundant J-chain are the ones that preferentially home to exocrine sites. IL-2, IL-5 and perhaps IL-6 are presumed to up regulate expression of J-chain where as IL-4 exerts an opposing effect (Reimann *et al.*, 1995).

The other possible out come is one that leads to immunotolerance. The oral administration of antigen in gastrointestinal tract (GIT), although expected to induce IgA responses in GIT and other distant mucosal effector sites, in reality this mode of delivery results in either poor or short-lived antibody responses. Furthermore, oral administration of large doses of protein often induces unresponsiveness to peripheral immunization (oral tolerance). That might be due to the fact that the human intraepithelial lymphocytes with 80-90 % of CD8+ (cytotoxic\suppressor) T cells, and the rest $\gamma\delta$-T cells. The former down regulate the systemic and local pro-inflammatory IgG and IgE antibody responses and DTH responses from CD4+ T-cells. The fact that the epithelial cells lack co-stimulatory molecules B7, CD40 also causes CD4 response suppression.The $\gamma\delta$-T-cells clean infected or damaged cells through interaction with heat-shock proteins (Born *et al.*, 1990); NK cells through production of cytokines that inhibit antibody formation and B-cell proliferation contribute their share. On induction of tolerance these cells seem to play a role for example by inducing villus atrophy and increased proliferation of crypt cells. Contrary to that super-antigen activated lamina propria T-lymphocytes produce IL-2 and INF-γ and thus cause tissue damage. As long as the wall is intact monomeric and polymeric IgA containing immune complex are able to suppress attraction of neutrophils, eosinophils and monocytes and inhibit their generation of oxygen intermediates. They also down regulate the secretion of pro-inflammatory cytokines such as TNF-α and IL-6 thereby avoiding inflammatory mediators (Wolf, 1994).

The gut effector limb consists of numerous T cells distributed in the lamina propria and in the epithelium and the IgA plasma cells. Mucosal T cells can be divided into two subsets: the $\alpha\beta$-TCR, CD3+ lymphocytes that arise from antigenic stimulations at the mucosa (Guy-Grand *et al.*, 1991) and that home back in lamina propria to serve as helper CD4, and

cytotoxic cells CD8 at the epithelial layer (Cerf-Bensussan *et al.*, 1993). The next subset originate from bone marrow and undergo thymus independent route to develop an $\alpha\beta$ and $\gamma\delta$ receptors but also FcϵRIγ chain bearing T-cells exhibiting cytotoxic properties mainly forms the intra epithelial lymphocytes (IEL). The lamina propria lymphocytes are 40-90 % T-cells, and of these CD3+CD4+ cells account for 65-80%. In contrast to the extrathymic derived IEL. The lamina propria T-cells, get induction by antigens encountered in Peyers patches and the lymphoid follicles. They then enter the circulation and home back to lamina propria. Phenotypically they are 66-96% memory type, (CD45O+), in contrast to peripheral blood (PB) that only express 20-30%. The lamina propria lymphocytes (LPL) though are CD45O+ lack expression of CD29, the chain of VLA antigens, and CD11a/CD18, α and β-chain of LFA-1 compared with PB T cells. On the other hand 40% of the IEL express $\gamma\delta$ TCR whereas only 3% of CD3+ LPL express this form of TCR the rest 95% as in the PB bear an $\alpha\beta$ TCR (Ullrich *et al.*, 1990). Both PB and LP-T-cells express CD2 and its ligand CD58 (Makgoba *et al.*, 1992). The percentage of CD8+ LPL expressing CD11 phenotype associated with suppressor-effector function is the same or lower than Peripheral blood. Lamina propria T-cells (CD4 and CD8) compared to PB, splenic and mesenteric lymphocytes express significantly higher IL-2R. It enhances helper functions of CD4 but not CD8 suppressor functions suggesting another regulatory mechanism for CD8 cells. Other activation markers found on lamina propria $\alpha E\beta 7$ T-cells are MHC class II, transferrin receptors, and in about 40% of them integrin although can be induced by TGF-β it is less than 2 % normally found on PBL. Thus TGF-β produced by epithelial cells and PP lymphocytes can alter the expression of lymphocyte adhesion molecules to IEL (E-cadherin), and facilitate mucosal homing. The $\alpha 4\beta 7$ marker increase on LPL during acute inflammation.

All activation processes in mucosal walls are regulated by T-helper cells secreted cytokines: INF-γ,IL-2 and TNF-α produced by TH1 (Cooke, 1995; Rabinovitch *et al.*, 1995) and IL-4, IL-5, IL-10 by TH2. The generation of s-IgA is a combined result of both types of T-helper cytokines. TH1 cytokines support IgG2a synthesis in mice whereas TH2 cytokines support IgA, IgG1 and IgE switching. TGF-β and IL-5 enhance IgA formation while IL-2, IL-4, IL-5 and IL-6 regulate the terminal differentiation. The ratio of IL-5 and INF-γsecreting cells in LPLs is 3:1 suggesting a TH2 domination. Unlike the PBL LPL has enhanced proliferation and cytokine secretion in response to CD2/CD28 stimulation (Targan *et al.*,

1995) and low response on CD3 stimulation. The notion behind this observation is that B-cells that bear CD2, CD28, LFA-3 and B7.1/B7.2 may activate LPL that is anergized through TCR delivery. Thus activated B-cells might be crucial in reactivating and inducing cytokine secretions in LPL. That provides help to cytotoxic cells to get rid off infected epithelial cells in the presence of B-cells (Targan *et al.*, 1995).

1.3 Immunology of schistosomiasis

Schistosomiasis is essentially an immunopathological disease with a wide spectrum of clinical manifestations each caused by the different stage of the parasite. The disease manifestations are dermatitis by the cercaria, cough, fever and pneumonia by schistosomula as it travels through the circulatory and the lymphatic system to the lung. The adult deposits eggs that lodge in the liver and peyer's patches of the intestine where it causes a characteristic granulomatous lesion with inflammatory cells (macrophages, lymphocytes and eosinophils) accompanied by recruitment of fibroblasts and collagen around the eggs. It causes bloody diarrhoea, dysentery and fibrogenetic granuloma, in severe cases, obstruct the portal blood flow using elevation of portal pressure and esophageal varices leading to impaired circulation, bleeding and organ malfunction (Nash *et al.*, 1982).

schistosomiasis haematobium is caused by eggs deposited in the wall of the bladder and ureters. It occurs as recurrent painless haematuria in most individuals. In about 30% of cases it involves the kidney and results in hydronephrosis or hydroureter which in very minor groups could chronically develop to include the bladder and develop to cancer. the areas of fibrosis and calcification around the dead eggs appear as a sign of past infection (Pike, 1986). During the chronic phase of infection the hepatosplenic granulomatous reaction around the deposited eggs recedes in size and the newly formed granuloma are modulated with minimal inflammation. However, the basis for the dynamics of vigorous granulomatous inflammation in the acute phase of the disease and spontaneous down-sizing of it in chronic phase and the relationship of intensity of infection to age are not fully understood. Recent studies done in previously non-infected communities in Senegal and Gambia have shown that this age-related pattern in prevalence, intensity and susceptibility to reinfection is not exposure dependent (Stelme *et al.*, 1994).

The complexity of the immune response arises from the fact that different stages of the parasite travels through different organs and compartments of the immune system at the same time. The schistosomula from the skin and the lung; the adult worms from the veins of the portal system and the eggs from liver, mesenteries and the gut mobilize both systemic and mucosal immune systems. The massive numbers of inflammatory cells stimulated from different corners (macrophages, lymphocytes and eosinophils), accompanied by recruitment of fibroblasts and collagen, develop around the ova in the liver while the same ova cause bloody mucoid diarrhoea in the intestine. Which one contributes what and in what capacity is still an issue where clarity is lacking. The tools to differentiate the causes from the effects are inadequately developed. Nevertheless, the fact that humans develop resistance to reinfection after chemotherapeutic cure and down regulates granulomatous reactions suggests that development of vaccine may be feasible (Bergquist, 1995).

Studies in mice have established that T cell mediated immunity is responsible for induction of resistance. However, when it comes to the sub-types of T-cells, evidence shows that both TH1 and TH2 could effect protection. The activated macrophage and INF-γ which are TH1 cell response products and cytokines like IL-12 that promote TH1 and suppress TH2 and reduce tissue fibrosis following vaccinations with irradiated cercaria suggests a role for vaccines that induce TH1 cell response (Wynn *et al.*, 1994a&b; Wynn, 1996). This was further confirmed by abrogation of resistance with MoAbs against INF-γ and IL-12. In contrast, other experiments have demonstrated that repeated vaccination while still protective is associated with TH2 (Caulada-Benedetti *et al.*, 1991). These protective immunities, mediated by multiple vaccination, or induced by irradiated larvae which fail to migrate from the skin are transferable in serum to naive recipient mice (Dean *et al.*, 1995). In contrast to this, Oswaldo *et al.* (1994) demonstrated that TH2 type response in mice does not play a protective role. Thus there seem to be several immune effector mechanisms with parasite killing capacity.

In rats the parasite does not reach full maturity. Antibody dependent cytotoxicity mediated with IgE, IgG subclasses and IgA in conjunction with effector cells (Macrophages, Eosinophils and Platlets) are reported to kill the larvae. The MoAbs (IgE and IgG2a) in rats could adoptively transfer resistance to infection (Capron and Capron, 1994) and IgE depleted sera reduces resistance in neonate rats (De-Clerk *et al.*, 1992). Upon adherence to

schistosomulum the cells from an immune complex release degranulated, toxic, cationic granules and enzymes while the platelets release soluble toxic mediators.

In humans based on the similarity of DTH response to primary hepatic lesion and the presence of IL-2, TNF and INF-γ in the tissue, at the height of the disease, the TH1 responses were for long considered the major contributors to disease initiation. Egg deposition, which is the major stimulus for the production of TH2 response comes later in the course of the disease and hence was associated with modulation. The cytokine IL-4 induces IgE; IL-5 induces eosinophilia and IL-3 and IL-10 induce mastocytosis, and down-regulate INF-γ and IL-2 (Sher *et al.*, 1990). Contrary to the view that assumes TH1 response as the source of pathology and TH2 as modulator, another line of thought holds TH2 response responsible for the pathology (Wynn *et al.*, 1994a; Andrade *et al.*, 1993). Evidence to this comes from the suppression of IL-12 which reduces INFγ and NK cells resulting in marked enhancement of granuloma formation. Addition of r-IL-12 significantly inhibits primary granuloma formation and increased INF-γ, IL-2 and IL-10 (known to have a counter effect INF-γ) (Wynn *et al.*, 1994a; Andrade *et al.*, 1993). In support of this Oswaldo (1994) has shown that following sub-cutaneous injection of schistosome eggs into the foot pad of mice, DTH develops and it is the TH2 cells that appear in the popliteal lymph nodes. The induced TH2 response can then be suppressed by the addition of IL-12 as evidenced by decrease in IL-4, IL-5 and IL-10 and an increase in INF-γ levels. In contrast to this, Couissinier-Paris and Dessein (1995) and Roberts *et al.* (1993) have demonstrated the association of *S. mansoni* resistance to reinfection to the presence of IL-4 producing T-cell clones in humans.

Furthermore, to these CD8+ T-cells known for their cytolytic activity and production of INF-γ and TNF-α, have been shown to play a double effector/regulatory role like CD4+ T-cells. They lose the CD8 antigen expression and cytolytic function and produce the TH2 cytokines IL-4, IL-5 and IL-10 and induce B-cells to synthesize Igs (Stadecker, 1994b). However, since CD8 cells are class I restricted, they would be reacting to different antigens than would the CD4+ TH2-cells.

TNF-α mediates schistosomulicidal activity and induces oviposition. A decrease in inflammatory response during the chronic phase of infection could be the appearance of

less inflammatory macrophages, that is, the granulomatous macrophages that do not produce IgG and IgG2b specific Fc receptors. They instead posses twice as much prostaglandin E2 (PGE2) which is suppressor of INF-γ production (Van-der-Pauw-Kraan *et al.*, 1995).

T-cells in addition to the primary MHC-peptide-TcR complex have two other receptors (CD28 and CTLA-4) that interact with a co-stimulatory molecules B-71/B72 which are found in B-cells and monocyte/macrophages (Linsley *et al.*, 1995). Exposure of specific TH-cell clones to these non-functional antigen presenting cells (APC) imparts a state of anergy, unresponsiveness or hypo-responsiveness on them (Zhu *et al.*, 1994; Stadcker, 1994a). IL-10 inhibits macrophage APC function as has been determined by decrease in lymphokine production by TH1 cells. This could be a way of down regulating co-stimulatory molecules such as B-7. The lack of these stimulatory molecules on the granuloma macrophages is one of the reasons for down regulation of liver granulomatous reaction in schistosomiasis (Chensue *et al.*, 1995).

At the mucosal level the normal immune homeostasis within the GALT and BALT is dependent on differential recognition, processing and presentation of food antigen from those of potential pathogens that results in tolerance to most food antigens and production of sIgA antibody specific to the pathogens. The phenomenon of immuno-modulation is described as a process of immunotolerance, a systemic unresponsiveness to an antigen one is orally exposed. This is presumed to be due to the large number of eggs deposited at the GALT after infestation of the liver. The systemic stimuli the eggs cause the granulomatous hypersensitivity reaction at the liver. This hypersensitivity reaction however presumed to be modulated at the chronic stage by the immune response stimulated at the Peyer's patch of the gut. At the mucosal level egg antigens that reach the PP are processed by APC (macrophages, B-cells and Dendritic cells) and presented to T-cells leading to the activation of B-cells that leave the PP and migrate to the mesenteric lymph nodes (MLN) and enter the blood circulation. These cells recirculate and home in the lamina propria where they mature to mainly sIgA but also sIgM-producing plasma cells. The cytokine IL-12 from macrophages and B-cells are essential mediators of the protective host responses against possibly the final effector cells in the infection process. TH2 response where IL-4, IL-10 and TGF-β from the bystander cells become dominant was associated with modulation. Local production of TH1 cytokines is reduced and TH2 cytokine response to soluble egg antigen and T-helper

producing both TH1 and Th2 cytokines (THO) to worm antigen increase in a similar pattern in kinetics to the modulation of granuloma size (Williams, 1994; Williams *et al.*, 1995). However attempts to better understand the molecular and cellular factors associated with granuloma formation have revealed the involvement of a number of other mechanisms whose relative contributions vary with host and /or parasite factors in influencing the DTH reaction (Stadecker, 1994b).

A shift from TH1 cytokines (IL-2, INF-γ and TNF-α which support macrophage activation, DTH- responses and immunoglobulin isotype switching to IgG2a to TH2- dominated responses where IL-4, IL-5, IL-6, IL-10 and IL-13 provide efficient help for B-cell activation for switching to the IgG1 in mice, IgG4 and IgE in humans has been reported (Lundgren *et al.*, 1989). This, however, depends on the dose and route of antigen intake, the type of antigen presenting cells involved and the involvement of MHC class II types. The fact that clones of T-cell injected with antigen into the footpads of naive mice produce TH1 specific cytokines and DTH resembling reactions at the acute phase of schistosomiasis confirm that the TH1 which is a cellular response is responsible for the pathogenesis. Nevertheless, T-suppressor/effector factor (Tef) produced by CD4+, CD8+ and total spleenocytes from chronically infected animals have also been identified (Fidel and Boros 1990; Noya *et al.*, 1995). It suppresses T-cells and IL-2 production. Addition of exogenous rIL-2 has been shown to completely ablate Tef production without any influence on the already produced ones (Mathew *et al.*, 1990).

By using available knowledge on the immunology of schistosomiasis mansoni, several endeavours to produce vaccines, targeting different stages of the disease have been attempted. However, as yet there is no safe vaccine developed to the stage of application in humans, although a lot of evidence and success stories indicating the feasibility of vaccine from experimental animals and evidences for acquired immunity from epidemiological observations in humans living in endemic areas are documented (Butterworth *et al.*, 1992; Grzych *et al.*, 1993; Capron and Capron, 1994). The first success story on vaccination comes from calves immunized with irradiated cercaria of *S. bovis* where 80% were partially protected (Bushara *et al.*, 1993; Capron *et al.*, 1995; Reist *et al.*, 1994). This was followed by another successful experiment that optimized the irradiation doses, the adjuvant effect, the route of administration of the vaccine (James and Sher, 1990).

Intra-nasal administration of killed shistosomula failed to induce protection but intradermal immunization with 20 Krad irradiation and BCG as adjuvant resulted in strong protection against challenge infection in mice. Attempts to identify the parallel response in humans revealed the induction of specific IgE antibody to adult worms and schistosomula (Hagan and Gryseels, 1994; Capron and Capron, 1994; Demeure *et al.*, 1994; Dunne *et al.*, 1992) and IgA antibody to parasite enzyme antigen, glutathione S-transferase. Many candidate vaccines either of purified native antigens like paramomycin, 14kD fatty acid and more recently nucleic acid vaccines encoding membrane protein Sm23, of *S. mansoni* are reported (Waine and McManus, 1995; Reynold *et al.*, 1992).

With the improved knowledge of mucosal immunity and identification of several ways of vaccine delivery the efficiency of mucosally directed immunity has increased. A good example is the successful oral vaccine composed of formalin killed vibrio and recombinant-B subunit cholera toxin that was developed against *Vibrio cholera* (Holmgren *et al.*, 1977). The problem of low immunogenecity of antigens with oral vaccines, as compared to live infection has been alleviated by the construction of adjuvants of various formulations: cholera toxin and cholera toxin-B (CTB). Cholera toxin induces IgA in the gut and IgG1, IgG2a and IgM responses in the circulatory system. More important, when given as an adjuvant, it enhances the effect of unrelated antigens at the systemic as well as the mucosal levels (Akhiani *et al.*, 1993). CTB, the non-toxic component of CT, besides serving as an adjuvant, induces selective s-IgA antibody production in the gut and a state of tolerance at the systemic level (Sun *et al.*, 1994).

This concept has been utilized in treatment of autoimmune diseases like autoimmune encephalitis and has successfully prevented auto-inflammatory reactions (Sun *et al.*, 1994); a process, that has similarities with schistosomiasis pathology. Although the balance between active immunity and suppression/tolerance varies with the nature of the antigen, types of accessary cells and the lymphocytes involved,immune response generated by cholera toxin has repeatedly been shown to promote both mucosal and systemic antibody response and even abrogates established resistance (Holmgren *et al.*, 1993).

From these developments in mucosal immunity on the one hand and the findings of Weinstock *et al.* (1985) and Weinstock and Blum (1987), where they demonstrated that animals sensitized with soluble egg antigens at the mucosa, unlike subcutaneous and

peritoneal exposure, diminish granulomatous response in schistosomiasis, we proposed the possibility of using mucosal route of immunization for immunotherapy to prevent the effect of the disease. However, the fact that they used granulomas formed in the lung as a result of egg-injection through the tail vain of mice puts some doubt on wether the cellular composition would be the same to that produced in the liver under natural infection. Therefore, it might be proper to test the system by utilizing the oral route of immunization to induce an anti-infectious and anti-inflammatory responses against schistosomiasis mansoni. It is well known that generation of sIgA at the mucosal surface plays a major role not only in protection of mucosal surfaces from being colonized by microorganisms but also suppressing systemic immune responses that are potentially harmful to the host. Induction of such responses is attractive from the standpoint of vaccine production. The generation of these responses however requires large quantities of the antigens given repeatedly over a long period of time and even then the response lasts only for a very short time. The knowledge concerning potential regulatory and /or accessory mechanisms has been scarce. Studies have been confined to the analysis of antibody responses in mucosal fluids (lavage fluid, saliva and gingival) and serum, primarily due to lack of appropriate methods that allow the induction, characterization and assessment of antigen-specific mucosal immune responses. With the advent of (i) transmucosal carriers like cholera toxin that are capable of inducing an antigen specific sIgA responses to both the carrier and the mitogen they are bound to, while at the same time abrogating systemic immune responses to the conjugated antigens; (ii) enzyme dispersion techniques that allow the isolation of lymphoid cells from biopsy specimens of human mucosal tissues and (iii) development of ELISPOT to determine immune response at a single cell level, a more precise investigation has become feasible (Czerkinsky *et al.*, 1988a&b).

