Molecular and Cellular Biochemistry **297:** 9–19, 2007.
DOI 10.1007/s11010-006-9316-2

The role of a heat shock protein from *V. cholerae 0139* in the gut immune response

Shibnath Mazumdar,[1] Shalmoli Bhattacharyya,[1] Sujata Ghosh,[1] Siddhartha Majumdar[1] and Nirmal. K. Ganguly[1,2]

[1]*Department of Experimental Medicine and Biotechnology, PGIMER, Chandigarh 160012, India;* [2]*Indian Council of Medical Education and Research, New Delhi India*

Received 25 April 2006; accepted 25 August 2006

Abstract

An immunodominant heat shock protein (Hsp 24) was purified from *Vibrio cholerae O139* at 42 °C and used as an immunomodulator for studying the gut immune response. T cell clone and T cell line specific for the Hsp 24 were generated from the lymphocytes of lamina propria and intra-epithelial lymphocytes of mice orally infected with *V. cholerae O139*, respectively. The T cell clone was TCR $\alpha\beta^+$, $CD4^+$ and appeared to play an important role in the functioning of gut B-lymphocytes. The T cell line had heterogenous population of CD8+ and CD4+ cells, most of which were found to be TCR $\alpha\beta^+$ and a minor population was TCR $\gamma\delta^+$. The lymphokine profile of T cell line showed IFN-γ to be the most abundant lymphokine followed by IL-2 and IL-4. The possible involvement of alternative pathway of activation for T cell clone was also addressed in this study. The splenocytes showed an up-regulation of their CD2 receptor expression on stimulation with the Hsp-24. The pattern of lymphokines released by splenocytes stimulated with the Hsp-24 showed no particular cell type to be responsible for mounting immune response. Thus, there is involvement of both, mucosal and peripheral arm of the immune system.

Key words: cholera, T cell line, T cell clone, immunization

Introduction

The disease, cholera, caused by *Vibrio cholerae* is still an important public health problem in many of the underdeveloped as well as developing countries of the world and the battle to completely control this deadly disease continues. There are many reports in literature regarding the mode of transmission of this disease and its etiologic agent. However, several questions regarding the host parasite interactions involved during the transition of vibrios from the naturally occurring viable but non-culturable stage to the infective vegetative stage within the human host remains yet to be answered.

Till the early nineties, the world knew about the two biotypes of vibrios with epidemic potential – the classical and the ElTor biotype. However, in 1992, cholera-like epidemic was reported from India as well as its adjoining parts and the causative agent was identified as *V. cholerae* of non-01 serogroup 0139 (139th serotype in the typing scheme for *V. cholerae*). Preliminary epidemiological reports as well as genetic analysis have assessed the fact that this serogroup may represent the etiologic agent of a new eighth pandemic [1]. In fact, in 2002, the serotype 0139 caused an estimated 30,000 cholera cases in Dhaka (Bangladesh) exceeding the number of cases associated with ElTor during any short period [2]. Hence, this new strain became the subject of active research in the field of cholera biology throughout the world.

Since *V. cholerae 0139* strains are relatively new entrants into the cholera scenario, very little is known about their

Address for offprints: S. Ghosh, Department of Experimental Medicine and Biotechnology, PGIMER, Chandigarh 160012, India (E-mail: shalmoli@glide.net.in)

immunobiology. Due to the non-invasive nature of the infection, host defense mechanisms in cholera has been studied elaborately in connection with the humoral immune responses and the information regarding cell mediated response of the host against cholera is limited. In order to have a proper understanding of the immune responses generated against *V. cholerae*, it is important to study the localized (gut) cell mediated immune responses.

Thus, the main aim of the present study was to characterize the immunomodulatory effects of a highly immunogenic low molecular weight heat shock protein (Hsp) from *V. cholerae 0139* on mucosal T cells obtained from orally immunized murine gut. This study may help us to understand the role of the immunodominant Hsp in the interplay of different immunocompetent cells present in the gut.