In this study, attempt was made to fill the gap in methodological ability to characterize the phenotypes of antibody secreting cells and to determine the homing mechanisms in the development of immune responses in humans. It has also been endeavoured to apply the recent knowledge on mucosal immunity with the view to better understand the strategy for a vaccine to schistosoma mansoni infection in mice. Selected members from the various surface markers including adhesion molecules and homing receptors were employed in the study. Using this system the immune response to the candidate vaccine (Sm28GST) (known to induce significant degree of protection in permissive hosts such as baboons, hamsters and rats and also strongly reduces egg related granuloma (Balloul *et al.*, 1987) was characterized.

To analyze cell mediated immunity on one hand and immune suppression on the other, dissection of the immune responses generated at the mucosa from those induced systemically would be necessary.

There has been substantial interest to phenotype the expression of the cell surface markers and the cytokines secreted for diagnosis, defining the state of disease progression and prognosis after vaccination or treatment. Studies along this line however had suffered from lack of appropriate techniques. Such studies cannot be done serologically as serology does not reflect the local immune status of the host especially when the stimulus is encountered at mucosal surfaces. The situation in schistosomiasis patients, where massive eggs are deposited in intestinal tract or the bladder is one good example. Furthermore, the information gained through the conventional immunohistological procedures or through in vitro cell culture technology, for assessment of B, T, and accessory cell functions are not reliable enough either.

The characterization of spontaneously antibody secreting cells as they reflect the course of events in B-cell immunity would be a more precise measure of the immune status of the host. These are migratory cells en route to their destination and thus appear in circulation for a short period after intentional immunization as in vaccines or following natural infection. They produce specific antibodies immediately after isolation and without any further stimulation. They persist as long as the stimulation continues as in the chronic infections, thus making the detection of these cells very relevant as a means of assessing the immunogenicity of vaccines and as a proxy-diagnostic measure of an on going infection. However, the phenotypic characteristic, degree of activation and the anatomical commitments and weather such characteristics can be used to differentiate antibody forming cells activated at systemic sites from those stimulated at mucosal is not well understood (Grogeon, 1996; Poggi, 1996; Kishimoto *et al.*, 1990). The advantage and potential utility of mucosal routes rather than parenteral for vaccine delivery is attractive alternative from both logistic and biomedical considerations. Its advantage of being simple, requiring less skill, and more important the fact that it can cover both systemic and mucosal immune systems makes it ideal.

Accordingly the present work was primarily focused on improving the available test system, the ELISPOT technique, which has the merit of detecting responses at a single cell level but does not allow the phenotypic characterization of the migrating ASC. Furthermore, its

sensitivity decreases when more than 100,000 cells are seeded in each ELISPOT wells. When one considers the fact that immunologically active cells are not so abundant in the circulatory system one finds it difficult to make any degree of association between the disease statuses. Thus the need to improve the sensitivity of the ELISPOT has been recognized.

To address the general problem, both in the methodology and the fundamental issues of induction of host protective immunity against schistosomiasis, the following objectives were set.

Objectives:

I. General

a. To develop appropriate method that increase the sensitivity of ELISPOT technique and characterize the phenotype of antibody secreting cells in humans.

b. To sue contemporary knowledge in mucosal immunity to develop potential vaccine against schistosomiasis.

II. Specific

i. Develop a method that combines magnetic beads with ELISPOT to sort and phenotypically characterize antigen specific antibody secreting cells.

ii. Evaluate the method in known systems using human mucosal (Cholera toxin) and Systemic (Tetanus toxoid) vaccines.

iii. Validate the developed method under natural infected schistosomiasis mansoni patients.

iv. Assessment of tonsil as a potential induction site:

   a. Frequency of vaccine specific ASC appearing in palatine
   b. Comparison of intra-tonsillar route to other routes of immunization
   c. Percentage of ASC expressing homing receptor to the tonsil.

v. Using cholera toxin-B bound glutathione transferase (Sm28GST) to induce protection against both the infectious organism and the granulomatous reaction produced as a result.

II. MATERIALS AND METHODS

2.1 Study subjects

2.1.1. Swedish vaccinees

Three groups of volunteer subjects were recruited for the study. Peripheral blood samples from 5 male and 5 female adults were first taken to develop the method and screen the surface markers that could optimally be used to isolate the ASC. Then 23 other subjects of which only 15 were additionally vaccinated for tetanus were both considered 7 days after the last vaccination. The comparative study of immune responses following systemic versus mucosal immunization. And finally 4 groups of patients, each consisting 5-7 individuals were added for nasopharyngeal mucosal study. All vaccinations were done with the approval of Ethical Research Committee of medical Faculty of the university of Gotenborg.

2.1.2 Schistosomiasis patients

The study population for schistosomiasis consisted of forty individuals all living in Wonji, a known endemic site for Schistosomiasis mansoni. Twenty nine schistosomiasis patients shedding ova at the time of examination, and 11 negative controls selected after preliminary screening for the presence or absence of the parasite by Kato thick smear and having confirmed their consent to participate in the study. These groups were then physically examined by a physician and their histories of schistosomiasis, malaria and other diseases of the preceding three months were recorded. The controls consisted of healthy individuals, who had never had infection, from the hospital staff and their children, but also those that were cured after treatment with praziquantel and those that have had malaria treatment as well. For screening of cell surface markers, samples from 8 other schistosomiasis positive patients, from those that attended hospital were examined.

2.2 Preparation of antigens

S. mansoni egg and adult worm antigens were prepared from the Wonji strain of parasites maintained at the laboratory at Addis Abeba University in *Biomphalaria pfeifferi* and Swiss albino mice. The parasite strain used in vaccine trial in Sweden was a Puerto Rican strain of *S. mansoni*, whose life cycle was maintained in *Biomphalaria glabrata* and Syrian hamsters. The adult worm was collected by simple perfusion with 0.1M Phosphate Buffer Saline (PBS) pH-7.2 (Hepatic portal vein was cut-open with scissors and PBS slowly injected

into the left ventricle of the heart pushing out the adults from mesenteries and sinuses of the liver. The adult worms were sorted from the washes collected on petri-dishes). The eggs were recovered from the liver by digestion of the granuloma with trypsin as follows: chopped liver tissue was incubated in 1% trypsin (Sigma St. Louis Mo., USA) in PBS solution for 2 hours at 37°C. The isolated eggs were enriched by passing them through 150 μm mesh size sieves. They were then washed three times with PBS at 1500 rpm. The clean eggs were collected from the bottom of the segment and were kept at -20°C until used. Both were separately homogenized in sterile PBS with glass homogenizer. The soluble fractions were collected in the supernatant after high speed centrifugation 35,000 xg for 30 minutes. Their protein content was determined by the Lowry method (Lowry *et al.*, 1951). The supernatant were then filtered through 0.45μm membrane filter and stored at -70°C.

2.3 Preparation of CTB-conjugated antigens

CTB used in this work was devoid of CT contaminants (Department of Immunology, Gotenborg University) (Table 1). It was produced from mutant *Vibrio cholera* that had lost its cholera toxin genes and was transfected with a plasmid encoding CTB. It was then purified by gel-filtration chromatography through sephadex G-100 column (Pharmacia, Uppsala, Sweden) (Friman *et al.*, 1994).

CTB-rSm28GST and CTB-SEA conjugates were prepared using a bi-functional coupling reagent N-succinimydyl (3-[2-pyridyl] dithio) propionate (SPDP) according to the manufacturer's recommendations (Pharmacia, Uppsala, Sweden). Briefly, CTB and the antigens were separately derivatized with SPDP at 1:5 molar ratios. After thorough mixing and letting it stand for one hour at room temperature, excess SPDP was removed by gel filtration through Sephadex G-25 columns (Pharmacia). The SPDP-derivatized antigens were reduced with dithiothreitol (DTT) and the resulting preparation was freed of excess DTT and pyridine-2-thione by sephadex G-25 chromatography. SPDP derivatized CTB and antigens were mixed at 1:5 ratio and incubated for 16 hours at room temperature. The CTB-antigen conjugates were purified by gel filtration through columns of Sephacryl S-300. Purified conjugates were shown to contain GM1-binding capacity and to retain CTB antigen serological reactivity as detected by solid phase ELISA using GM1 as a means of capture and enzyme labelled antibodies as detectors.

Individual wells were coated with 3 μM solution of GM1 gangliosides (Sigma Chemical Co.) in PBS for overnight at 4°C. The next day they were washed three times and 100 $\mu\ell$ of 1μg/ml of the conjugate and 1μg/ml bovine serum albumin for controls were added. Unabsorbed proteins were cleaned and 150 μl of horse radish peroxidase labelled anti-CT antibody were added. After washing with PBS-Tween, the antibody was determined by addition of its substrate that consisted of 0.3 mg/mℓ of 3-amino-9-ethyl carbazole (Sigma Chemical Co.) and 0.015% (vol/vol) H_2O_2 in 0.1M sodium acetate pH 5. After 30 minutes of incubation the absorbance was measured by a multi-channel spectro-photometer (Lab Systems Multiscan PLUS) at 405 nm and results expressed as a mean absorbance of duplicate wells after subtraction of the background values.

2.4 Specimen collection from the study subjects

Stool specimens from schistosomiasis patients and heparinized venous blood samples from all subjects were collected just after the physical examination. The stool specimens were immediately checked by direct method and double kato slides were prepared and transported to the laboratory for quantitative determination. Those in which eggs were not detected in both searches were registered as negative.

About 10mℓ of heparinized blood samples, mononuclear cells (MNC) were isolated by gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and washed several times with cold 0.1M PBS, pH-7.2 supplemented with 1% heat inactivated Fetal Calf Serum (FCS). The MNC were then counted and re-suspended in RPMI medium supplemented with 5% FCS and 100 μg/mℓ gentamicin (Gibco) (complete medium) and kept over ice until use.

2.5 Test systems

2.5.1 Enzyme Linked Immunosorbant Assay (ELISA)

The wells of Polyvinylchloride plates (Immunoplate II; Nunc, Roskilde, Denmark) were sensitized with 100μl of optimal concentrations of pertinent antigens in $NaHCO_3$ and were kept at 4°C over night. The plates were then washed three times in PBS, pH-7.4 with 0.05 % PBS-Tween.This was followed by blocking with 0.1 % bovine serum albumin (BSA) for 1 hour at ambient temperature. The test plasma diluted 1:50 in 0.05 % PBS-Tween and supplemented with 0.1% BSA was incubated for two hours at 37°C. After repeated washing

in PBS-Tween alkaline phosphatase labelled sheep anti human IgG antibody, (heavy chain specific) (Sigma Chemical CO., T.1063) supplemented with 0.1 % BSA at optimal dilutions were added to the wells and incubated at 37°C. Alkaline phosphatase activity was assayed by adding 100 μl of 0.006M P-nitro-phenyl-phosphate in glycine buffer with 0.001 M MgCl2 (pH-10.5). The reaction was stopped by adding 50μl of 1.5M H_2SO_4 acid after 30 minutes. Optical density was read at 490 nm in a micro-titter plate reader (Labsystem Multiscan MCC/340).

For determination of antibody subclasses, plates were coated with 50$\mu\ell$ of mouse monoclonal antibodies to human IgG1-4 diluted in PBS with 0.05% Tween-20 and incubated for one hour at 37°C. Sera diluted 1:200 were added in triplicate after blocking with BSA. Then it was developed against peroxidase-conjugated rabbit anti-mouse IgG1-4 diluted with optimal dilutions in PBS with 0.05% Tween-20. 100 μl of the substrate solution, O-penylene diamine (10 μg/30 ml) in 0.1M phosphate citrate buffer pH-5, with 5 μl of 30% H_2O_2 was added to each well. The reaction was stopped after 30 minutes by adding 50 μl of 1.5M H_2SO_4 and the optical density read at 405 nm.

2.5.2 Sorting of MNC by phenotypes and Quantification of Antibody Secreting Cells

The fractionation of B-cells from the MNCs was done on the basis of expression of surface markers using a combination of immunomagnetic cell sorting and ELISPOT techniques. Briefly, paramagnetic microspheres (Dyna beads, Dynal, Oslo Norway) coated with sheep anti-mouse IgG antibody (Dynabeads; Dynal Oslo, Norway) were incubated for over night at 4°C with mouse MoAb specific to the desired surface markers. The anti-CD19 coated beads were obtained directly from Dynal (Oslo, Norway). In the case of CD38+ cells, removal of CD2+ cells before hand and incubation of the cells with monoclonal antibody rather than beads was performed in the laboratory. Optimal beads-to -antibody and beads-to-cell ratio ranging from 6:1 to 10:1, for each set of marker, was employed. In schistosomiasis mansoni patients antibodies to three surface markers HLA-DR, CD38 and CD19 were tested for their relative efficiency in picking antigen specific ASC by using 10:1 ratio.

For determination of cell surface expression of CD44, Integrin $\alpha4\beta7$ and L-selectin similarly the sheep anti mouse antibodies coated beads were incubated overnight with mouse MoAbs, Hermes 3, ACT-1 or Dreg-56 specifying CD44, Integrin a4$\beta7$ and L-selectin respectively.

Coated beads were washed and mixed with the MNC suspensions at optimal bead to cell ratio of 10:1 (CD44, $\alpha 4\beta 7$) and 5:1 (L-selectin)

Beads and cells were pelleted by centrifugation for 1-2 minutes at 800 RPM in a centrifuge (Beckman Instruments, Inc. Palo Alto, CA.) and kept at 4°C for 45 minutes. Then they were gently mixed and left for 30 minutes. Cells bound to the immunomagnetic beads were then captured by magnetic bar to be refereed as positively selected and the non bound cells refereed as negatively selected. The resulting positively and negatively selected cells were re-suspended in equal volume of ISCOVE complete medium (Gibco). For controls, beads coated with irrelevant mouse IgG MoAb to *Streptococcus mutans* protean-Ag I/II were used and no ASC could be detected attesting to the specificity of the method.

The positively and negatively fractionated and unfractionated MNCs were assayed for ASC by two-colour ELISPOT technique. The cell fractions were separately washed in RPMI-1640 complete medium and diluted to the desired cell concentrations in RPMI-1640 complete medium. They were then seeded in duplicate into nitrocellulose-bottomed 96-well plates (Millipore, Bedford, MA) that were coated with 100 μl of crude egg antigen (5 μg/ml) or Adult Worm Antigen (AWA) (8 μg/ml). The cells were incubated at 37°C and 7.5% CO2 for 4 hours. The secreted antibodies appeared as red and blue spots when horse radish peroxidase and Alkaline phosphatase labelled Goat anti-Human IgA, IgG and IgM antibodies 1:5000, 1:10000 and 1:10000, respectively were applied for an hour and developed by their respective chromogens (Czerkinsky *et al.*, 1988a). The spots expressing each class were counted under microscope and their concentration per million calculated.

2.6 Immunization and sampling protocols

2.6.1 Humans

With the approval of Ethical Clearance Committee of the Medical Faculty of the University of Gotenborg, a total of 33 healthy swedish volunteers who had consented to be part of the study were enrolled in the study. Samples from 5 males and 5 females, were given only booster subcutaneous injection of tetanus toxoid and their samples were used for methodological studies. The other 23 subjects were first primed with a single oral dose of cholera vaccine which was produced at the Department of Medical Microbiology, University of Gotenborg. The vaccine was composed of 1 mg of purified cholera toxin B (CTB) and

10^{11} of killed whole cell *Vibrio cholera* organisms vaccine in 150 ml of bicarbonate buffer (Holmgren *et al.*, 1977). The same individuals were boosted with the same dose of the oral vaccine 3 weeks later. At the second vaccination 15 of these subjects were also injected with one dose of tetanus/diphtheria vaccine containing 2 Lf of tetanus toxoid (TT) and 7.5 Lf of diphtheria toxoid (SBL, vaccine, Stockholm, Sweden). Each of the volunteers had received several prophylactic immunizations with tetanus vaccine during childhood and youth, with the last of these immunization being performed at least 2 years before the present study. Vaccination protocol was as shown in Fig. 5.

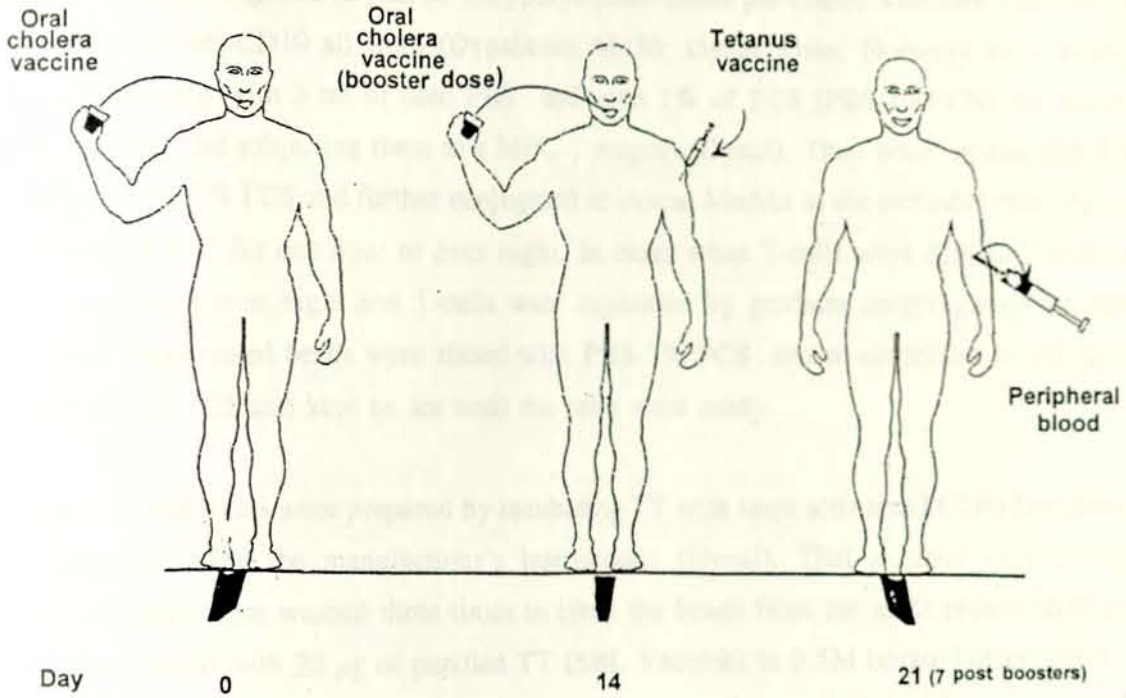


Fig. 5. Cholera toxin and tetanus toxoid vaccination protocol.

Heparinized blood samples were collected at the 7th and 8th days post oral booster immunization, a time point corresponding to the peak of both anti-CT and anti-TT ASC response in the circulation in vaccinated individuals (Tarkowski *et al.*, 1985). The samples from and schistosomiasis patients were taken from those that came for treatment at the Wonji Sugar estate hospital, Wonji, following their informed consent. In all cases, 5-10 ml of heparinized venous blood was drawn from each individual patient.

2.6.2 Isolation and fractionation of mononuclear cells (MNC) and leucocytes

Peripheral blood MNCs were isolated by standard gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Isolated MNCs were washed three times with PBS (0.01M phosphate buffered 0.15 M NaCL, PH 7.4, (Gibco Europe, Edinburgh, UK) containing 100 μg/ml of gentamicin (Gibco) and kept on ice until further fractionation or analysis. The isolated cells were depleted of T-cells by step wise sheep red blood cell rosseting (Perussia *et al.*, 1983).

The mono-sized magnetic (Dynal M-450) polystyrene beads pre-coated with sheep anti mouse Ig or human anti-CD19 all from (Dynabeads M450; Dynal, Oslo, Norway) were washed three times each with 3 ml of cold PBS and with 1% of FCS (PBS-1%FCS), by placing them in tubes and subjecting them to a MPC-1 magnet (Dynal). They were re-suspended in 300 μl of PBS-1% FCS and further conjugated to mouse MoAbs to the pertinent markers and incubated at 4°C for one hour or over night. In cases when T-cells were depleted, rosetted cells were kept over night and T-cells were separated by gradient centrifugation the next morning. The treated beads were rinsed with PBS-1% FCS, and re-suspended in 200 μl of cold PBS-1% FCS and kept on ice until the cells were ready.

Antigen coated beads were prepared by incubating TT with tosyl activated M-280 Dynabeads prepared following the manufacturer's instructions (Dynal). That is, four million tosyl activated beads were washed three times to clean the beads from the azide preservative and were then mixed with 20 μg of purified TT (SBL Vaccine) in 0.5M borate buffer, pH 9.5, and incubated for 2-4 hours at room temperature or over night at 4°C. Beads were saturated with 1 % BSA in PBS and washed with borate buffer as described above. Binding of TT to magnetic beads was confirmed by ELISA using human hyperimmune serum from a tetanus vaccinee as source of primary IgG antibody and enzyme-conjugated anti-human IgG as detection reagent. For control purposes, tosyl-activated beads were coated with Streptococcus mutans protein Ag I/II (a gift from Dr. Mike Russel, University of Alabama) exactly as described for TT-coated beads.

The isolation of CD28 and CD38 expressing cells was performed by first incubating MNC with the anti-CD28 (0.5μg/10^6 cells) and B-cell enriched fractions by depletion of CD2+ cells and treating them with anti-CD38 MoAb (0.4 μg/10^6 cells) for 45 minutes in 300 μl of ice cold Iscove complete medium. For the rest of the markers, 0.25μg MoAb/mg of beads

were used. Thereafter, cells were washed once in cold PBS-1 % FCS by centrifugation and incubated with dynal beads. CD19 coated beads were purchased and used directly.

The efficiency of the cell separation procedures was assessed by comparative flow cytometric analysis of unfractionated and negatively sorted cell suspensions. For this purpose, cells expressing the pertinent markers were stained with PE-or FITC conjugated MoAbs from Becton-Dickinson were used at optimal concentrations. Cells at $10^5/100\ell$ of 0.1% BSA in PBS were incubated with the different MoAbs for 20 minutes over ice and thereafter washed thoroughly in 0.1% BSA in PBS before analysis on a FACS Scan (Becton Dickinson). The flow cytometric analysis was used to identify and rapidly count the number of different cells in the tissue samples. In order to identify the distinct live lymphocyte populations by the flow cytometry it was necessary to measure in addition to cellular antigens, visualized by antibodies linked to fluorescent dye, physical properties of a cell, such as size internal structure and nuclear to cytoplasm ratio. Based on the formed and side scatter characteristics the live lymphocyte was identified and gauged for analysis from the dead cells.

Typically the selected cell population were stained directly by PE-labelled antibodies to the pertinent surface markers. Cells in suspension were made to flow in a laminar manner by a suitable hydrodynamic system such that they line up in a single file, like beads on a string. The cells move in this manner through a beam of illuminating light from a laser. Once a cell has intersected a laser beam, the light which is subsequently transmitted is composed of two types: scattered light and fluorescent light. These signals are collected by lenses and focused on photo-multiplier detectors. The electrical pulses generated by these detectors were then amplified, measured and displayed on a screen.

Tosyl activated beads were also coated with tetanus toxoid 40 μg/ml antigen for 4 hours or overnight depending on the temperature, washed and cleaned in cold PBS-1 % FCS before mixed with cells. The procedure was shown to provide efficient separation of the cells expressing anti-TT antigen. Similarly the two step procedure was more efficient for both CD28 and CD38. Ficoll-Hypaque isolated MNC were first depleted of T-cells by incubation with anti-CD2-coated Dynabeads, at a bead: MNC ratio of 10:1, according to the manufacturer's instructions (Dynal AS, Oslo, Norway). Unbound cells were then further fractionated as above.The MoAbs specifying the rest of cell surface markers: HLA-DR-, HLA-DP, and HLA-DQ, CD-44 (H-CAM, Putative receptor for Peyer's patch high endothelial venules); LECAM-1 (Leu 8, putative receptor for peripheral lymph nodes HEV), Igs kappa and lambda chains, CD20 and CD71 (transferrin receptor) but also CD28, CD38,

were used. The immunoparamagnetic separation procedure used several steps to fractionate the MNC (Fig. 6).

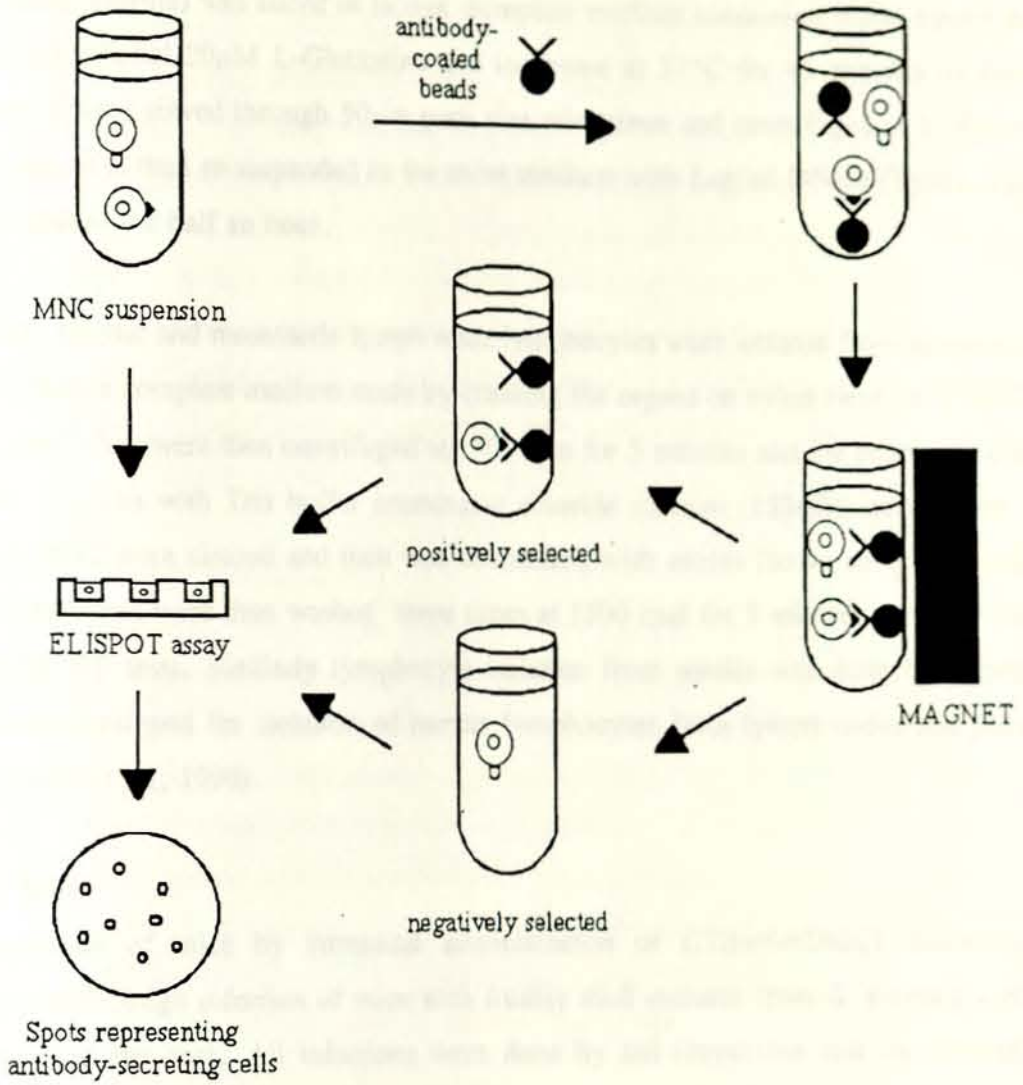


Fig. 6. Cell sorting scheme using immunomagnetic beads coated with pertinent Moabs.

The beads and cells that were mixed in a round-bottomed glass tube in a total volume of 300 μl complete medium, pelleted by centrifugation at 1,000 RPM for 5 minutes were then gently re-suspended and incubated for an hour at 4°C and then kept for 15 minutes with gentle mixing on ice. For an optimal beads to cells ratio determination, a range of 20:1 to 100:1 was employed. Cells rosseting the dynal beads, i.e., expressing a given cell marker were then captured using MPC-1 Dyna magnet. Unbound cells were removed from the tube by aspiration. Both the positively selected cells and the negatively selected cells in suspension were re-suspended to the original volume of Iscove complete medium and assayed for the number of vaccine or antigen specific ASC.

Isolation of leucocytes from liver was done by collagenase/Dispase digestion of pooled and, weighed whole liver tissues after mechanically crashing them on nylon sieve. 40 U/ml collagenase (Sigma) was added in Iscove complete medium containing 10μM Hepes 50μM 2-Mercaptoethanol 20μM L-Glutamine and incubated at 37°C for 45 minutes on rotating agitator. l were sieved through 50μm pore size wire-sieve and centrifuged at 1500rpm for 5 minutes. It is then re-suspended in the same medium with 1μg/ml DNase (Type1, Sigma) and incubated for half an hour.

Similarly splenic and mesenteric lymph node lymphocytes were isolated from suspension of cells in Iscove complete medium made by crashing the organs on nylon sieves of 150-50 μm pore sizes. They were then centrifuged at 1500 rpm for 5 minutes and the pellet was cleared of RBC by lysis with Tris buffer ammonium chloride solution (155mM) added drop wise until all RBC were cleared and then was neutralized with excess Iscove complete medium. Cell suspensions were then washed three times at 1500 rpm for 5 minutes each and seeded for ELISPOT tests. Similarly lymphocyte isolation from tonsils was done by dispersion technique developed for isolation of human lymphocytes from lymph nodes and intestine (Nordström *et al.*, 1990).

2.6.3 Mice

Immunization of mice by intranasal administration of CTB-rSm28GST following an experimental design infection of mice with freshly shed cercaria from B. glabrata kept for one week in the dark. All infections were done by tail immersion and the counting of cercaria used for infection and those left at in the test tube after infection were checked by staining them with iodine under a binocular microscope.

EXPERIMENTAL DESIGN

INFECTION	INTRA NASAL-TREATMENT	EFFECTS
70 cercaria	CTB-GST CTB-SEA 10 μg x 3 IN	Parasites Histopathology Immunity
Day 0 Days 21 ⇑	28 ⇑ 35 ⇑ Days	47 and 51 ⇑ ⇑

a. Intranasal immunization

Female Balb/C mice of 4-5 weeks old were infected with 70 cercaria each by tail immersion with Puerto Rican strain of *S. mansoni*. Starting from the 4th week post infection, 10 μg of CTB-rSm28GST or CTB-SEA conjugate vaccines were administered intranasally to each group of mice, for three consecutive weeks. The controls were similarly treated with equal volume of saline, CTB- and rSm28GST alone. The effects of vaccine treatments were measured using parasitological (egg count), histopathological (granuloma size around eggs) and immunological parameters (Lymphocyte proliferation and INF-γ and IL-4 determination). Worm burden, liver egg counts/gm were made after one hour incubation in 4% KOH at 37°C. Samples of the same lobe of liver were collected for histological processing and stained with hematoxylin and eosin. Inflammatory areas and diameter of granulomas in liver were measured by image analyzer connected to a leitz DN microscope. The sizes of the granulomas were calculated as the percentage of the whole section.

b. Other routes of immunization

To study vaccine specific ASC in palatine tonsil peroral, intra nasal, systemic (subcutaneous), Intra tonsillar injections were used. Tonsillar materials and venous blood samples were collected from adult volunteers scheduled for tonsillectomy indicated by recurrent tonsillitis, tonsillar hypertrophy, or severe snoring problems from hospitals in Gotenborg, Sweden. The volunteers were organized in groups of 5-7 individuals and each group according to one of the following 4 different immunization schemes (i) one single injection in the right palatine tonsil with (CTB), and tetanus toxoid (ii) two intranasal immunizations with CTB (iii) two peroral immunizations with CTB as in oral cholera vaccine and (iv) two oral immunization with CTB followed by booster dose of TT intracutaneously.

c. Enumeration of ASC

Fractionated as well as unfractionated MNC suspensions were assayed for the specific ASC by micro-modification of the ELISPOT technique (Czerkinsky, 1988). Nitrocellulose-bottomed 96 well plates (Millipore, Bedford, MA) were coated directly with appropriate quantities of the respective antigen: 20 μg/ml of purified TT. Crude schistosome egg antigen (SEA), at 2.5 μg/ml and Adult Worm Antigen (AWA), at 4μg/ml were used. The binding of CT is facilitated by first coating the nitrocellulose well with 3 μM of GM1-ganglioside over night. After blocking it with 150μl of Iscove complete medium for 30 minutes at 37°C, the wells were incubated with various numbers of MNC from 4-12 hours at 37°C in 200μl of complete medium. The solid phase bound Ab secreted by individual MNC were revealed as spots by stepwise addition of horseradish peroxidase labelled goat Ab to human IgG, IgA, IgM (Southern Biotechnology Associates, Birmingham, AL) diluted 1:5000, 1:10000 and

1:10000 in PBS-Tween and a suitable chromogen substrate prepared by mixing 0.015 % (vol/vol) H_2O_2 and O.3 mg/ml of 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, Mo) in 0.1 M sodium acetate, pH 5. The percentage of ASC expressing a certain marker was calculated by dividing the number of spots in the positive fraction with the number of spots in the unfractionated MNC suspension assayed at the same cell density. The corresponding percentage of surface negative cells was calculated.

To ascertain that binding of beads or the MoAb applied did not interfere with spot formation at the concentrations used in the experiments, a series of control experiments were performed. MNCs isolated from an immunized donor were incubated together with beads coated with MoAb to CD19, CD20, light chains, control IgG1, or with corresponding MoAb alone in TT-coated ELISPOT wells. Addition of Ab coated beads to unfractionated MNC was found to have had no effect. Cells bound to beads coated with control MoAb did not contain any detectable Ig-secreting cells further attesting to the specificity of the separation method employed.

d. Lymphokine determination

Production of INF-γ and IL-4 were determined by reverse ELISPOT. Spleen and liver cell suspensions (10^5 cells /well) prepared as follows: Spleen and liver were gently forced through a nylon sieve in to Iscove medium (gibco BRI, Life Technologies Ltd. Middlesek, UK). The cell suspensions were centrifuged and the pellet suspended in 2ml on top of which 4ml of 70% and 4ml of 40% percol were layered and centrifuged at 2000 for 10 minutes. The cells at interface of 70% and 40% were collected washed and re-suspended in cold Iscove complete medium before used for test. In the case of liver additional collagenase /dispase treatment was necessary. The isolated cells from mice intranasally immunized with CTB-rSm28GST, were cultured in nitrocellulose base 96 well Millipore plate (Millipore Corp. Bedford, MA, USA) coated with rat anti mouse (IgG1) INF-γ, IL-2 (JES6-1A12), IL-4 (bvd4-1D11) and IL-5 (TRFK5). They were incubated with 10 μg of CTB-rSm28GST for 20 hours at 37°C in 100 μl of Iscove complete medium. Zones of solid phase bound cytokines secreted by individual cells were developed as spots by polyclonal rabbit anti-mouse IFN-γ and biotinylated rat anti-mouse IL-2 (JES6-5H4), IL-4 (BVD6-24G2), IL-5 (TRFK4) (PharMingen) were added. These were further incubated with HRP-conjugated avidin (Sigma) in PBS containing 0.05 % Tween 20 and 1 % FCS was added and the plates incubated at 37°C for overnight. The reaction was developed by the addition of substrate chromogen consisting of 10 mg 3-amino-9-ethyl carbazole (AEC) (Sigma) dissolved in 1 ml of dimethyl-formamide and diluted in 30 ml of 0.1 M sodium acetate (pH 5). The solution was filtered through 0.2 μm membrane filter before the addition of 0.015% (vol/vol) H_2O_2.

The reaction was stopped by rinsing it with tap-water after the spots appear. The number of spots/10^6 cells were calculated from a count made under a microscope.

e. Lymphocyte transformation

Single cell suspensions were prepared from pooled spleen, liver and mesenteric lymph nodes of mice. They were seeded in round bottomed 96 well culture microtiter plates (Nunc) at 10^5 cells /well in 200 μl of Iscove or RPMI-1640 containing 10% AB+ human serum, 100 μ/ml penicillin and 100 mg/ml Streptomycin. Some wells were left un-stimulated for the determination of background proliferation. Cultures were then incubated at 37°C in a 5 % humidified atmosphere containing 5% CO_2. After 72 hours of incubation, cultures were pulsed with 1 μCi/well of [3H] thymidine (Amersham International, Amersham, UK). Cells were collected 16-18 hours later on a glass-fiber filter and the level of radioactivity determined using an automated argon activated β scintillation counter (Inotech, wohlen, Switzerland). Data were collected from triplicate samples and results were expressed as mean count/minute $\pm$SD after subtraction of background values of triplicate samples. Results of transformation assays for mice were expressed as the stimulating index (SI), defined as the ratio of the mean thymidine incorporation of rSm28GST stimulated cultures divided by that of un-stimulated cultures.

Delayed-type hypersensitivity (DTH)

For DTH response determination to GST, four mice in each group were primed by footpad (right) injection of GST, *Schistosoma mansoni* crude AWA and SEA emulsified in fruend complete adjuvant (FCA). Three and six days after priming animals were intranasally administered with 10μg of CTB-bound Sm28GST CTB-ova, AWA, SEA twice to each group. Six days after the last intranasal treatment, DTH reactivity was tested by measurement of the thickness of the reaction at the left footpad 24 hours after the challenge injection.

2.7 Data analysis

Data regarding the difference in the amount of antibody expressing cells and classes of antibodies were analyzed by Wilcoxon rank sum test. Comparison of means at P values <0.05 were taken as significant differences.

Table 1. Major chemicals and reagents used in the experiments.

Reagents	Sources
MoAb (CD5, CD20, CD22, CD23, CD25, CD28, CD37)	Immunotech (Marseille, France)
MoAbs PE-conjugated Ab to CD5, CD19 and CD25	Becton, Dickinson and CO. (San Jose, CA)
Hermes-3, (LECAM-1)	Department of pathology, Stanford University
Dreg-56, (HCAM)	Collaboration of Dr. E. Butcher
Putative MoAb for CD44, (mucosal) and L-selectin, (peripheral)	Department of pathology, Stanford University
MoAb to CD71	Dr.M. Egan (University of Albama, Birmingham, AL)
MoAb anti-kappa and Lambda-chains of human Ig (clones 6056 and 6054)	Dr. C. Reimer, centre for Disease Control (Atlanta, GA)
FITC-labelled F(ab')2 fragments of rabbit Ab to mouse Ig	Dakopatts AS (Glostrup, Denmark)
Horseradish peroxidase and alkaline phosphatase conjugated goat Ab to human IgG, IgM and IgA	Southern Biotechnology Associates (Bermingham, AL)
Magnetic beads (Dynabeads) coated with Ab to mouse IgG, IgM and to human CD19	Dynal AS (Oslo, Norway)
Oral cholera vaccine, tetanus/diphtheria vaccine, and purified tetanus toxoid (TT)	BSL (Stockholm, Sweden),
Cholera toxin (CT)	List Biological Laboratories, Inc. (Campell, CA)
CTB	Department of Immunology, Gotenborg University
Ganglioside GM1	Sigma (St. Louis, MO)

3.1 Methods development: Adaptation of immunomagnetic beads sorting to ELISPOT

From preliminary experiments, it was known that seven days after tetanus vaccination large numbers of circulating TT-reactive IgG ASC (arithmetic mean 370 ASC/10^6 MNC, range 42-1700, n=10 appeared in peripheral blood).