Materials and methods

Bacterial strains and growth conditions

The *V. cholerae 0139* strain (Strain 776) used in this study was a clinical isolate obtained from the stool samples of a clinically diagnosed patient admitted in the Institute (PGIMER, Chandigarh) hospital. The strain was animal passaged and maintained on nutrient agar slants. For [^{35}S]-methionine labelling,the bacteria were grown in the medium MM9 as described by Chakravarti and Ghosh [3]. *Salmonella typhimurium* (Strain 11828) was a kind gift from CRI Kasauli, India.

V. cholerae was grown to log phase in tryptone soy broth (Hi Media, India, TSB, pH 8.2–8.4) at 30 °C and then subjected to heat shock at 42 °C as well as 47 °C by transferring aliquots to a set of flasks maintained at desired temperature in a water bath. Bacterial survival was determined by withdrawing aliquots at different time intervals followed by plating on nutrient agar plates. Cell survival immediately prior to heat shock was taken as 100% value.

Induction of Hsps

Bacteria were grown to logarithmic phase at 30 °C in MM9 medium. To analyze the Hsps, aliquots of culture (1 ml) were transferred to vials containing 15 μCi [^{35}S]-methionine (specific activity15 mCi/ml, BRIT, India) followed by incubation at 42 °C for varying time periods. Labelling of cells was terminated by chilling to 0 °C. The labelled cells were washed (10,000 rpm, 5 min, 4 °C) and suspended in 1 mM Tris/HCl (pH 7.3) containing 10 mM each of EDTA, leupeptin, pepstatin, and PMSF (Buffer A). Finally, the cells were lysed, the membrane and the cytosolic fractions were separated by centrifugation (37,000 rpm, 1 h, 4 °C). The protein estimation was done according to Bradford [4] and the amount of radioactivity incorporated was measured by counting in a liquid scintillation counter (LKB1214RACK BETA). The fractions were analyzed by 10% sodium dodecyl sulphate polyacryamide gel electrophoresis [SDS-PAGE] [5] followed by autoradiography [6].

Identification and purification of immunogenic proteins

V. cholerae 0139 was grown in TSB (pH 8.2) at 30 °C to mid exponential phase and then heat shocked at 42 °C for optimum time. On termination of stress, the bacteria were harvested (10,000 rpm, 10 min at 30 °C), suspended in Buffer A and lysed. The membrane and the cytosolic fractions were separated (37,000 rpm, 1 h, 4 °C).

The solubilized *V. cholerae* membrane proteins were run on 10% SDS PAGE, transferred to nitrocellulose membrane [7] and the immunogenic protein bands were identified using pooled sera from convalescent (day 21) as well as acute (day 1) stage patients separately as the primary antibody. The HRP-conjugated antihuman immunoglobulin (Dakopatts) was used as the secondary antibody. Infant human sera obtained from the same endemic region served as control primary antibody in this experiment.

The band corresponding to the most immunodominant Hsp was eluted out from the gel [8] and further subjected to SDS-PAGE. The SDS content in the eluted material from the gel was minimized by passing it through AG1148 column (1 × 40 cm, BIORAD) according to the manufacturers' instructions. The fractions were extensively dialysed against deionized water. Finally, the extent of SDS removal from the protein was estimated by basic fuschin method [9] and the protein content was determined by the method of Bradford [4].

Immunological experiments

Six days old Balb/c mice were orally immunized with live *V. cholerae* (1×10^6/100 μl PBS, LD50 dosage) under sterile conditions. The surviving animals were sacrificed 21–30 days post immunization. Subsequently, the lymphocytes were isolated from Peyer's patches, intestinal intraepithelial, lamina propria and spleen by the method of Davies and Parrot [10] with slight modifications.