3.1.1 Direct binding of antigens to beads

With direct TT-antigen bound beads, antibody secreting cells were isolated from parenterally immunized tetanus vaccinees. It was possible to capture 79% of the specific antibody forming cells (Fig. 7). Although there were variations in total responding cells among individuals the percentage of ASC picked by this method was constant.

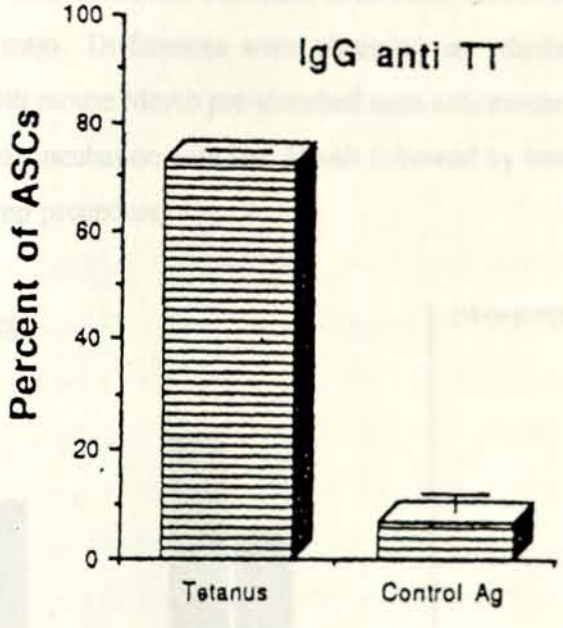


Fig. 7. Antigen specific binding of human ASC from TT-vaccines. Data generated from 5 individuals in each group.

3.1.2 Concentration of MoAbs and beads to cell ratio

For each of the given cell surface markers the optimal conditions were determined by checker-board titration experiments taking into account the concentration of the MoAbs employed, and the relative concentration of beads to cell ratios. Results indicated that these factors vary from markers to markers. For example, higher concentration of MoAbs was

needed to sort cells expressing CD28 marker than it was to CD20. Moreover, CD28 expressing cells were depleted better when the MoAb was primarily bound to the cells rather than to beads (Table 2).

Table 2. Comparison of fractions of CD20 and CD28 which were bound to the beads (left side) or to the cells (right side).

	Beads									Cells	
bead: target	20:1			50:1			100:1			100:1	
μgAb/mg beads	0.25	0.5	2.0	0.25	0.5	2.0	0.25	0.5	2.0	2.0	4.0
Anti-CD20	4[a]	2	2	6	7	8	ND	ND	ND	ND	ND
Anti-CD28	ND[b]	ND	ND	95	73	41	85	67	43	0	3

[a] Percent of cell surface positive cells remaining in the negative population after fractionation; [b] Not determined

3.1.3 Separation procedure

The efficiency of the cell separation was found to be more related to the order of steps rather than beads to cell ratio. Differences were observed on whether suspensions had been incubated directly with mouse MoAb pre-absorbed onto anti-mouse Ig coated beads (one step procedure) or by prior incubation with free MoAb followed by treatment with anti-mouse Ig coated beads (two-step procedure) Fig. 8.

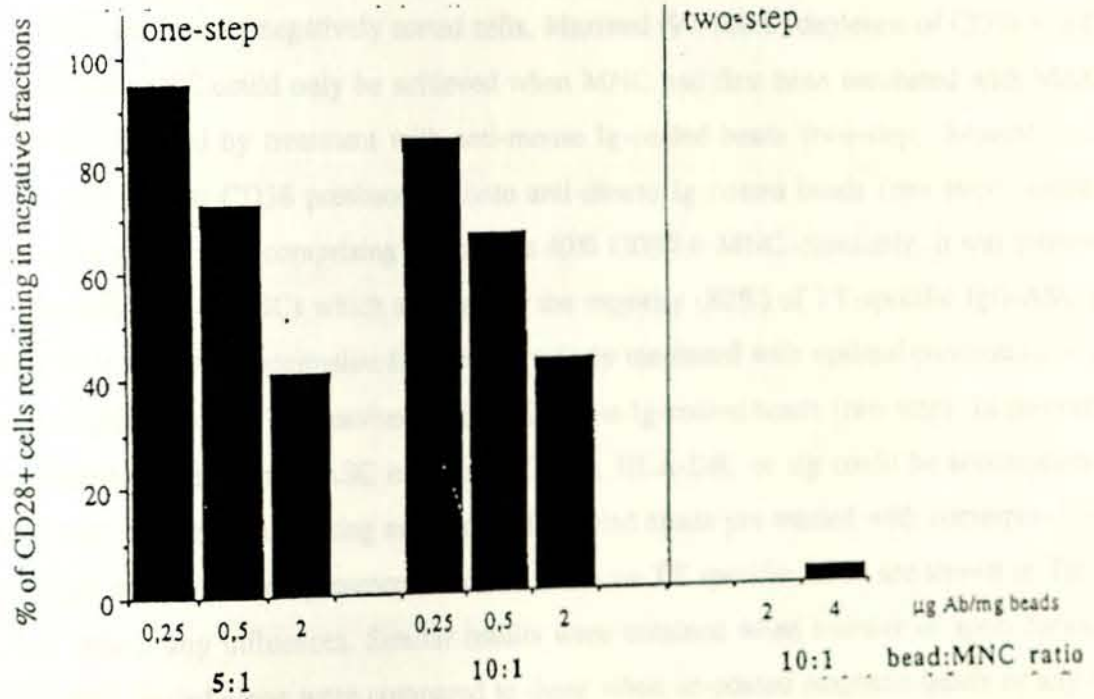


Fig. 8. Percent of CD28 cells left in the negative fraction after different combination of factors.

3.1.4 Optimization of conditions for separation of antigen specific IgG secreting cells

The optimal conditions for CD19, CD20, CD28, CD38, HLA-DR, and Igλ and Igκ was determined using TT specific IgG ASC in human peripheral blood. The optimal MoAb concentrations, beads:target cells ratio and separation protocols employed are listed in Table 3.

Table 3. Optimal conditions for separation of TT-specific IgG ASC in Human PBMNC.

Surface marker	μg Moab/mg beads	bead:MNC	separation procedure[1]
CD19	0.5	10:1	one step
CD20	0.5	10:1	one step
CD28	2	10:1	two-step
CD38	0.5	15:1	two-step
HLA-DR	1	10:1	one step
Ig k light chain	1	5:1	one step
Ig λ light chain	1	5:1	one step

[1] MNC were incubated either directly with anti mouse Ig-coated beads per-complexed with MoAb to the indicated cell surface marker (one step) or first with MoAb followed by anti-mouse Ig-coated beads (two-step).

Figure 9 illustrates the results of a typical experiment aimed at determining by means of flow cytometric analysis of negatively sorted cells. Maximal (97-100%) depletion of CD38+ cells from blood MNC could only be achieved when MNC had first been incubated with MoAb to CD38 followed by treatment with anti-mouse Ig-coated beads (two-step). Separation of MNC with MoAb CD38 preabsorbed onto anti-mouse Ig coated beads (one step), yielded negatively sorted cells comprising as many as 40% CD38+ MNC. Similarly, it was possible to separate CD28+ ASCs which account for the majority (80%) of TT-specific IgG-ASC in blood after systemic vaccination from MNC priorly incubated with optimal concentration of MoAb CD28 followed by treatment with anti-mouse Ig-coated beads (two step). In contrast, a high degree depletion of ASC expressing CD20, HLA-DR, or sIg could be accomplished with a one step procedure using anti-mouse Ig-coated beads pre-treated with corresponding The effect of MoAbs and presence of coated beads on TT specific ASCs are shown in Table 4, had little if any influences. Similar results were obtained when number of spots formed from MNC seeded alone were compared to those when un-coated magnetic beads or any of the above MoAb were added. Cells bound to magnetic beads pre-treated with control IgG1

MoAb, which accounted for approximately 6-10 % of the total MNC seeded, as determined by counting of isolated cells, did not contain any detectable ASC, nor did cells pre-incubated with control MoAb and thereafter isolated with anti-mouse Ig-coated beads.

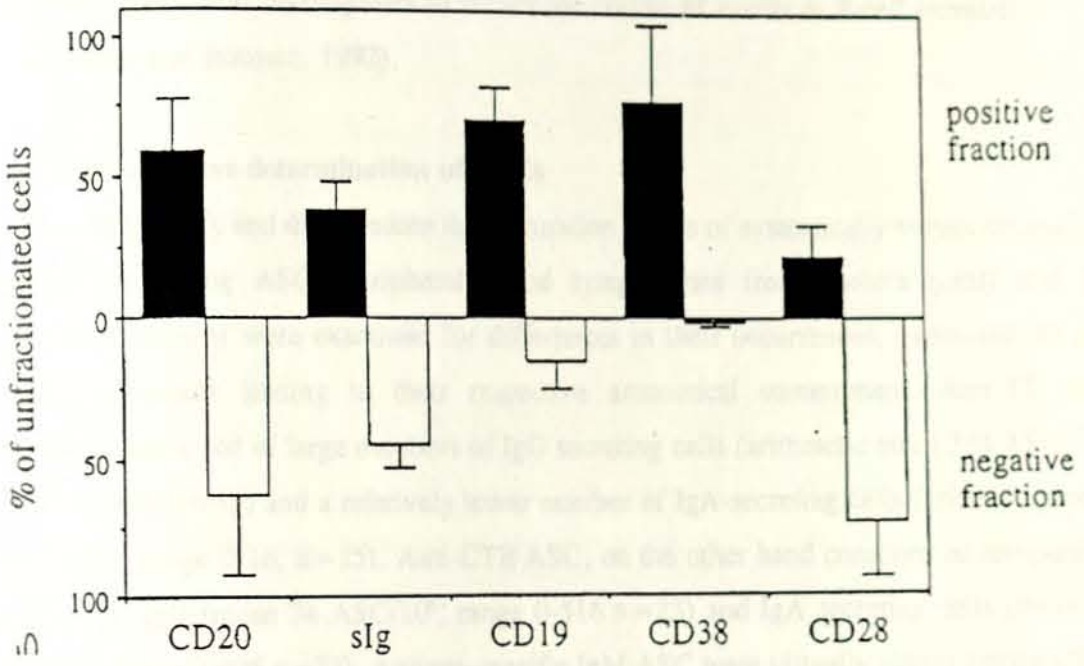


Fig. 9. **Comparative efficiency of immunomagnetic cell sorting of MNC expressing CD20, sIg, CD19, CD28 and CD38 using 10:1beads to MNC ratio and 0.5μg/mg beads.** (Results are pooled from 10 individuals and expressed as arithmetic mean percentage of ASC).

Table 4. Evaluation of the effect of MoAb-coated beads or MoAb alone on the spot forming capacity of TT-specific ASC.

bead:target cell ratio	αCD19 coated beads	αCD20 coated beads	ακ+αλ coated beads	control IgGl coated beads	αCD19 beads	αCD20 MoAb	ακ+αλ MoAb
5:1	99[a]	99	86	83	98	80	96
20:1	85	92	96	115	129	76	126
50:1	115	98	115	115	85	112	90
100:1	129	92	99	110	80	87	112

[a] Percent of TT-specific in beads or MoAb-treated cell suspensions compared to untreated cells. Among the untreated cells, ASC numbers varied between 62 and 137 % with a standard deviation of 21%.

3.2 Application of the method: Functional characterization of human ASC after mucosal versus systemic immunization and their differential commitments

The process of antibody secretion is a result that follows the clonal expansion of antigen specific cells upon exposure to antigenic substances. These expanding cells have been implicated by several investigators to reflect the course of events in B-cell immunity (Banchareau and Rousset, 1992).

3.2.1 Quantitative determination of ASCs

In order to identify and differentiate the maturation stages of systemically versus mucosally, induced circulating ASC, peripheral blood lymphocytes from cholera (oral) and TT (systemic) vaccines were examined for differences in their occurrences, expression of cell surface molecules leading to their respective anatomical commitment. Anti-TT ASC responses comprised of large numbers of IgG secreting cells (arithmetic mean 384 ASC/10^6, range 5-1700, n=15) and a relatively lower number of IgA-secreting cells (arithmetic mean 8 ASC/10^6, range 0-16, n=15). Anti-CTB ASC, on the other hand consisted of comparable numbers of IgG (mean 74 ASC/10^6, range 0-516 n=23) and IgA secreting cells (mean 85 ASC/10^6, range 2-406 n=23). Antigen-specific IgM ASC were virtually absent among CTB- as well as TT-specific ASC. Because of these major differences between magnitude and isotype composition of blood ASC responses to TT and CTB, cell suspensions isolated from each vaccinee were assayed at several densities to allow a more precise comparison of anti-TT and anti-CTB responses. Overall, 93 % of the volunteers responded with greater than 10 TT-specific IgG-ASC/10^6 MNC and/or more than 5 TT specific IgA ASC/10^6, MNC, and 87 % with greater than 10 CTB-specific IgA or IgG ASC/10^6 MNC.

3.2.2 Determination of maturation stages of systemically versus mucosally induced circulating ASC

A combination of ELISPOT and immunomagnetic cell sorting techniques enabled characterization of the differentiation stages of vaccine-specific circulating ASC. Circulating MNC collected 7 days after parenteral immunization with TT-vaccine were sorted according to cell surface expression of differentiation markers. The cell sorting analyses indicated that the majority of TT-specific IgA and IgG ASC did not express the plasmocyte marker CD28 but did express CD38, a marker expressed on both plasmablast and plasmocytes. In addition, virtually all ASC detected expressed cell surface HLA-DR, sIg, and CD19 (Fig. 10a).

Furthermore, the majority of TT-specific ASC did not express detectable levels of cell surface CD5, CD22, and CD37 molecules which are normally lost during the transition of plasmablasts to plasmocytes (Fig. 10a, Table 5).

Table 5. Expression of early and intermediate B cell differentiation markers on CTB and TT-specific ASC.

	% of positive ASC[a]			
	anti-CTB		anti-TT	
Surface marker	IgA	IgG	IgA	IgG
sIgD	4±2	3±4[b]	18±6	25±4
CD5	4±2[b]	6±6[b]	45±15	28±14
CD20	20±8	38±16	52±29	50±13
CD22	8±8	8±6[b]	24±9	21±7
CD23	11±4	19±8	46±19	46±17

[a] Percentage of ASC expressing the indicated cell surface marker (arithmetic mean ± standard deviation). Data are pooled from 5 to 13 individuals; [b] $p < 0.05$, as compared to anti-TT IgG ASC

Parallel examinations performed on CTB-specific ASC on the same individuals, after oral immunization detected CD5, CD22 and CD37, and a minority of them expressing CD20 and CD23 (Fig. 10b, Table 5). As with TT-specific ASC responses, the large majority of CTB-specific ASC had acquired CD38 but not CD28 and retained HLA-DR, sIg, and CD19 (Fig. 10b). In addition, mucosally derived circulating CTB-ASC seem to be considerably more homogenous than corresponding TT-ASC with regard to differentiation stage, being of a transitional plasmablast/plasmocyte type. In anti-TT both ASC populations although of only borderline significance, higher numbers of IgA-secreting cells than of IgG-secreting cells had Ig on their surface, but these differences showed only borderline significance. A similar difference in Ig expression between IgA and IgG-secreting cells was also noted among total immunoglobulin-secreting cells (ISC), irrespective of antigen specificity.

A distinctive pattern of HLA class II antigens was expressed on blood ASC. Thus virtually all TT- and CTB-reactive ASC expressed HLA-DR and the majority also expressed HLA-DP. On the other hand, relatively fewer ASC expressed HLA-DQ (Table 6).

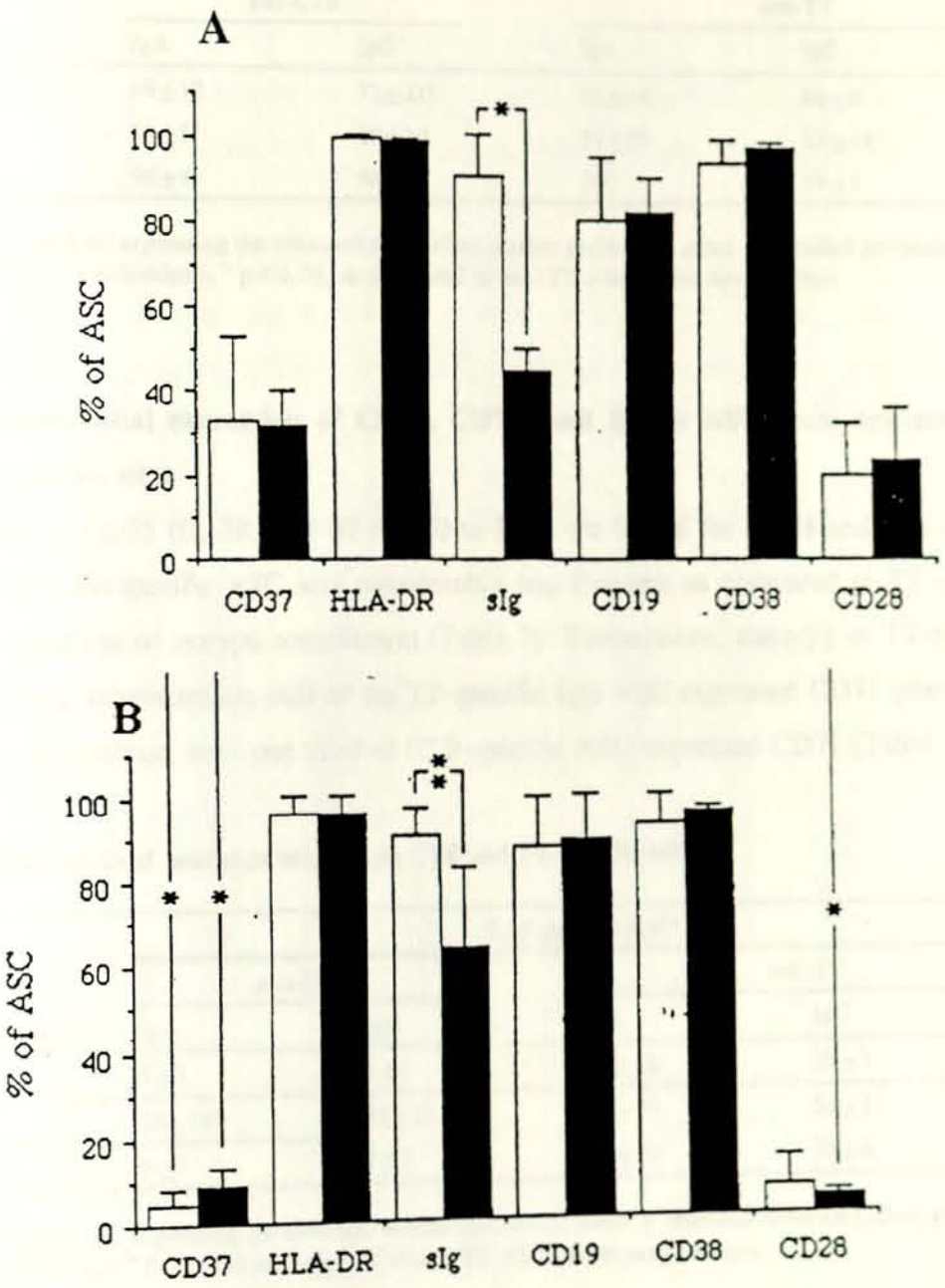


Fig 10. **Differential expression of cell surface associated molecules on peripheral blood CTB and TT-specific ASC after peroral and parenteral immunizations.** Cell suspensions obtained 7 days after booster immunizations with oral cholera and parenteral tetanus vaccine were fractionated and assayed for ASC numbers to CTB (panel A) and TT (panel B). Open bars indicate percentage of IgA and filled bars IgG. Data expressed as arithmetic mean + SD. The level of significance between CTB and TT-specific ASC of a given isotype and between IgA and IgG ASC of a give specificity are indicated by * ($p < 0.05$).