For the isolation of Peyer's patches lymphocytes (PPL), the Peyer's patches were removed from the small intestine with two 26G needles and pushed through the stainless steel mesh. The resulting cell suspension was layered on Ficol-Histopaque solution and centrifuged (1400 rpm, 30 min). The mononuclear cells thus obtained, were de-

pleted of erythrocytes and monocyte/macrophage population. The non-adherent cells were passed through a nylon wool column, preequilibrated with RPMI-1640 (1 h, 37 °C). The T-cells and the B-cells were eluted out with warm RPMI-1640 and chilled RPMI-1640, respectively. The B-cells thus obtained were washed, incubated with 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells (SRBC) in the ratio 1:10 and left overnight at 4 °C. Subsequently, the cell suspension was layered on Ficol-Histopaque and the B-cells were collected from the interface. The purity of B-cells was assessed under the UV microscope after staining with FITC conjugated anti Thy1.2 (Pharmingen). The splenic lymphocytes were obtained from the spleen of the infected mice by methods as described for PPL.

For the isolation of the intestinal intraepithelial lymphocytes (iIEL), the small intestine was removed from the mice and properly washed in calcium–magnesium free-Hanks Balanced Salt Solution (CMF-HBSS). These were then opened longitudinally, cut into small pieces and incubated in HBSS containing 5 mM EDTA for 30 min with constant stirring. This was repeated till a clear supernatant was obtained. The cloudy supernatants were pooled in RPMI-1640 and passed through a sterile glass wool column to remove the debris. The filtrate was layered on Ficol-Histopaque, centrifuged (1400 rpm, 30 min) and the mononuclear cells were collected from the interface. The cell suspension was incubated in a prewarmed wet nylon wool column for 1 h and the T-cells were eluted out with warm RPMI. The T-cells were incubated with AET-SRBC (1:10) overnight at 4 °C to further enrich the purity of eluent.

The T-cells of infected mice were obtained by methods described earlier for iIEL. After the complete recovery of iIEL, the gut pieces were washed with CMF-HBSS and RPMI containing 2% FCS (cRPMI) for the isolation of Lamina propria (LP). This was followed by 20 min incubation in 20% FCS with gentle stirring to inactivate the residual EDTA. The intestinal pieces were digested at 37 °C in cRPMI containing 20 units/ml of collagenase (Type IV). The T cells were purified as mentioned before.

Development of T cell line and T cell clone

For the development of T cell line, the intestinal intraepithelial T cells were suspended in cRPMI containing recombinant IL-2 (rIL-2, 35U/ml) and distributed into 24 well tissue culture plates at a concentration of 1.5×10^6 cells/ml. The lymphocytes were stimulated routinely with the specific heat shock protein (2.5 μg/ml) in the presence of γ-irradiated (3000 rad) syngenic feeders and rIL2 after every 4th day for 15 days. Finally, the antigen specificity of T cell line was determined and the lines propagated for further immunological characterization. The Hsp specific T cell line from LP was developed in the same way but without the addition of rIL2.

For the development of antigen specific T cell clone, cells comprising the Hsp specific T-cell line from LP were plated in 96U bottom tissue culture plate with Con A stimulated rat splenocyte suspension (RSS). To each well, cRPMI and irradiated feeder cells (5×10^5) were added. Prior to their usage as feeders, the syngenic splenocytes in all the experiments were treated with a mixture of anti thy 1.2 (Pharmingen), anti CD4 (Pharmingen) and anti CD8 (Pharmingen) for 45 min on ice, washed and incubated for 30 min with low toxicity baby rabbit complement to remove the residual T cells. Additional medium containing 10% FCS, 2.5 μg/ml of antigen alongside 50 μl RSS was added to each positive well on every 5th day.

Antigen specificity

In order to determine the antigen specificity of the clone, all the antigens [the heat stressed formalin fixed killed *V. cholerae* 0139 (10^4/ml)/*V. cholerae* Hsp (2.5 μg/ml)/ *S.typhimurium* (diarrheogenic) total membrane protein (2.5 μg/ml)/ cholera toxin (CT, 1 μg/ml)/ BSA (25 μg/ml)/ Con A (25 μg/ml)] were all diluted in RPMI 1640. Control wells had cells only. Plates were incubated in humidified CO_2 (5%) incubator at 37 °C for 72 h and 0.5 μCi of ^{3}[H]-Thymidine (BRIT, Bombay) was added to each well prior to last 18 h of culture period. The cells were harvested with automatic cell harvestor and the incorporated radioactivity was detected in liquid scintillation counter. To the cell line, 35 U/ml of murine rIL-2 was also added alongwith the antigens. All other assay conditions were kept unchanged for the cell line. Data were represented as mean cpm of triplicate culture ± SD.