Table 6. Expression of HLA-DP, DQ and DR on CTB and TT-specific ASC.

	% of positive ASC[a]			
	anti-CTB		anti-TT	
surface antigen	IgA	IgG	IgA	IgG
HLA-DP	69±12	72±11[b]	71±18	86±6
HLA-DQ	16±9[b]	20±10	51±29	33±14
HLA-DR	96±4	96±4	100	99±1

[a] Percentage of ASC expressing the indicated cell surface marker (arithmetic mean ± standard deviation). Data are pooled from 5 individuals; [b] $p<0.05$, as compared to anti-TT ASC of the same isotype.

3.2.3 Differential expression of CD25, CD71, and B7 on ASC from systemic and mucosal sites

Expression of CD25 (IL-2R) and B7 (CD80 or BB1, the ligand for CD28 and CTLA-4 on T-cells) by CTB-specific ASC was considerably less frequent as compared to TT-specific ASC, irrespective of isotype commitment (Table 7). Furthermore, majority of TT-reactive IgA ASC and approximately half of the TT-specific IgG ASC expressed CD71 (transferrin receptor). In contrast, only one third of CTB-specific ASC expressed CD71 (Table 7).

Table 7. Expression of activation markers on CTB and TT-specific ASC.

	% of positive ASC[a]			
	anti-CTB		anti-TT	
surface marker	IgA	IgG	IgA	IgG
CD25	4±3	3±3	18±18	20±2
CD71	32±11[b]	38±14	77±20	56±17
B7	5±7	3±5	36±10	35±6

[a] Percentage of ASC expressing the indicated marker (arithmetic mean ± standard deviation). Data are pooled from 13 individuals; [b] $P < 0.05$ as compared to anti-TT ASC of the same isotype.

3.2.4 Differential expression of homing receptors induced by systemic and mucosal immunization

CTB-specific IgA ASC and IgG ASC comprised significantly ($P < 0.01$) less proportions of L-selectin bearing cells compared to TT-specific ASC which comprised approximately 85% of cells expressing L-selectin. Only half of CTB-specific IgG ASC expressed L-selectin, and even less IgA ASC were detected as L-selectin positive cells. The expression of $\alpha 4$ integrin chain, and CD44 that are implicated in lymphocyte trafficking to mucosal sites was looked into. Results indicated that both CD44 and $\alpha 4$ which can associate with either the $\beta 1$ or the $\beta 7$ integrin chain were expressed by virtually all CTB-and TT-specific ASC (Fig. 11).

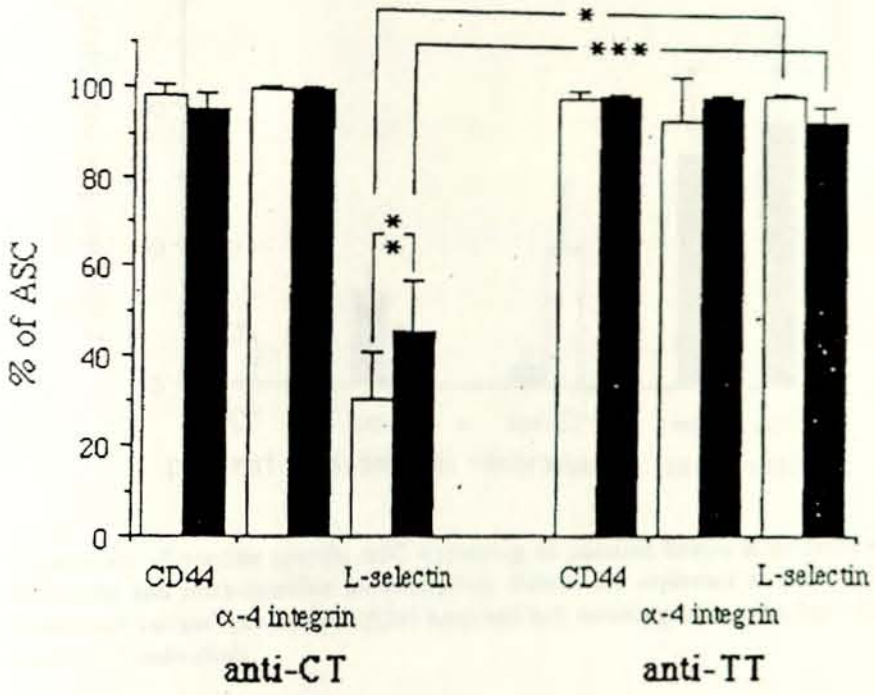


Fig. 11. **Expression of homing receptors on mucosally and systemically derived blood ASC.** Cell suspensions obtained 7 days after booster immunizations with oral cholera and parenteral tetanus vaccines were fractionated and assayed for ASC numbers to CTB and TT. Bars indicate the percentage of IgA (open bars) and IgG (filled bars)-ASC expressing the indicated homing receptor. Data were pooled from ten individuals and expressed as arithmetic mean + SD. The level of significance between CTB and TT specific ASC of a given isotype and between IgA and IgG-ASC of a given specificity are indicated by-* $p<0.05$; **-$p<0.01$ ***- $p<0.001$.

3.2.5 Induction of local immune response at the tonsillar level

The dynamic and isotype commitment of specific immunoglobulin producing cells in tonsil was explored to check the possibilities of using tonsils as induction sites. Different types of antigens were injected into palatine tonsil and the results displayed that IgA and IgG secreting cell-pattern and magnitude at the palatine tonsil was more when immunization was done from the tonsil itself compared to all other routes (intranasal, peroral,and parenteral). Although the intranasal stimulation was dominated by IgA response, all routes were able to induce both IgG and IgA responses (Fig. 12).

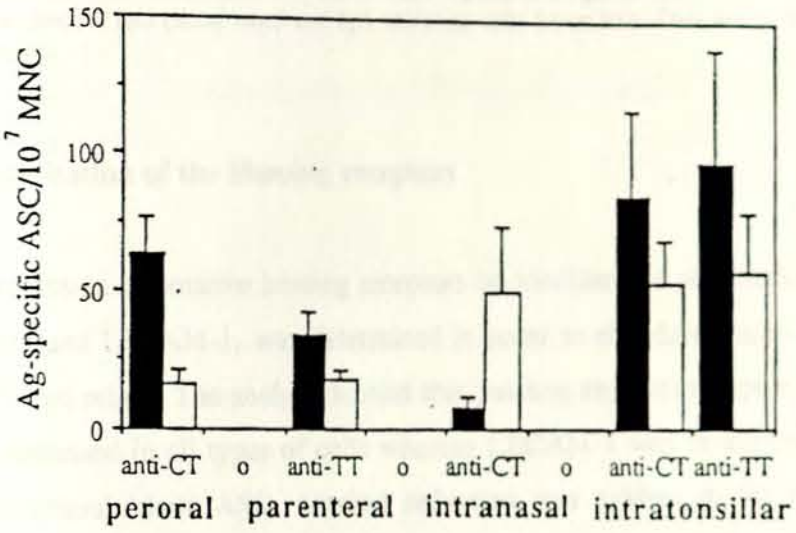


Fig. 12. **Frequencies of vaccine specific ASC appearing in palatine tonsils after peroral, parenteral, intranasal and intra-tonsillar immunization.** Results are expressed as geometric mean + SD numbers of vaccine specific IgG (filled bars) and IgA secreting cells (open bar). Data are pooled from 5 to 7 individuals.

Comparison of the effect of the intra-tonsillar route with the peroral and subcutaneous vaccination on peripheral response similarly produced IgG and IgA response. In both cases although IgA was also present, IgG was dominant and IgM could only be detected occasionally. The peripheral blood response after single intra-tonsillar response was equivalent to booster peroral immune response to CTB. In TT immunized subjects although IgA was also present, IgG was the dominant response. On the other hand the intra-tonsillar and peroral immunizations of CTB produced equivalent number of IgG and IgA ASC in peripheral blood (Fig. 13).

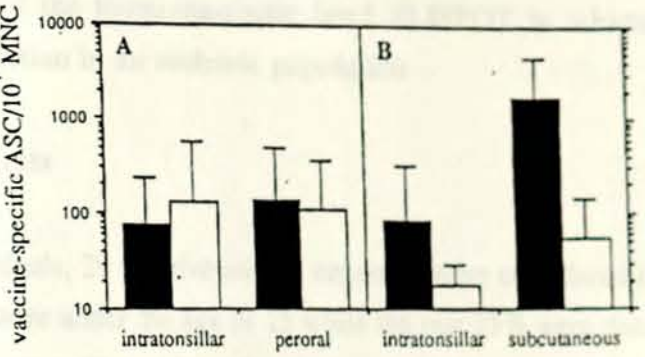


Fig. 13. **Frequencies of: A) CTB specific ASC appearing in peripheral blood after peroral and intra-tonsillar immunization; B) TT-specific ASC appearing in peripheral blood after intra-tonsillar and subcutaneous immunization.** Results are expressed as geometric mean + SD numbers of vaccine specific IgG (filled bars) and IgA secreting cells (open bar). Data are pooled from 5 to 7 individuals.

3.2.6 Characterization of the Homing receptors

Differential expression of putative homing receptors on tonsillar and peripheral blood ASC, H-CAM integrin and LECAM-1, was determined in order to elucidate possible differences on cells of different origin. The study exhibited that putative mucosal receptor CD44 ligand H-CAM was expressed in all types of cells whereas LECAM-1 was co-expressed by large majority of peripheral blood ASC. Marked reduction was evident in the expression of LECAM-1 on tonsillar ASC. The large majority of peripheral blood ASC co-expressed both markers, whereas most tonsillar IgG and IgA secreting cells did not express the LECAM-1 molecules (Fig. 14).

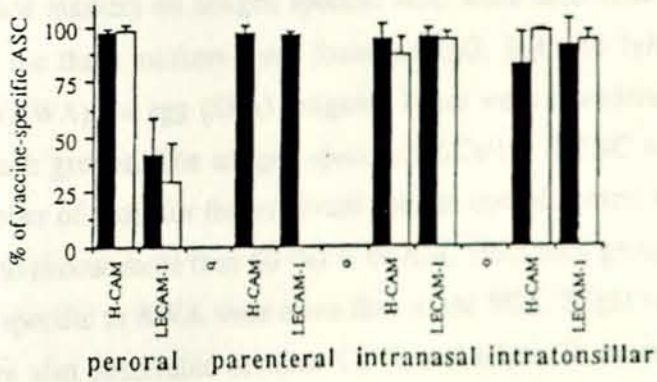


Fig. 14. **Percentage of vaccine specific ASC expressing the H-CAM and LECAM-1 surface molecules on immunization.** Results are expressed as geometric mean + SD numbers of vaccine specific IgG (filled bars) and IgA secreting cells (open bar). Data are pulled from 5 to 7 individuals.

3.3 Validation of the immunmagnetic bead ELISPOT in schistosomiasis mansoni natural infection in an endemic population

a. The study subjects

A total of 40 individuals, 29 positive and 11 negative, were considered for this study. More than 75% of them were under the age of 15 while the rest 25% were distributed between the ages of 16 and 45 years. The male to female proportion was nearly 4:1 (Table 8).

Table 8. Age and sex profile of the study subjects.

	Study Group			Controls
Age group	Male	Female	Total	
0-5.5	1	1	2	-
5.5-10.5	6	4	10	4
10.5-15.5	14	5	19	4
15.5-20.5	1	1	2	-
25.5-30.5	1	1	2	-
30.5-35.5	2	1	3	1
35.5-40.5	2	1	3	1
40.5-45.5	1	0	1	-
45.5-50.5	1	1	2	1

b. Determination of different cell surface markers

Expression of surface markers on antigen specific ASC were determined for CD19, CD38 and HLA-DR. All the three markers were found on IgG, IgA and IgM ASCs-specific to crude Adult worm (AWA) and egg (SEA) antigens. There were considerable inter individual variations within each groups. The antigen specific ASCs/10^6 PMNC was calculated after subtracting the number of spots for the irrelevant antigen coated control beads. All the three markers were able to choose more than 60 -80 % of ASC from each group. However, on the average, the ASC, specific to AWA were more than to the SEA. Slight variation in IgM and IgA antibodies were also observable between CD19 and HLA-DR markers (Fig. 15). IgM antibody secreting cells specific to AWA were found expressing more CD38 markers. The negatively selected ASC specific to adult worm and egg antigens were less (20%) for CD38

coated compared to 30-40 % in CD19 and HLA-DR sorted ones. The relative quantity of expression between the classes of antibodies in each group was similar.

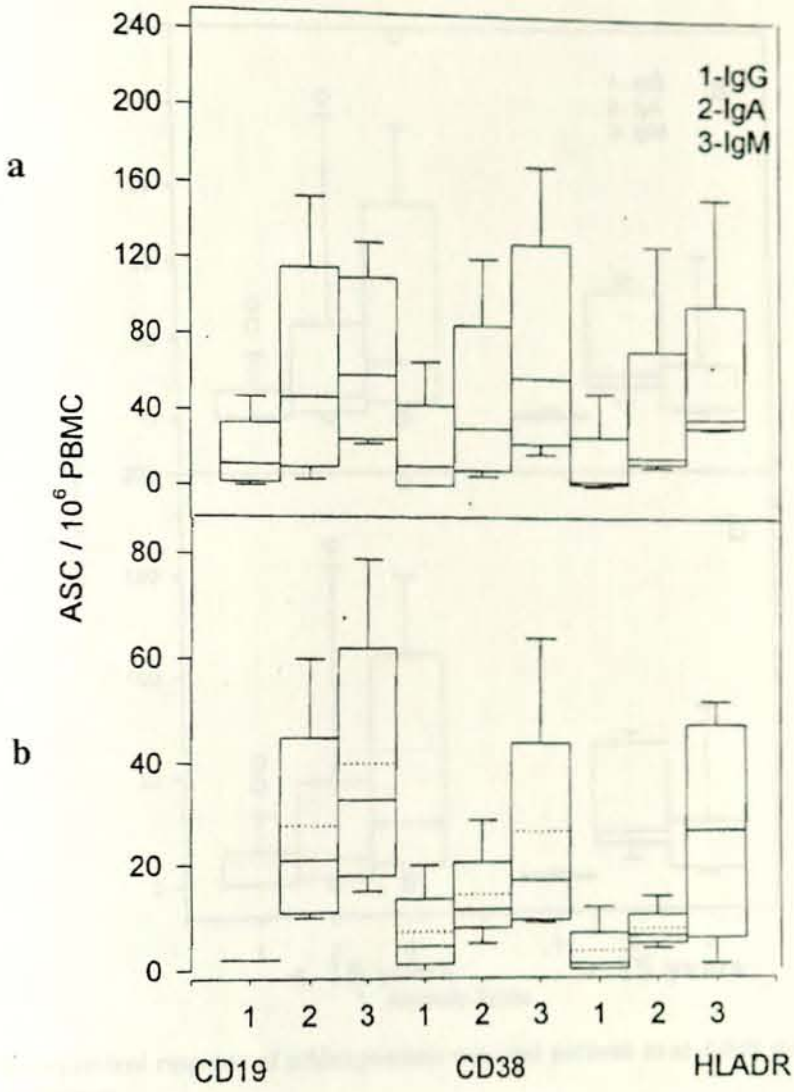


Fig. 15. **Expression of surface markers by specific IgG, IgA and IgM antibody secreting cells to *Schistosoma mansoni*.** a) Adult worm antigen (AWA); b) Egg antigen (SEA). (Data from eight schistosomiasis patients).

c. Age related immune response to schistosomiasis

Comparison of *Schistosoma mansoni* infected patients' responses by age and intensity of infection, to that of the controls showed a clear distinction as characterized by the difference in the egg output and that the magnitude of response changes with intensity in the secretion pattern of IgM and IgA. Analysis by age revealed that AWA and SEA specific IgM antibody

secreting cells were significantly higher in young (0–15 years old) age groups while IgA antibody was significantly higher in older age groups (Fig. 16a and b).

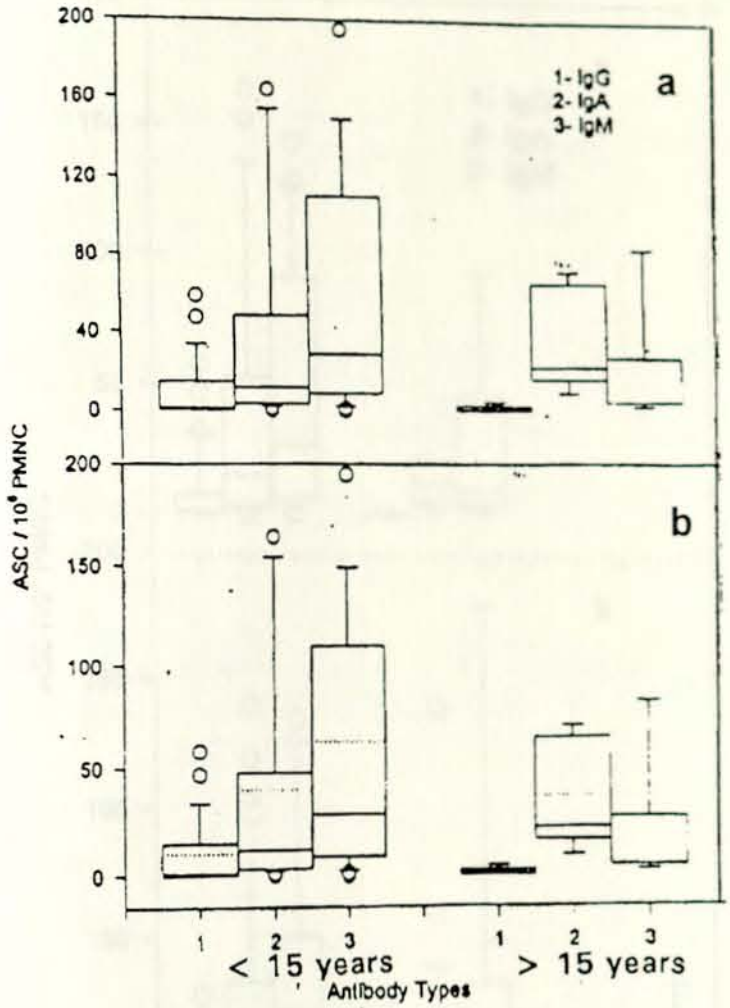


Fig. 16. **Age dependent response of schistosomiasis mansoni patients to a) Adult worm antigen and b) Egg antigen.**

d. Intensity of infection and the immune response in schistosomiasis

The analysis of immune response by intensity of infection revealed that the pattern of response in infected individuals is different from the non-infected ones. The infected individuals were categorized by the intensity of infection as characterized by the difference in the magnitude of egg output. Individuals with high intensity of infection (> 100 eggs/gm), responded to AWA and SEA in a much lower magnitude. As the intensity of infection increased (> 200 eggs/gm) (Fig. 17a), the response to AWA was rather very low (Fig. 17b). When IgM ASC are considered, the control groups had very little if any IgM ASC. It was

evident that the intensity of infection increases as the response level decreases. The relative isotype distribution was nevertheless, of similar pattern (Fig. 17).