Phenotyping

The T cells were separated from the irradiated splenocytes over Ficol-Histopaque, washed twice in cRPMI-1640, enumerated and adjusted to 1×10^6 cells/ml in PBS. The cells were stained with Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and phycoerythrine (PE) conjugated anti-CD8/FITC-conjugated anti-TCR$\alpha\beta$ (H57-597; Pharmigen) and PE conjugated anti-TCR$\gamma\delta$ (GL3; Pharmigen) for 45 min on ice. After washing twice, the cells were enumerated and analyzed under FACScan (Becton Dikinson). The intestinal intraepithelial T cells were stained in identical manner. The other antibodies used were anti CD5 (FITC) and antiCD45RO (PE).

Lymphokine profile

The LP-derived T cell clone (1×10^5) comprising the Hsp-specific T cells were plated out with irradiated syngenic feeders (5×10^5) in 200 μl of cRPMI in each well of a 96 well U bottom tissue culture plate. To each well, specific Hsp (2.5 μg/ml) was added. The control wells had cells only. All the assays were set up in triplicate and culture supernatants were collected at different time intervals and stored at −70 °C till further assays were performed. The detection and quantification of IL-2, IL-4, IL-6 and IFN-γ were carried out with ELISA kits (Genzyme). The lymphokine profile of iIEL derived T cell line was carried out in a similar manner.

Role of T-cell clone in antibody production

Cell proliferation assay for B-cells
T cell clone (5×10^4/well) was irradiated (2000 rad) and co-cultured with PP derived B-cells (1.5×10^5/well) from mice infected with *V. cholerae 0139* in presence or absence of the specific Hsp to which the clone was raised. After 54 h incubation, [^{3}H]-thymidine (0.5 μCi/25 μl of cRPMI) was added to each well and the cells were incubated for an additional 16 h. The amount of radioactivity incorporated was taken as a measure for B-cell proliferation. Data was expressed as mean CPM ± SD of triplicate wells.

Role of LP derived T cell clone on immunoglobulin synthesis
The clonal T cells (2×10^6) were co-cultured with PP derived B cells (2×10^6, obtained from syngenic mice orally immunized with live *V. cholerae*) with or without RSS (50 μl). The cells were stimulated with specific Hsp (2.5 μg/ml). The supernatant was collected after 5 days and 7 days of culture and checked for the presence of Hsp specific IgA and IgG using HRP conjugated anti mouse IgA (1:30,000; Sigma) and anti mouse IgG (1:5000; Sigma), respectively in ELISA. All the assays were set up in triplicate and data represented as mean of triplicate OD ± SD.

Mucosal and systemic immune response

Lymphocyte transformation test in splenocytes
Splenocytes (1×10^5) were obtained from the mice orally immunized with *V. cholerae 0139*.The erythrocyte population was removed by hypotonic shock. The lymphocytes (1×10^6cell/ml) were washed and plated in 96-well U bottom plates in cRPMI (100 μl). These were stimulated for 72 h at 37 °C with heat killed whole bacteria/specific Hsp (2.5 μg/ml)/BSA (25 μg/ml)/ConA (25 μg/ml). About 0.5 μCi of ^{3}[H]-Thymidine (BRIT, Bombay) was added to each well prior to the last 18 h of culture period. The cells were harvested with automatic cell harvester and the incorporated radioactivity was detected in liquid scintillation counter.

Lymphokine profile
Splenocytes (1×10^5) were obtained as above and plated in 96 well U bottom plates. These were stimulated with the Hsp 24 and the cell supernatants were collected at 24, 48 and 72 h, respectively following the antigenic stimulation. The levels of IL-2, IL-4, IL-6 and IFN-γ in the supernatants were detected with help of ELISA kits.

Modulation of alternative pathway (Rosette formation)
The T cell rosettes were developed and enumerated as described by Wybran and Fundenberg [11]. Briefly, T cell clone (1.5×10^6/ml) was plated out with irradiated syngenic feeders (5×10^6/ml) in 2 ml of cRPMI in 24 well tissue culture plates and incubated with the Hsp for 48 h. The T cells were washed and separated on Ficol histopaque and adjusted to 2×10^6 cells/ml. These were incubated with an equal volume of 0.5% AET treated SRBC (45 min, 4°C). The resulting cell suspension was gently resuspended and the percentage of rosette forming cells was enumerated. Lymphocytes with >3 erythrocytes were counted as rosettes. A minimum of 300 lymphocytes was enumerated and results were expressed as percentage of total T cell population. Similar experimental protocol was maintained for studying CD2 modulation in splenocytes.

Statistical analysis

Paired '*t*' test was used to analyze antibody production in control and experimental observations. Anova and Turkis test was used to determine the level of significance in T cell line and the splenocytes proliferation experiments.

Results

The bacterial viability was found to be reduced to 15% within 5 min following an increase in temperature from 30 to 47 °C and by the end of 10 min practically no bacteria remained viable. There was very little change in cellular viability when the *vibrios* were incubated at 42 °C even for a period of 1 h. However, when *vibrios* were incubated at 42 °C for 20 min and then exposed to 47 °C there was a significant improvement in their cellular viability. Thus, exposure of the vibrios to an intermediate temperature elevation could induce thermotolerance, which helped them to survive when exposed to a lethal temperature elevation.

Optimal temperature for the induction of Hsps in *V. cholerae* was selected as 42 °C as there was a progressive decrease in the synthesis of Hsps at higher temperatures. Analysing the membrane associated Hsp expression at different time intervals in ^{35}S-methionine, it was found that maximum number of Hsps of molecular weight (M_r) 11.5–160 kDa were expressed after 20 min of heat stress at 42 °C and thus, this time period was considered optimal for subsequent experimental works. Figure 1 shows the autoradiogram of the Hsps of *V. cholerae 0139*.

Western immunoblot experiment revealed several immunoreactive bands and these were identified as Hsps by overlay of the autoradiograph on the Western blot (Fig. 2). The protein of Mr 24 kDa (Hsp 24) was selected to study the immunomodulatory effect on gut T lymphocytes as it appeared as an intense band on immunoblotting as compared to other Hsps. SDS-PAGE of the immunodominant Hsp eluted from gel revealed a single major band thus confirming the purity of the eluted Hsp-24 (Fig. 3).

The T cell line specific for the Hsp24 (EMB1), developed from iIEL of mice orally infected with live *0139 vibrios* was found to be IL-2 dependent. Maximum cellular proliferation occurred in presence of heat stressed whole bacteria followed by the Hsp-24. Moderate proliferation of T cells was observed with the membrane protein of *S. typhimurium* however, the cell line proliferated poorly in presence of CT (Fig. 4a). The Hsp24 specific T cell clone (NKG-1) developed from the LP of mice orally immunized with *V. cholerae 0139*, showed maximum proliferation with Hsp-24. In each set of experiment, cellular proliferation in presence of ConA was taken as the positive control while that with BSA was taken as negative control (Fig. 4b).

In view of phenotypic analysis, EMB1 was found to be heterogeneous in nature. Around 76.01% of the cells were $CD8^+$, while 13.15% of the cells were found to express the $CD4^+$ phenotype. Moreover, the T cell line appeared to be essentially composed of TCR $\alpha\beta^+$ cells (72.13%) along with a minor population (10.02%) of $\alpha\beta$ receptor bearing cells (Fig. 5a). In addition to these, around 65% of cells representing EMB1 were found to be $CD5^+$ and around 48% of them were expressing CD45RO (Fig. 5b). The T cell clone was found to be TCR$\alpha\beta^+$ $CD4^+$ (Fig. 6).