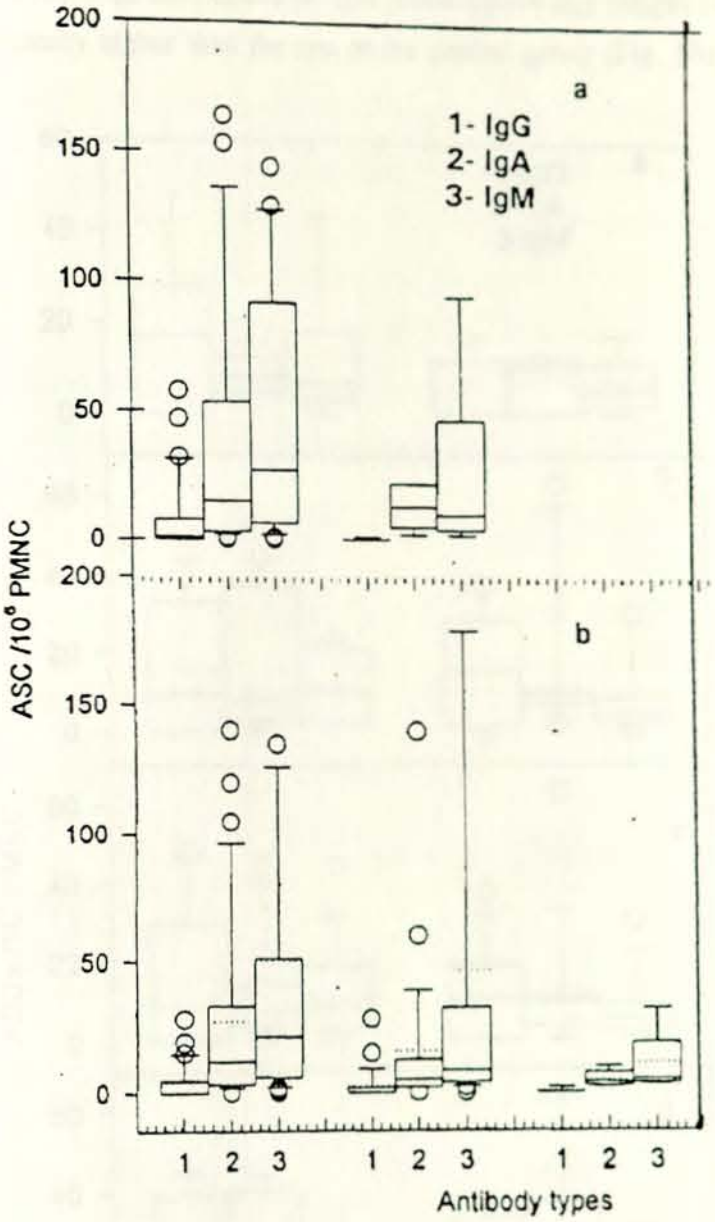


Fig. 17. **Frequency of IgG, IgA and IgM ASC specific to a) Adult worm antigen (AWA) b) Egg antigen in schistosomiasis patients at different intensity of infection as defined by egg out put.** Left all positives, >100 eggs/gm and right >200 eggs/gm.

e. The control group

The control groups consisted of a total of 11 individuals not shedding ova of *Schistosoma mansoni*. Five of them had been treated for *S. mansoni* a year before, three were treated for malaria 1-3 months before they were examined. Three others were neither infected nor treated for *S. mansoni* or malaria. Analysis of their response to AWA revealed higher IgG

than IgM in non-ova producing individuals (Fig. 18a). IgA response in malaria treated groups and the schistosomiasis-treated and cured controls responses to AWA were higher than in the non-infected ($P < 0.05$) (Fig. 18b, c, and d). IgA ASCs against egg antigen in malaria treated groups was significantly higher than the rest of the control group (Fig. 18d).

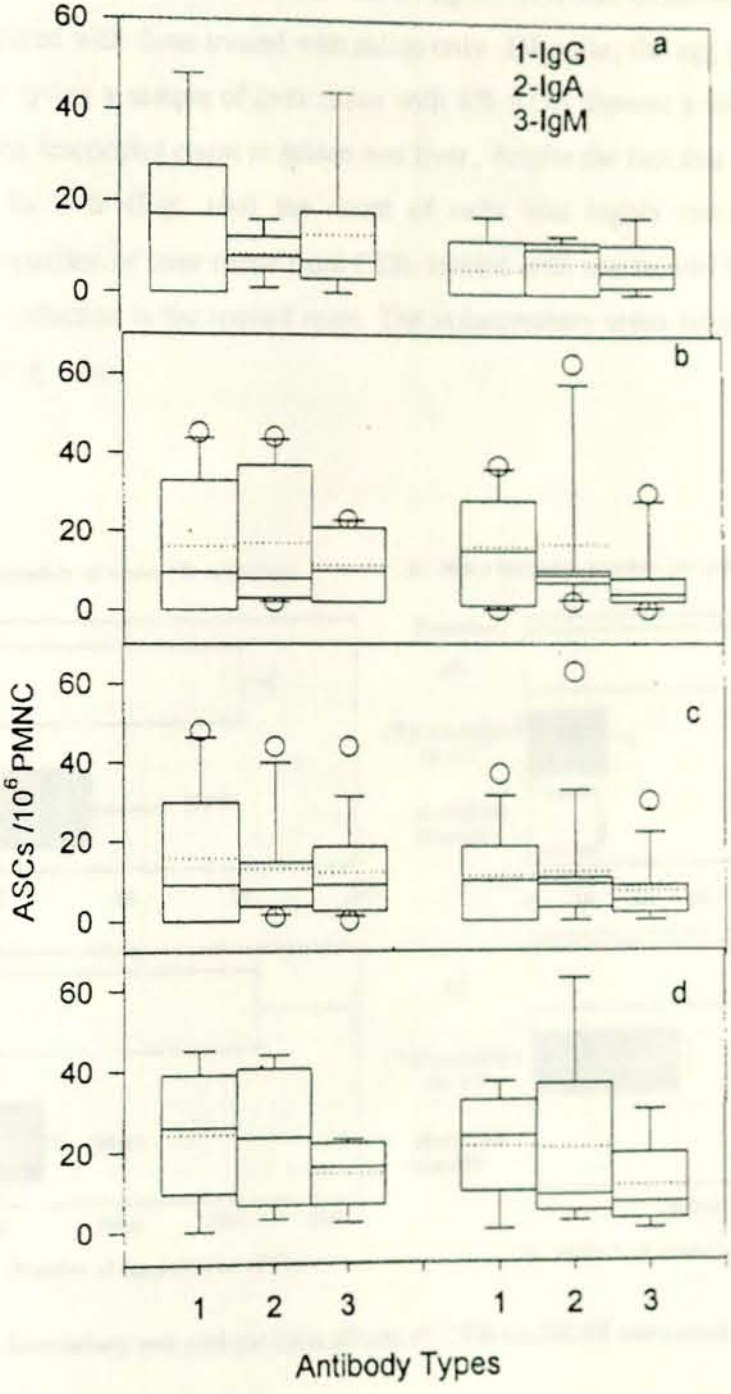


Fig. 18. **Antibody secreting cells in: a) All control subjects (Non-ova shedding); b) Treated and cured of schistosomiasis; c) Non-infected non treated controls and d) Controls that were treated for malaria 1-3 months before the study period.** Responses to AWA are on the right panel and to SEA are on the left panel. * $P < 0.05$.

3.4 Specific immune intervention in *S. mansoni* infection in murine model system

3.4.1 Anti-parasite immunity following intranasal immunization with CTB-Sm28GST in mice

The number of worms in mice immunized with 10 μg of CTB-was diminished by 66 % Fig. 19a) when compared with those treated with saline only. Likewise, the egg per gram of liver determined after lysing a sample of liver tissue with 4% KOH showed a reduction by 84 % Fig. 19b. And the leucocytes count in spleen and liver, despite the fact that there were some inflamed areas in liver (Fig. 19d) the count of cells was highly reduced (Fig. 19c). Histological comparison of liver tissue from CTB- treated with non treated but infected mice showed a 66 % reduction in the treated mice. The inflammatory areas around each egg was much reduced (Fig. 19a).

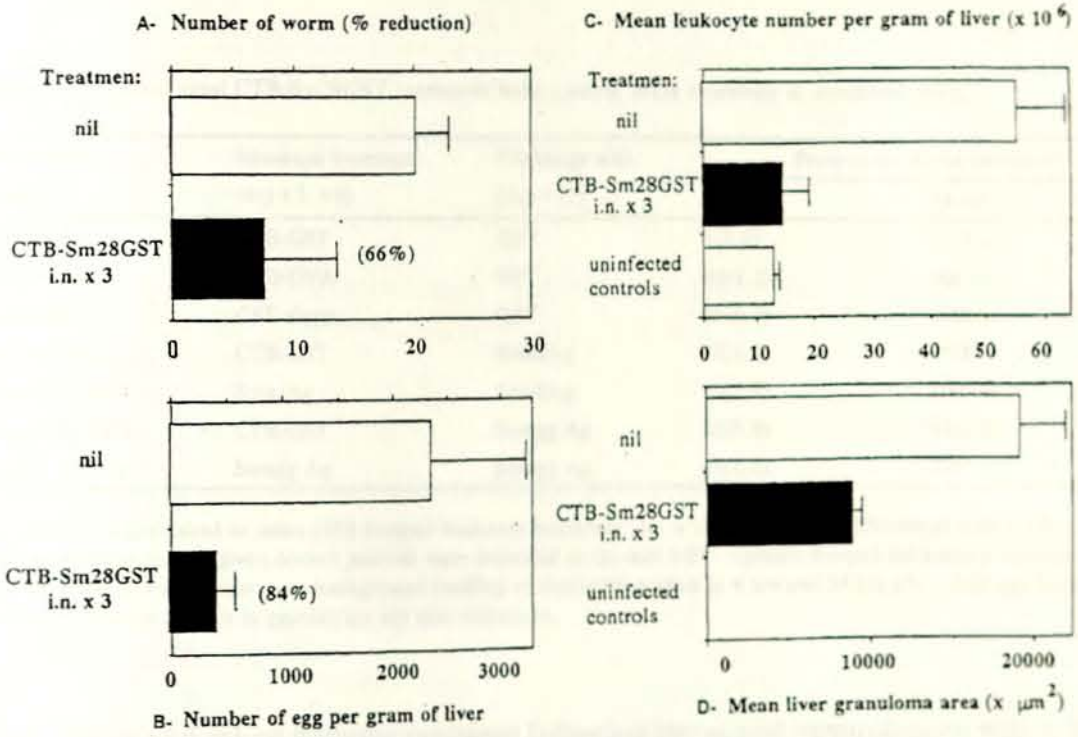


Fig. 19. Anti-inflammatory and anti-parasitic effects of CTB-Sm28GST intranasal immunization.

The proliferation of liver and spleen T-cells from CTB- treated mice compared to non-immunized controls was markedly reduced. The response of spleen T-cells were relatively better responding to Sm28GST antigen than those of the liver (Fig. 20). The DTH response

after intranasal treatment with 10 μg of CTB-Sm28GST conjugate and two times in three days interval with GST/CFA had markedly reduced DTH responses to GST compared with animals treated with irrelevant conjugate CTB-ova or GST alone as shown in Table 9.

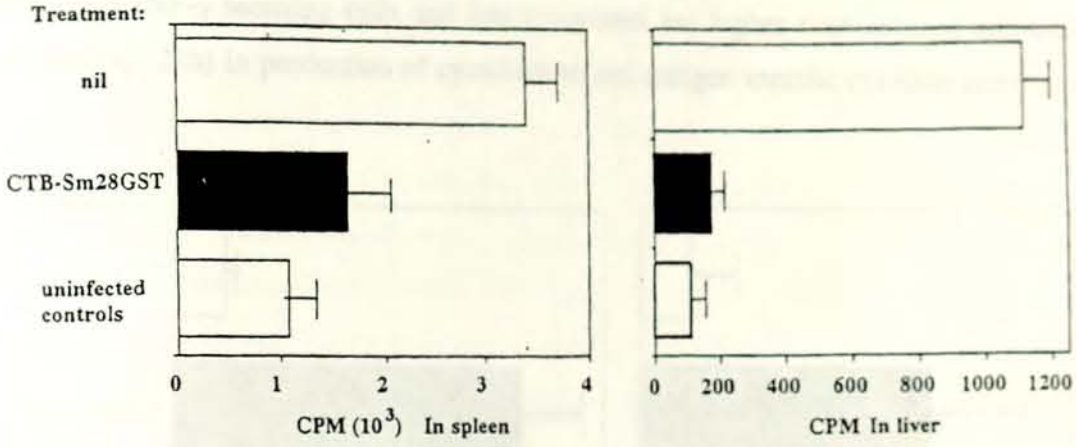


Fig. 20. **Proliferative reactions to Sm28GST of splenic and hepatic leukocytes from *S. mansoni* infected mice after intranasal treatment.**

Table 9. **Low dose nasal CTB-Sm28GST treatment halts specific DTH reactivity in sensitized mice.**

Sensitization (day 0)	Intranasal treatment (day+3,+6)	Challenge with (day+12)	Footpad thickness increment	
			4 hrs	24 hrs
GST/FCA	CTB-GST	GST	5(5.1)*	11(4.2)*
GST/FCA	CTB-OVA	GST	45(4.2)	40(11)
GST/FCA	GST alone	GST	44(8.6)	29(6.2)
SmadAg/FCA	CTB-GST	SmadAg	48(4.8)	25(5.2)
SmadAg/FCA	SmadAg	SmadAg	52(2.6)	30(6.6)
Smegg Ag/FCA	CTB-GST	Smegg Ag	25(0.9)	15(1.5)
Smegg Ag/FCa	Smegg Ag	Smegg Ag	41(1.2)	25(4.3)

The results are expressed as mean (SD) footpad thickness increment, cm x 10^{-3}. Significant differences with CTB-OVA-given and uncoupled-Ag-given control animals were indicated as marked with*. Specific footpad thickness increment was calculating by subtracting the mean background swelling of unprimed animals at 4 hrs and 24 hrs after challenge from the swelling of primed animals to provide the net skin responses.

3.4.2 Liver and spleen immune reactions following intranasal immunization with CTB-Sm28GST in mice

Nasal treatment can also partially suppress the DTH reactivity to Sm28egg antigen challenged, although it did not affect the responses to schistosomes adult worm antigen.

Liver leucocytes of intranasally treated mice upon stimulation with the antigen produced less IL-4 and INF-γ, than infected control animals. The results were the same when tested either spontaneously or was stimulated with GST (Fig. 21a). Where as, spleen leucocytes produced higher of INF-γ secreting cells when cultured with GST produced markedly increased numbers of INF-γ secreting cells and less prominent but higher than infected non-treated controls (Fig. 21b) In production of cytokines to test antigen specific cytokine secretions.

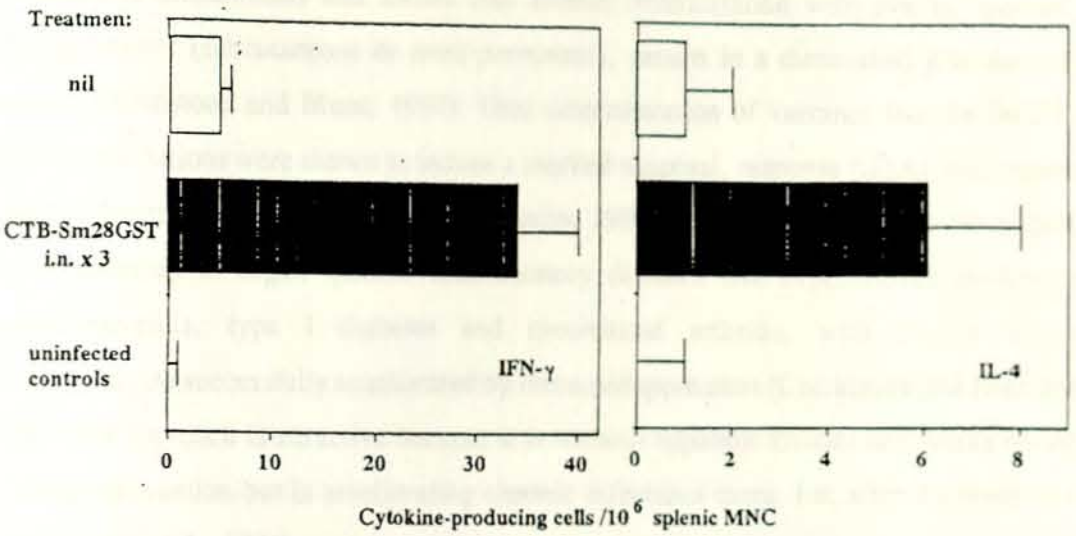


Fig. 21a. *In vitro* Sm28GST-induced cytokine production in the spleen after intranasal treatment of *S. mansoni*-infected mice with CTB-GST.

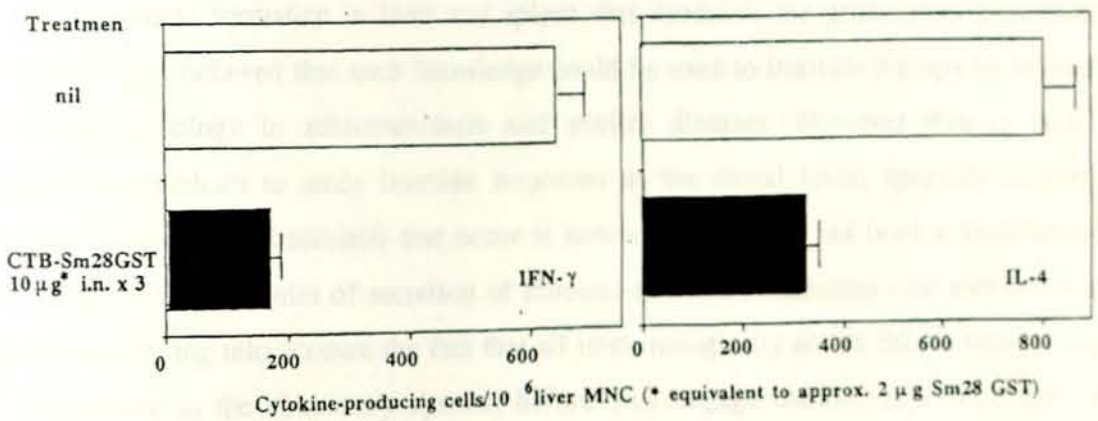


Fig. 21b. Spontaneous Cytokine-producing cells /10^6 MNC a) hepatic leukocytes b) splenic lymphocytes, after intranasal treatment of *S. mansoni* infected mice with CTB-Sm28GST.

IV. DISCUSSION

In this work an attempt has been made to use the recent knowledge and developments in mucosal immunity to address some of the gap in the understanding of host immune response to schistosomiasis mansoni, a disease which essentially is due to an immunological hyper-reaction. The main goal was to better characterize the antigen-specific granulomatous hypersensitivity reaction which is the pathological hallmark of schistosomiasis. Experiments on murine schistosomiasis had shown that enteric immunization with ova as opposed to systemic, routes (subcutaneous or intra peritoneal), results in a diminished granulomatous response (Weinstock and Blum, 1987). Oral administration of vaccines like Sm28GST in various formulations were shown to induce a marked mucosal, response (sIgA), accompanied by significant reduction in worm burden (Capron, 1995). On the other hand the development of oral vaccines to organ specific inflammatory diseases like experimental autoimmune encephalomyelitis, type I diabetes and rheumatoid arthritis, with similar immune mechanisms, are successfully ameliorated by immunosuppression (Czerkinsky and Holmgren, 1995). This approach is attractive because it is without apparent toxicity and works not only in disease prevention but in ameliorating chronic infectious cases, i.e, after the onset of the disease (Sun *et al.*, 1994).

These findings are the results of the manipulation of the fact that animals fed with soluble antigen(s), develop an antigen specific state of unresponsiveness. On the basis of the findings reported by Weinstock and Blum (1987); and McCurley *et al.* (1986) it was hypothesized that in schistosomiasis it is the same egg antigens that through systemic stimulation produce the acute granuloma formation in liver and spleen that modulate the granuloma upon enteric exposure. It is believed that such knowledge could be used to institute therapy to cellularly mediated pathology in schistosomiasis and similar diseases. However due to lack of appropriate methods to study immune responses at the clonal level, specially in human diseases such as schistosomiasis that occur at non-accessible sites has been a hinderance to the study on the dynamics of secretion of immuno-reactive metabolites like antibodies and cytokines. Taking into account the fact that all immunologically active cells involve a stage of occurrence in the circulatory system, before they engage themselves as effectors, we thought of trapping this cells from the peripheral blood, characterizing their phenotypes and using them to evaluate the immune status in humans. To that end, that is, to evaluate the

immunity at the time of vaccination or prognostically a method that combines ELISPOT technique and dynal beads was developed.