The lymphokine profile of the T cells comprising the T cell line (EMB1) generated in response to the Hsp 24 indicated the presence of both Th1 and Th2 type of cells in the cell line, since IL2, IFN-γ along with IL4 could be detected in the culture supernatant. Interestingly, the level of

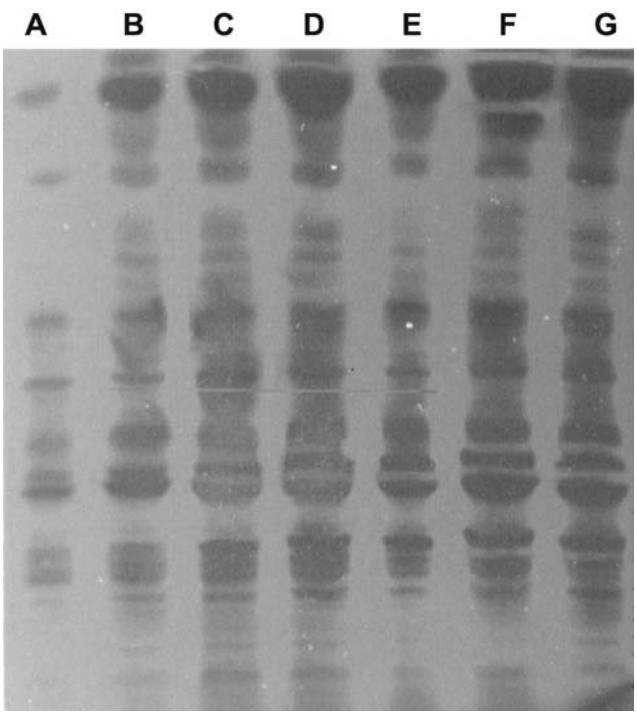

Fig. 1. The autoradiogram of *V. cholerae 0139* (strain 776) heat shock proteins (Hsp) at different time intervals. Lane A: Control, Lane B: 5 min, Lane C: 10 min, Lane D: 20 min, Lane E: 25 min, Lane F: 30 min, Lane G: 35 min.

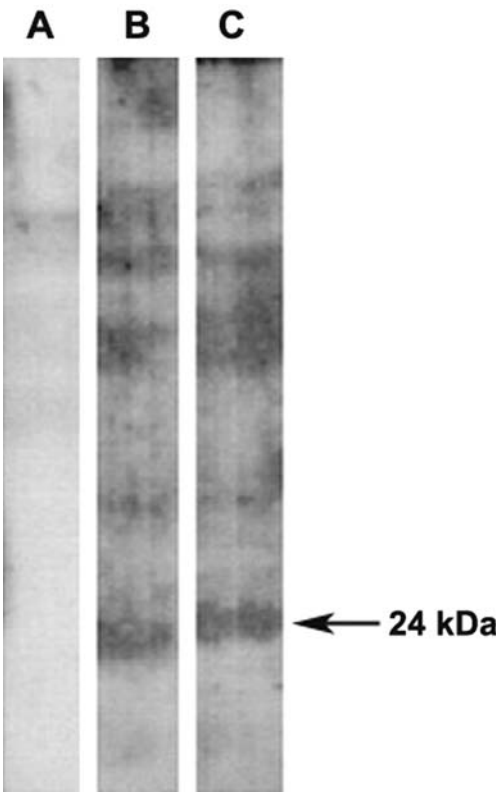

Fig. 2. Western immunoblot of *V. cholerae 0139* heat shock proteins. (A) Neonatal serum as primary antibody. (B) Serum from convalescent patient as primary antibody. (C) Serum from acute stage patient as primary antibody.

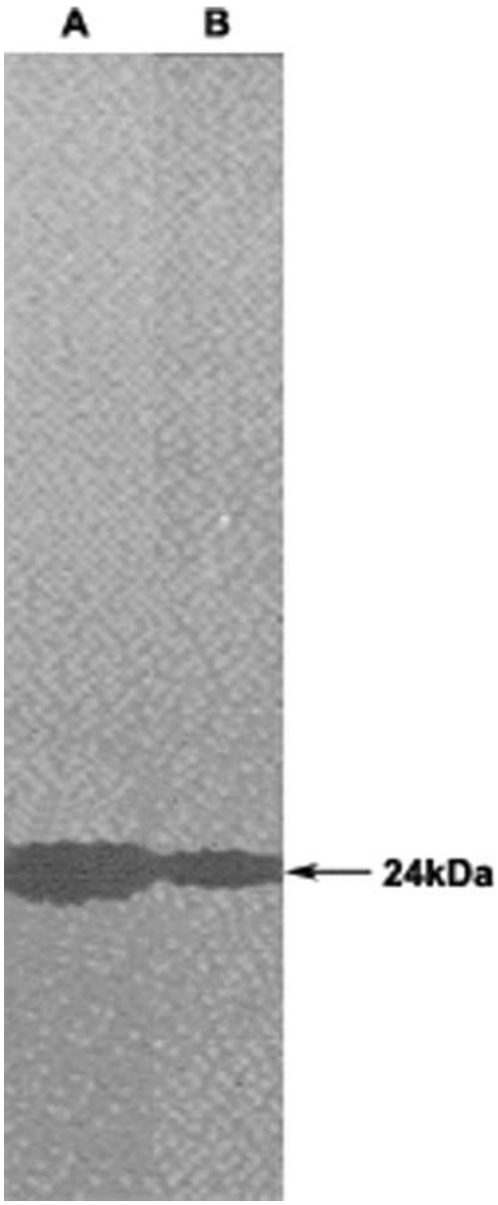

Fig. 3. SDS-PAGE of the purified 24 kDa Hsp from *V. cholerae 0139*. Lane A: Purified 24 kDa Hsp Lane B: molecular weight marker.

IFN-γ attained its peak during the first 24 h and gradually decreased following 72 h of stimulation. The T cell clone (NKG1) appeared to be of Th2 lineage as significant levels of IL-4 and IL-6 could be secreted in the culture supernatants, but no IL-2 or IFN-γ could be detected (Table 1).

It was observed that T cells (NKG1) could enhance the proliferative potential of syngenic B cells obtained from the PP of immunized mice. Since, the T cells were irradiated prior to setting up the co-culture experiments; the proliferation that occurred was solely associated with B cells.

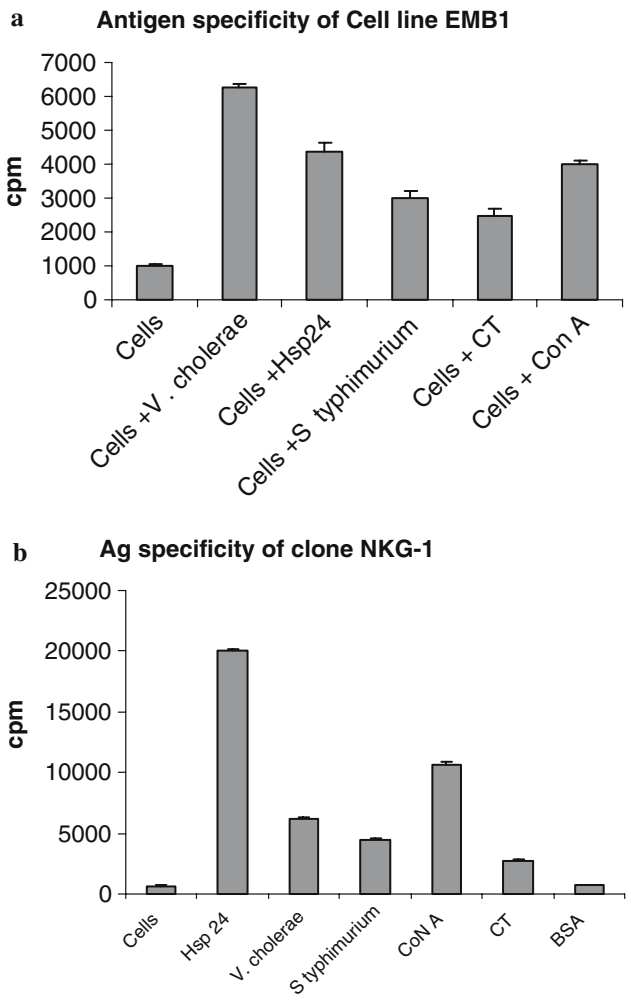

Fig. 4. (a) Antigen specificity of the T cell line (EMB1). Results are mean ± SD of 3 set of experiments. (b) Antigen specificity of the T cell clone (NKG1). Results are mean ± SD of 3 set of experiments.