The first isolation of antigen specific MNC was done using Tetanus toxoid coated tosyl activated beads. The intention was to determine the frequency of ASC specific to the different fractions of antigens (Vaccine or infectious agents) in an infected individuals. It was tried on a known vaccine system (Tetanus/diphtheria) IgG secreting cells 7 days after vaccination. The result obtained clearly demonstrated that such approach is feasible. It was possible to isolate 79 % of tetanus toxoid specific ASCs. The method could not be used as intended to determine the contribution of each fractions of schistosome antigens in the over all immunity. It was the first approach allowing phenotypic characterization of functional ASC. It is simple and useful in screening antigen fraction and determining the frequency of the different clones of effector cells and their relative role in the over all immune responses.

The other approach was, an attempt to identify MNC expressing a given differentiation marker, by immunomagnetic isolation or depletion, and then determining the presence of ASC in the resulting cell fractions by an ELISPOT assay. The enrichment process was influenced by the relative beads to cells ratio and differences in their requirements for optimization that is, those that are optimally concentrated directly by beads pre-adsorbed onto anti-mouse Ig coated beads (CD20, Ig-light chains) and those that needed prior incubation with free MoAb before incubation with anti-mouse Ig-coated beads (CD28 and CD38). These could be due to differences in marker density and/or affinity. But it could as well be that prior binding of cell surface markers does not allow the non-specific up-take of MoAbs by IgG Fc receptors bearing bystander MNC, thus making the antibodies relatively more available for combination. The latter, however, does not seem to be the case because cells bound to magnetic beads pre-treated with control IgG1 MoAb, which accounted for approximately 6-10 % of the starting MNC did not contain any detectable ASC, nor did cells pre-incubated with control MoAb and there after isolated with anti-mouse Ig coated beads.

In an endeavour to determine the degree of influence of each factor on spot formation, free beads, beads coated with MoAbs to CD19, Ig light chains, or control mouse IgG1 MoAb were co-incubated with MNC from a TT-immunized volunteers and results were compared with MNC alone. The results were similar, whether the MNC were kept alone, with un-

coated or coated beads or any of the above MoAbs. Thus demonstrating that binding of beads and/or MoAb to ASC do not appear to interfere with spot formation.

From the sequentially expressed surface molecules on B-cells and different role they play in activation of cells, a few markers were chosen to characterize the course of events in B-cell immunity. Accordingly κ and λ markers that mark the transition of B-cells from Pre-B-cells to mature naive B-cells were chosen. Their expression is a molecular events in B-cell lymphopoiesis. It is after their expression that the B-cells gain power to interact with antigens. Sixty-six percent of anti-TT-specific IgG1 antibody secreting cells expressed these markers indicating that they are activated cells. Transferrin receptor (CD71), and CD5 were less expressed. The former indicates that the part of the circulating antibody secreting cells were actively engaged in proliferation, for transferrin receptor is detrimental in the supply of iron for DNA synthesis. It increases when the cells are most rapidly proliferating and diminishes when they terminally differentiate. CD5 phenotypes were least expressed. They appear during germinal centre proliferation and thus are associated with self replenishing memory cells. They however, in a normal individual exists in a low density and are under-regulated by T-suppressor cells.

The absence of both markers suggest that these cells are actively dividing and relatively differentiated cells. This is in conformity with earlier works (McHeyzer-Williams *et al.*, 1993; Mackay *et al.*, 1992a) where they showed that mucosally induced ASCs are less active compared to the heterogenous population of cells by parenteral immunization.

The expression of MHC Class II antigens (HLA-DR, HLA-DP and HLA-DQ) involved in activation of B-cells through T-cell dependent mechanism is increased by cytokines from activated T-cells to which they present antigens to further activate T-helper cells to produce more B-cell activating factors. Thus, up- regulating the antibody responses. The presence of these surface markers and the absence of markers expressed at less differentiated B-cells (IgD, CD37, CD20, CD22, CD23) also confirm that the ASCs are at advanced or proliferative stages.

Under optimal conditions, CD20 and sIg, which are expressed not only on early and/or mature B-cells but also on majority of B-cell blasts were detected on variable (40-60 %),

numbers of vaccine specific ASC. In contrast, most ASC, expressed HLA-DR and CD19, both of which are cell surface molecules present on mature B-cells and B-cell blasts, but not on plasma cells. Finally, CD38 specifying terminally differentiated B-cells and plasma cells, was expressed by virtually all TT-specific IgG-ASC. In keeping with previous reports (Brieva *et al.*, 1991), these observations suggest that human circulating ASCs are at an advanced stage of B-cell differentiation into plasma cells but are not plasma cells yet capable of spontaneously secreting antibodies.

To characterize the phenotype of various ASC populations in both the human and animal systems alternative approaches have been described. Measurement of Ig produced by cultured human B-cells is normally done on cells fractionated by negative selection on MoAb-coated dishes (Brieva *et al.*, 1991). That however requires relatively long culture periods (a week or more), and most importantly does not allow direct clonal size estimates to be made on the corresponding cell population. Fluorescence-activated cell sorting of detectable murine ASC has been described (Lalor *et al.*, 1992) but this approach relies on tedious and time consuming preparative multi-parametric cell-sorting and requires equipment which is considerably more sophisticated. The possible use of immunomagnetic rossetting of plaque-forming cells to characterize specific subpopulations of Ig-secreting cells has also been described (Jones, 1990). Although similar in principle to the approach described here, the later technique requires prior removal of T-cells, and employs an haemolytic plaque assay as the indicator system for identification of plaque forming cells with attached beads. Apart from being less sensitive than the ELISPOT assay, haemolytic plaque assays rely on the tedious evaluation of plaque forming cells and their accuracy is influenced by factors such as the density of lysable indicator red cells. The variation in the density of lysable red cells may explain why low (1:1) bead : cell ratio were employed when assaying plaque forming cells attached to magnetic beads (Jones, 1990). The low cell to beads ratio does not allow optimal separation of ASC expressing certain cell surface markers.

The accuracy and sensitivity of the ELISPOT assay is inherently limited by the surface area of the solid phase employed (Czerkinsky *et al.*, 1988a). The major advantage of the immuno-magnetic sorting technique described here is that it permits the assay of relatively low numbers of cells, resulting in increased sensitivity. This feature should be particularly advantageous in situations where only very few ASC are present, such as at the very early

stage of an immune response, or in situations where only very few ASC are present. That is in situations such as at the very early stage of an immune response, or where ASC of a given specificity and isotype represent only a negligible fraction of Ig-secreting cells such as IgE-ASC or during a chronic infection. Particularly informative in this regard were the results of experiments involving detection of blood ASC to *L.major* crude promastigote antigen in chronically infected patients. In three out of the six patients examined, negligible numbers of parasite specific ASC could be detected among unfractionated blood MNC, even when assaying as many as $4x10^5$ cells per (9 mm diameter) well, i.e., a cell density close to confluence. In these three patients, prior immunomagnetic depletion of CD2+ cells followed by separation of MNC expressing CD38 yielded subpopulation enriched in parasite specific ASC which were readily detected when assaying considerably lower numbers (10^4 cells/well) of cells per ELISPOT well. In three other patients where ASC could be detected when assaying unfractionated MNC, the immunomagnetic cell sorting procedure resulted in a 50 to 600 fold relative enrichment in detectable ASC as compared to the corresponding unfractionated MNC. Thus, the immunomagnetic cell sorting procedure is a novel approach that overcomes major limitations of ELISPOT assay, inability to characterize the ASC on the bases of the cell surface markers. Simple, sensitive, and high reproducibility of this approach should prove useful as a tool to isolate and characterize cells secreting a variety of immuno-reactive substances, including possibly cytokine-producing cells. The coating of antigen directly on tosyl activated beads is rather an interesting approach towards identification of fractions of proteins and the corresponding clone of cells.

Characteristics of ASC following systemic and mucosal immunizations was done taking advantage of the fact that systemic immunization in humans results in a transient appearance in blood of B-cells that are en route to their final destination in systemic lymphoid tissue (Kantele *et al.*, 1986) where they transform into high Ig-secreting plasmocytes (Roldan and Brieva, 1991; Roldan *et al.*, 1992). Similarly vaccine-specific ASC have also been detected in blood after enteric immunization, confirming the notion of mucosal immune system (Czerkinsky *et al.*, 1987; Czerkinsky and Holmgren 1994; 1995). According to this concept B-cells activated at mucosal inductive sites, like the intestine enter the lymphatics, undergo further differentiation in mesenteric lymph nodes, and eventually reach the circulation through the thoracic duct (Brandtzaeg, 1985; 1987). They eventually migrate primarily to the initial sites of antigen exposure but also to other mucosal-associated glands (Czerkinsky and

Holmgren, 1994). Lymphocytes homing in different tissues are assumed to be initiated after interaction of cell surface adhesion molecules on lymphocytes and tissue-specific counter receptors expressed on vascular endothelial cells in the target organ (Butcher, 1991, Picker and Butcher, 1992; Springer, 1994).

Although a lot has been done to characterize the activation and maturation processes of B-cells, due to technical limitations only little has been done to characterize human circulating antigen-specific ASC with regard to phenotype, activation stage and homing commitments. The novel approach based on the combination of immunomagnetic cell sorting and ELISPOT technique, that allows the partition of subpopulation of functional lymphoid cells according to expression of cell surface molecules was applied to characterize human blood ASC elicited by mucosal and systemic immunizations. It has also been demonstrated that ASCs that appear spontaneously and secrete antibodies are differentiating B-cells that have not yet fully progressed to the plasmocyte stage. This study also indicated that these cells when appearing in the blood as a result of intestinal immunization with the prototype enteric immunogen, CTB, comprise a highly homogenous population of plasmoblasts. In contrast, circulating ASC induced by systemic immunization with the prototype systemic immunogen, TT, contained both B-cell blasts and plasmoblasts.

The vast majority of ASCs originating from either systemic or mucosal sites lacked surface expression of the plasmocyte marker CD28 (McHeyzer-Williams *et al.*, 1993), but they still expressed sIg, HLA-DR and CD19, all of which are lost immediately before or during the transition of plasmoblasts to plasmocytes (Banchareau and Rousset, 1992). Several cell surface molecules defining early, mature and blastic B-cells were not detected on the surface of the vaccine-specific ASCs (McHeyzer-Williams *et al.*, 1993). This was clearly supported by the observation that CD38, specifying terminally differentiated B-cells and plasmocytes were expressed by virtually all spontaneous Ig secreting cells, i.e., with unknown specifications and origin, are less mature than bone marrow Ig secreting cells, but exhibit a more differentiated phenotype as compared to germinal centre B-cells (Breiva *et al.*, 1991; Jones, 1990).

In line with this the present study indicated that vaccine-induced, enterically derived ASC constitute a highly homogenous population. TT-specific ASCs induced by systemic

immunization, on the other hand, were more heterogenous with regard to expression of maturation markers. Therefore, it can be suggested that at least two subpopulations of ASCs arise in the blood after systemic immunization; one major population consisting of plasmablasts, identified by their loss of markers defining early and mature B-cells and another minor population of blastic B-cells, still retaining many of these surface molecules.

The early, less differentiated B-cell surface markers sIgD, CD20, CD22, CD23 in the systemic and mucosal vaccine specific cells were obviously different. Expression of these activation markers, however was always detected on a smaller fraction of CTB-specific ASCs than on TT-specific ASCs. The higher expression of these markers on systemically derived TT-specific ASC population than on the mucosally derived CTB-specific indicates that the former population consists of less differentiated cells. The relative abundance of CD71 and CD25 on TT-specific ASCs suggests that they are in the process of proliferation (Breiva and Stevens, 1984; McHeyzer-Williams *et al.*, 1993).

The substantial numbers of TT-specific ASCs of CD5+ phenotype was unexpected. In adults, CD5 marker-expression is generally ascribed to a separate linage of B-cells that mainly produce polyreactive low-affinity IgM. Furthermore de novo expression of CD5+ has, however, been reported on in vitro activated B cells (Vernino *et al.*, 1992) and the present data suggest that this may also occur in vivo following activation. The increase in CD5+ cells among TT-specific ASCs than among CTB-specific ASCs might correlate with the relatively larger fractions of cells expressing activation markers in TT-specific ASC populations as compared to CTB-specific ASC.

Both ASC populations had a characteristic distribution of HLA class II molecules, in that virtually all cells expressed HLA-DR and the majority also co-expressed HLA-DQ. Earlier studies of leukaemic cells have suggested that MHC class II molecules are sequentially up-regulated during B-cell maturation (Guy and Heyningen, 1983; Direxler *et al.*, 1988). Based on the present results, it is possible that HLA class II molecules are also sequentially down-regulated during terminal B-cell differentiation, starting with HLA-DQ followed by HLA-DP and ultimately HLA-DR.

Furthermore, mucosally and systemically activated blood ASC appear to differ markedly with regard to their respective homing potentials. In all cases the process of selective lymphocyte homing is dependent on binding of organ specific homing receptors expressed on lymphocytes to their ligands, addressins, on endothelial cells. Generally cell adhesion molecules such as integrins, endothelial cytokines and their counter-receptors work coordinately to confer tissue specificity (Butcher, 1991; Picker and Butcher, 1992; Hamann *et al.*, 1991). On lymphocytes, L-selectin has been characterized as a peripheral lymph node homing receptor, interacting with carbohydrate ligands on high endothelial venules in non-mucosal lymph nodes (Picker and Butcher, 1992). L-selectin can also participate in lymphocyte homing to organized mucosal lymphoid tissues, such as the Peyer's patches in the mouse (Hamman *et al.*, 1991), where, specificity appears to be determined by the integrin $\alpha 4\beta 7$ (Berlin *et al.*, 1993). Importantly, however, L-selectin does not appear to contribute to the trafficking of gut -homing immunoblasts to the lamina propria.

In this study expression of L-selectin was clearly different between the ASC populations induced by intestinal and systemic immunizations, respectively. Almost all ASC activated at systemic sites expressed L-selectin, whereas only few gut-derived ASC expressed L-selectin. The expression of two other antigens that have been implicated in lymphocyte trafficking to mucosal sites, the $\alpha 4$ integrin chain and CD44 have been examined, $\alpha 4$ can associate with two different β chains on circulating lymphocytes, either $\beta 1$, forming VLA-4 involved in binding to VCAM-1 or $\beta 7$, forming Lpam-1, a receptor for the mucosal vascular addressin MAdCAM-1 (Berlin *et al.*, 1993) and selectively involved in lymphocyte trafficking to mucosal lymphoid tissues and lamina propria (Hamann *et al.*, 1994).

The expression of $\alpha 4$ by almost all TT-and CTB-specific ASC is consistent with their expression of one or both of these hetrodimers. Both populations also uniformly express CD44, a wide spread hyaluronate binding adhesion receptor that has been implicated in vitro models in lymphocyte homing to mucosal tissues, although its involvement in such trafficking remains to be demonstrated in vivo (Picker and Butcher, 1992). Also among memory T cells, tissue association seems to correlate with L-selectin expression, but not with expression of $\alpha 4$ integrins or of CD44 (Picker *et al.*, 1990). Therefore, it is possible that not only the presence of mucosal homing receptors, but also the lack of homing receptors, for peripheral tissues determines the anatomical location of a migrating mucosal lymphocyte. One could

argue that the differential homing receptor expression by intestinally and systemically derived ASC recorded in this study might be due to the use of different antigens, rather than the different sites of antigen delivery. The observations from the intra-tonsillar study, however, argue against this possibility, since circulating CTB-and TT-specific ASC induced simultaneously by intra-tonsillar immunization carry the same set of homing receptors.

The fraction of cells expressing L-selectin was significantly larger in the IgG-secreting ASC population from the gut than in the corresponding IgA-secreting population. This difference might indicate a certain disposition of intestinally derived IgA-secreting cells for migration to mucosal sites as compared to IgG-secreting cells. This notion is consistent with previous findings, that although enteric CTB immunizations induce comparable IgG and IgA ASC responses into the circulation, it is the IgA ASC that clearly dominate the B-cell-mediated responses in mucosal effector compartments such as the duodenal mucosa and the salivary glands (Czerkinsky *et al.*, 1991).

All in all, this study has demonstrated the feasibility of combining cell sorting and ELISPOT techniques to allow detailed phenotypic characterization of functional subsets of lymphocytes. With this approach, we could demonstrate that circulating ASC originating from both mucosal and systemic sites are terminally differentiating B-cells that have not yet fully differentiated into plasmocytes. Furthermore, ASC activated by mucosal immunizations utilize organ-specific recognition mechanisms distinct from those of corresponding systemic B-cells. This observation could explain both the unification of immune responses in diverse mucosal sites and the physiologic segregation of mucosal from non-mucosal immune mechanisms in humans.

Assessment of nasopharyngeal mucosa as a site of vaccination as seen from the response at the palatine tonsil level, is highly independent of the systemic and gut mucosal response. A single injection in the right tonsil induced large number of antigen specific ASC in that tonsil than did booster immunization by intranasal, peroral or subcutaneous immunization routes. The tonsillar ASC were mostly IgG secreting cells and when immunized with antigen directly they also developed antigen-specific IgA-secreting cells but no IgM was detectable. It does not seem to serve as an expression site for perorally or parenterally delivered antigens. The failure of the immune response induced at the right palatine tonsil to equally involve the left,

made this fact very vivid. From their location close to upper respiratory tract and sharing an architectural similarity with the gut associated lympho-reticular tissue (GALT) and lymph nodes one would expect the tonsillar glands (palatine, lingual and nasopharynx tonsils) to serve as induction and effectors sites. They are equipped with all types of cells to carry immunological reactions; the M-cells, APC (macrophages, dendritic cells and langerhans cells) and cells serving effector or regulatory functions for various types of immune response lymphocytes and natural killer cells. These features, qualify them well enough to mount a B-cell mediated response. In fact the tonsil is suggested to be the functional analogue to Peyer's patches in the GALT within the bronchial associated lymphoid tissue (BALT) (Korsrud and Brandtzaeg, 1981). It has many times been suggested to be an ideal spot to deliver antigens for the antigens could hardly suffer from enzymatic degradation before contacting the mucosal wall. This in keeping with the idea of common mucosal immune system and its being at the cross road was the reason for looking into its overall influence in induction and expression of systemic and mucosal immune responses. Nevertheless this was not true. Probably the role of tonsils is limited to the upper respiratory tracts for it had been possible to trace activated cells at the tonsil elsewhere in salivary glands and nasal mucosa and also systemic sites like spleen and lung (Ray *et al.*, 1990; Nadal *et al.*, 1991). The appearance of more numbers of ASCs at the primed tonsil compared to the non-primed tonsil in the same individuals on boasting suggest the production of local memory cells. This implies that at least a sub-population of B-cells had been to germinal centre follicule and migrated there from.

The attempt to trace the sources of tonsillar B-cells by the expression of receptors on ASCs residing in and migrating from the tonsils revealed that the frequencies of vaccine-specific IgG and IgA secreting cells were significantly higher in the right tonsil than in the left ($p < 0.05$). This suggested that it is only limited numbers of locally induced ASC that travel to reach the non-immunized tonsil. From these observations one can safely say that there is only a minute fraction of the tonsillar IgG and IgA that reach the circulation to be disseminated to other organs. This is in agreement with earlier reports that showed in vitro proliferation and antibody responses of tonsillar MNC are selective for upper respiratory tract antigens and not to distantly located antigens (Czerkinsky and Holmgren, 1995).

In contrast, intranasal immunization induced less ASC at the palatine tonsil but increased quantities of IgG and IgA antibody secreting cells at the nasopharyngeal tonsils. When it comes to induction of peripheral blood response the intratonsillar immunization,just like the response at the tonsil, was IgG dominated but IgA was induced at the peripheral level. It was equal in magnitude and isotype composition with perorally booster immunization which induced peripheral responses.

The peripheral responses to intratonsillar and subcutaneous TT were similarly dominated by IgG although more so in the latter. The differential responses to systemic (TT) and mucosal (CTB) antigens could be due to the presence of earlier activated B-memory cells in the tonsil. This would account for the results of studies indicating that the tonsils harbour B-cells with specificity for antigens previously encountered at enteric and systemic sites.

The appearance of substantial numbers of vaccine-specific ASCs in blood indicates that the activated B-cells leave the tissues and that the tonsil serves as a priming organ for B-cells. Because the locally induced tonsillar ASC does not go back to the neighbouring non immunized tonsils. The assertion that the destination of circulating antigen specific ASCs is said to be towards the BALT (Nadal *et al.*, 1991) warrants further investigation. Whereas, the intranasal induction by CTB induces comparable immune response to those of intra-tonsillar and peroral immunization both in yield and types, indicating that several routes of mucosal immunization could at least produce immune responses of equal magnitude in circulation. Although the tonsil as expected did not seem to serve as a site to reach the mucosal system outside itself, the intra-nasal has proved to be the best to induce both systemic and mucosal immune responses.

The expression and distribution of homing receptors following different routes of CTB-immunizations revealed that the tonsillar expression of the LECAM-1 was minimal. The pattern was in contrast to that induced by parenteral, intratonsillar and intranasal immunizations where the large majority expressed both H-CAM and LECAM-1. These findings indicate a marked degree of heterogeneity regarding utilization of homing receptors by B-cell immunoblasts activated in different mucosal organs. The tonsillar and intranasal stimulations as evidenced by the appearance of ASC in the respective organs as well as in the blood, suggests they could act as inductive sites. However the tonsillar lymphoid tissue

as shown in this experiment poorly serves as expression site for remotely induced mucosal or systemic immune responses.

The validation of the immunomagnetic sorting technique in tuberculosis and leishmaniasis has clearly demonstrated the efficiency of the method to monitor the diseases at the cellular level (Lakew *et al.*, 1997).

Characterization of antibody secreting cells in schistosomiasis immunity was first done.The purpose of identifying the surface marker molecules that best select the antigen specific ASC from the three surface molecules CD19, CD38 and HLA-DR that are involved in activation and proliferation of B-cells showed that all surface markers can equally be used to determine the relative abundance of antibody secreting cells specific to *S. mansoni* antigens. In general more cells secreting specific antibodies to AWA per million were captured than those to egg antigen. However, all the three markers successfully demonstrated the same ASC pattern (IgM > IgA > IgG). It seems that at this stage of B-cell development, the ASCs bear all the three markers. The slight variation in the quantity of cells picked by the different markers could be a difference in cell clones that recognize specific antigen but also in their binding efficiency to beads which depends on density of the markers (Patel and Rickwood, 1995). The long steps in CD38+ cell isolation, did not affect the positively selected but the negatively selected were much less compared to those of other markers. The occurrence of such clones of IgM producing cells in particular to polysaccharide egg antigen, have been shown in murine schistosomiasis suggesting that an association exists between the markers and IgM immune secreting cells in schistosomiasis patients (Dunne *et al.*, 1987; Dunne *et al.*, 1991).

The fact that CD19 is expressed throughout ontogeny of B cells and that the only other cell type expressing CD19 is dendritic cells (Banchareau and Rousset, 1992), makes the surface marker a strong candidate portraying the circulatory B-cells. However, since it is expressed by B-cells from germination until just before plasma cell formation, they are found in abundance and a large proportion are not at the stages capable of secreting antibodies i.e. they could be any where on the growth and differentiation scale and could also isotype-switch to produce different antibody types other than the ones at the sampling time. Whereas CD38+ cells are expressed only at early and late stages of B-cell development (Germinal

centre B-cells and Plasma cells), acting as an immunoregulatory ectoenzyme. However, its expression on differentiating T-cells as well makes prior clearing of the T-cells an additional procedure. Nevertheless, once the isolation is done, the likelihood of getting a higher proportion of ASCs from the selected cell population is high. Therefore, in situations where ASCs are very scarce, it might be useful to concentrate only on the desired population of cells using this marker.

Most important in the expression and modulation of multiple cell surface molecules are the local availability of different cytokines within the micro environment at any given time. They selectively promote the development of cellular events and thus the course of inflammatory process which are directly related to antibody production. In this respect it is well established that immunological responsiveness to viral, bacterial and parasitic immunogens depend on MHC molecules that are capable of binding and presenting immunogenic peptides (Wasson and Kelly, 1990). HLA-DR, is a MHC class II molecule, enabling mononuclear phagocytes to present antigens to T-helper lymphocytes leading to cytokine production and local recruitment of inflammatory cells. Pro-inflammatory cytokines (IL-2, IL-4, TNF-α and TNF-β) increase HLA-DR expression on monocytes but not on B-lymphocytes (Rothenberg, 1995). When it is expressed on B-cells it also serves as an indicator of active inflammatory response and hence is equally good as a CD19 marker. An interesting exercise would be to use double markers and optimize the markers that synergize efficiency of isolation. Probably one can use this conception to increase the sensitivity of the method by using specific ASC to different antigens of the same organism.

The immune response comparing the ASC specific to *Schistosoma mansoni* SEA and AWA showed that there is a statistically significant difference ($p<0.01$) in IgM ASCs between infected individuals and the controls. The finding is in agreement with other studies on the kinetics of antibody classes and subclasses during the course of *S. mansoni* in mice (Bout, 1980). They also indicated that IgG1 and IgM antibodies specific to AWA respectively appear in circulation after egg production starts and are followed by IgA production to carbohydrate epitopes expressed on schistosomula blocking in vitro killing of schistosomula (Bout, 1980). Butterworth *et al.* (1992) have also shown IgM and IgG2 blocking antibodies to be related to susceptibility to reinfection in humans. Khalife *et al.* (1986) and Dunne *et al.* (1987) have also reported that IgM antibodies to polysacharide egg antigens may interfere

with the binding of protective IgG antibodies directed against surface antigens of the schistosomulum.

In humans IgG and IgE isotypes in association with effector cell population such as macrophages, eosinophils and platelets have long been demonstrated to kill schistosoma larvae and modulate granuloma formation. IgM, IgG4 and IgG2 isotypes are correlated with susceptibility to reinfection. It is presumed that these antibodies may cross-react with schistosomulum surface glycoproteins and inhibit the binding of effector IgG or IgE antibodies that may be formed in response to glycoproteins released from the schistosomulum or adult worms. IgM secreting cells dominate the immune response of the young while IgA dominates the older age group in humans. IgA antibody to specific antigens as previously mentioned are responsible for the schistosoma induced modulation of the immunopathology in old ages (Ivanoff *et al.*, 1996), by down regulating inflammatory cytokines (Wolf, 1994). Wilson (1990), has documented that the pattern of response in patients is more specific to egg antigen than to adult worm antigens.

Thus the presence of high number of IgM secreting cells, could mean the blocking of the effector functions, a phenomenon that is also clearly established in the rat model (Balloul *et al.*, 1987). This phenomenon could be explained in terms of TH2 cytokines, IL-5 and IL-4, which through IgE mediation and suppression of TH1 response induce susceptibility (Grzych *et al.*, 1991) while TH1 associated cytokines like IL-12 induce immunity by decreasing IL-4, IL-5, and IL-13 and IgE production and tissue eosinophilia (Mountford *et al.*, 1996; Perussia *et al.*, 1992; Chensue *et al.*, 1995). Associated with the route of stimulation the presence of TGF-β, chemokines like RANTES and macrophage inhibitory protein (Kimata, 1996) are also known to regulate anti-parasitic activity (Williams *et al.*, 1995). The absence of IgG ASCs in the present study, considering the long persistence of IgG antibody in sera of patient is hard to explain. It could but be that the IgG ASCs may have infiltrated into the liver, spleen and the bone marrow where they actively release their products into circulation. Other reasons like the parasite mechanisms interfering into the antibody isotype switching could account for the IgM accumulation (Bout, 1980; Grzych *et al.*, 1984). Cytokines IL-7 and IL-13 for example are known to decrease the production of INF-γ by increasing MHC class II antigen expression on B-cells that are potent APCs for TH2 type cells (Wolowczuk *et al.*,

1989), that in turn has been documented to be responsible for induction of MHC class II, IgE and IgG4 isotype switching in B-cells (Gajewski *et al.*, 1991; Punnonen *et al.*, 1993).

The interesting feature of the present experiment is not only that IgM is significantly higher in infected patients than in the controls, but that the ASCs to both AWA and egg antigens decrease with the increase in intensity of infection. In all cases chronic, acute and more acute as determined by the number of eggs shed at the time of examination, showed a similar profile in the relative antibody types within each group of patients. However, the decrease in ASCs in those shedding more eggs is intractable from this observations. The possible reasons for inducing such quantitative changes in ASCs types are many : The type of antigen presenting cells involved, the MHC class II expression, route of stimulation (Williams *et al.*, 1995) and the type of antigens which in turn influence the way antigens are presented and T-helper cells produce cytokines. One can also speculate that it may be due to blockage of resistance inducing antibodies, (IgE, IgG1 and IgA) by IgG4 that is known to act as a blocking agent (Grzych *et al.*, 1993). Although the work here being rather descriptive could allow a further speculative answers for the observations from the literature a better understanding warrants further examination into both the antibody subclasses and cytokine profiles.

The other equally important observation in the present work is the age related phenomena. The risks of the young to higher prevalence and intensity of infection and susceptibility to reinfection in *S. mansoni* and *S. haematobium* is now well established in several human studies carried in Africa. Individuals with comparable exposure showed up to 100 fold increases in intensity of infection and susceptibility to reinfection and that it is positively correlated with age. The susceptibility of the young and patients at early stage of infection is attributed to a decrease in eosinophil counts, and lack of IgE mediated killing of the shistosomula. These observations suggest different immune mechanisms to be operating in the two groups. The fact that IgG4 and IgE responses in children and adults are affected differently by chemotherapy could support this fact (Grogeon, 1996). The high production of IgM to the same surface antigens (Polysaccharides or heavily glycosylated glycoprotein) targeted by IgG and IgE and that during early infection the blocking antibody IgM, dominates the potentially protective IgE response could possibly account. The second more recent elucidation takes the increase in IgE with age, independent of intensity of infection to be

responsible. The profile of ASCs in different age groups to egg antigens and AWAs are more or less in conformity with the former explanations. IgM secreting cells dominate the immune response of the young while IgA dominates the older age group. Specific antibodies like IgA to Sm28GST that are minimal at the acute stage of infection later shifting to a predominantly protective type of response are also discussed in the literature (Capron, 1995).

In the past, considerable efforts have been exerted to determine the parasite antigen(s) that induces and elicits T-cell mediated responses, granuloma formation and antibody secretion. The age influence, antibody restriction particularly in intensely infected individuals deserves further study but is an interesting finding of this investigation. The comparison between the different markers suggests that the IgM dominated response to schistosome antigens has been shown by all the three markers has substantiate that it is really parasite induced. Its disappearance in treated patients giving way to more IgG response confirms it. Among the non-egg shedding controls IgA seems to be due to cross reaction with egg and adult worm antigens for it was more prevalent in malaria patients while the non-infected controls are limited to AWA. Schistosomiasis-treated patients have IgG dominated response suggesting that the immune response due to infection is reversible (IgG > IgA > IgM). These results indicate the importance of the IgM response and its possible utility in monitoring both the disease progression and possibly vaccine efficacy.

Induction of tolerance is another phenomenon associated with schistosomiasis. The interesting feature about the MALT is its capacity to generate potent immune response at the mucosa on the one hand and to induce peripheral tolerance. Immunological tolerance at the thymus is against self reaction and is important for self integrity; whereas at the periphery it is against external antigens and could be the function of a specific T-cell response. The mucosal response produced by feeding or inhalation of antigens guards the mucosal surface through specialized effector mechanisms typified by sIgA, which is found in very high proportion at the mucosal layer (Xu *et al.*, 1994; Grzych *et al.*, 1993). IgA is an antibody that does not have a complement activating and relatively inefficient as opsonin thus represent a unique non-inflammatory specific immune effector molecule. It prevents bacterial and viral pathogens, potentially harmful effects of bacterial toxins, and molecules from ingested food items, by selectively neutralizing their effects through antibody-dependent cytotoxicity, a mechanism that utilizes macrophages, eosinophils and lymphocytes. In this effort, the

cytotoxic T-cells and natural killer cells play important role as a first line defense (Santos *et al.*, 1994). But these factors may as well play part in human interaction to the parasite.

The development of granulomatous reaction induced in schistosomiasis is a systemically induced type IV delayed hypersensitivity response. It is also transferred with T-cells from infected or schistosoma egg-sensitized animals and is inhibited by anti-T-helper cell antibodies. Moreover, the induction of IgA antibody specific to Sm28GST antigen in humans has been shown to increase resistance manifested by parasite fecundity and its pathological consequences (Capron *et al.*, 1995).

Taking these facts together the possibility of using mucosal stimulation combined with systemic tolerance as a therapy to suppress unwanted tissue damaging immunological reactions (Brandtzaeg, 1987), was evaluated. Simple mucosal exposure of an antigen induces systemic tolerance. But it requires large quantities of the antigen, stays for only short duration and it is more inducible in naive as compared to the immune host (Mestecky and McGhee, 1987). Thus in this experiment attempt was made to optimize conditions for maximal mucosal immune response (sIgA) while at the same time provoking systemic tolerance in a situations where chronic inflammatory disease has already been established.

The large requirement of antigen was reduced by coupling CTB, the non-toxic mucosal binding moiety of cholera toxin to Sm28GST-a known inducer of resistance in schistosomiasis patients through IgA production. Instead of the oral route intranasal was chosen for induction site because recent evidences have provided that CTB-linked to auto-antigens can inhibit systemically occurring inflammatory diseases like encephlomylitis and diabetes (Sun *et al.*, 1994; Bergerot *et al.*, 1996).

Results as anticipated produced a worm burden reduction of 66% which is superior to the 40 % produced with out the use of CTB-as carrier or-adjuvant (Balloul *et al.*, 1987). The egg which is responsible for induction of the pathology at the liver and intestine was reduced by 84%. Although viability test has not been done in this experiment considering the fact that 85% of them were not capable of hatching in animals immunized with SM28GST (Boulanger *et al.*, 1991), it is likely that a higher number would be non-viable. This is confirmed by the reduction of the mean granuloma area by <50%. Glutathione S-transferase when

administered with aluminium hydroxide, in baboons has been shown to produce a mean protection of 42% with 68% reduction in egg out put and 85% in their hatch (Grezeel *et al.*, 1993).

The mechanisms involved in the protection are probably many. Local immune effects are thought to be produced by CD4+ T-lymphocytes suppressor and mediated by CD8+ suppressor T-cells. The cytokines that affect lymphocytes as well as other cells of the immediate environment include IL-6, TGF-β, TNF-α and INF-γ (Weiner *et al.*, 1991). The role of immunosuppresor cytokines like IL-4, IL-10 and TGF-β (Chen *et al.*, 1995), is also implicated in changing the histopathological features of granuloma which occurs over the course of infection with schistosomiasis. The $\gamma\delta$-T-lymphocytes form the first line of defense at epithelial surfaces by destroying altered cells by infections, recognizing non-classical MHC proteins expressed on enterocytes, recognizing bacteria derived super-antigens and heat shock proteins (Haregewoin *et al.*, 1989). The possible regulatory role $\gamma\delta$ T-cells could play is supported by the panel of cytokines they produce (IL-2, IL-6, INF-γ, TGF-β). Intranasally administered antigens have been transferred by TcR$\gamma\delta$ intra-epithelial lymphocytes from airway epithelium.

TNF-α is another cytokine released from macrophage and is responsible for its accumulation and differentiation into epitheloid cells through chemotactic cytokines and adhesion molecule expression which is central to granuloma formation. Since macrophages cannot be activated without INF-γ in the liver might have failed to produce TNF-α. Macrophages from egg induced granuloma have no stimulatory effect but render them unresponsive through anergic processes.

The reduction in leucocytes count which form part of the delayed type hypersensitivity reaction is rather marking. In inflammatory conditions the antigen is first taken by APC and presented to IL-2 secreting T-cells at the lamina propria. IL-2 augments the synthesis of more IL-2, IL-2R and of other cytokines such as TNF-α and INF-γ by other T-cells. TNF and lymphotoxin stimulate venular endothelial cells to express adhesion molecules which increases the infiltration of monocytes, lymphocytes, neutrophils and eosinophils into the sites of antigen challenge. There may be several mechanisms involved in clonal anergy. It could be due to lack of critical ligands like β7, CD40, LFA-1 and LFA-3) by APC thus failing to

present antigen to TH-cells (Miller *et al.*, 1994). This is supported by the cytokine and proliferation assay results that showed non-detectable quantities of both INF-γ and IL-4 and minimal T-cell proliferation. In the absence of INF-γ the macrophage is unlikely to be activated enough to process and present antigen efficiently thus resulting clonal anergy. But the possibility of active suppression by CD4 and CD8 T-suppressor cells triggered through antigen-specific mechanisms like production of TGF-β and IL-10 can not be ruled out (McMenamin *et al.*, 1991; 1993; Chen *et al.*, 1995). The presence of IL-4 and INF-γ secreting T-cells at the spleen suggest that tolerance induction is localized at the liver and the bystander suppression can operate from CD8 T-cells for it is documented that suppression can be transferred from donor to recipient with splenic cells (O'-Doherty *et al.*, 1993).

It therefore, appears that in terms of schistosomiasis vaccine strategy, immunization with Sm28GST through mucosal route may be feasible as it leads to significant reduction in pathological consequences by reducing the worm burden and fecundity. The fact that the vaccine functions after infection also suggest that it might be possible to halt and possibly suppress the pathology which gives chance to use it in individuals that are already infected. This might require the identification of the right type of T-cell sub-populations involved in the suppression process. In general it has been possible to show through the present investigation that suppression of systemic and mucosal immunity at the same time or selective suppression of systemic immunity while augmenting mucosal immunity in schistosomiasis mansoni, is possible in the mouse model system. Regression of hepatic lesion has been achieved by many workers: Andrade *et al.* (1993) in *S. japonicum*; Amory-Soisson *et al.* (1992) and Williams (1994) in *S. mansoni*. However, the present approach offers a novel means to induce concomitantly anti-parasite and anti-inflammatory (pathological) immunity in an already infected host.

V. CONCLUSION AND RECOMMENDATION

A new and sensitive method that allows phenotypic characterization of ASC following vaccination or natural infection has been developed. Its application in detecting the immune process at cellular level in known human vaccine systems (Cholera Toxin-mucosal and Tetanus toxoid-systemic) and schistosomiasis mansoni infection was validated. The endeavour to discriminate mucosally induced cells from systemically induced ones has revealed the

expression of L-selectin to be a good marker at least in IgG secreting cells. The tonsil although claimed to be the Peyer's patches of the oral mucosa has been shown to be of little importance to serve as induction site to the mucosal immune system. Primarily because it induces IgG dominated response and is also highly localized. Instead the intranasal route was found to be more convinient, far reaching and IgA dominated at the peripheral and mucosal levels.

acombination of the above findings and the availability of a mucosal adjuvant CTB that allows the efficient delivery of antigens at the mucosal levelfacilitated the vaccine trial in murine schistosomiasis mansoni. Sm28GST documented as protective vaccine when given intranasally coupled to CTB at 10μg dose in mice protected mice against parasite infection and liver pathology. At the liver the infiltration of leukocytes the activation of TH1 and TH2 as evidenced by expression of cytokines INF-γ and IL-4 and their proliferative response is highly suppressed.

The above strategy of immunotherapy by tolerance induction will have an application in the control of other diseases with similar immune reactions like leprosy, leishmaniasis, tuberculosis and even HIV. Thus further investigations along this line is a worth a cosideration.

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Declaration

I, the under signed declare that this thesis is my original work and has not been presented for a degree in any other university.

Name: Mekuria Lakew

Signature: 

Place and date of submission: Addis Abeba University, July 1998