Moreover, the degree of proliferation of B cells obtained from unimmunized mice was several folds less than that of the B cells from immunized mice (Fig. 7).

In order to assess whether the activated T cell clone could not only deliver a signal to trigger B-cell proliferation but also induce B-cell differentiation, the supernatant obtained from the co-culture of cloned T cells and B-cells in presence of the Hsp-24 was analyzed for their content of IgG and IgA. It was observed that the level of IgA increased on addition of RSS to the co-culture and also the level of IgG was higher than that of IgA. (Table 2).

Although it is now well established that the mucosal and the systemic immune system are anatomically and functionally distinct, there appears to be a bi-directional flow of messages and cells between the two compartments. To determine the relative involvement of each arm of the

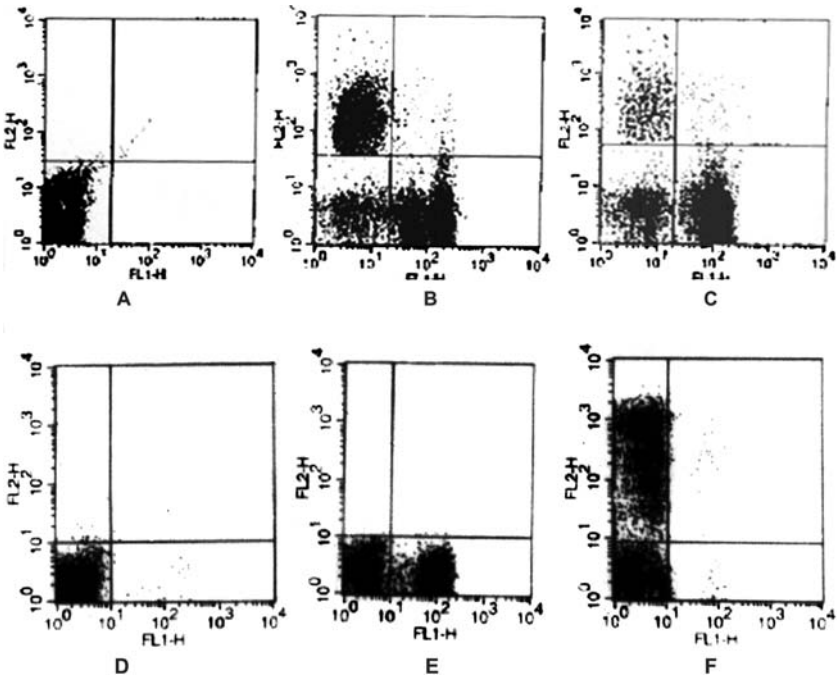

Fig. 5. (a) Phenotypic receptor expression of the T cell line (EMB1). A: Control cells. B: FL1-T cells incubated with FITC-labeled anti-CD8 antibody, FL2-T cells incubated with PE-labeled anti-CD4 antibody. C: FL1-T cells incubated with FITC-labeled anti-TCR$\alpha\beta$ antibody, FL2-T cells incubated with PE-labeled anti-TCR$\gamma\delta$ antibody. (b) Phenotypic receptor expression of the T cell line (EMB1). D: control cells. E: FL1-T cells incubated with FITC-labeled anti-CD5 antibody. F: FL2-T cells incubated with PE-labeled anti-CD45 antibody.

immune system a detailed characterization of the peripheral immune system was also carried out. It was found that the splenocytes obtained from mice orally immunized with live *V. cholerae 0139* proliferated maximally in presence of whole bacterial antigen (1×10^5 bacteria/ml) followed by the Hsp-24. Interestingly, the degree of proliferation observed with the splenocytes was much lower than that observed with mucosal T cell line or clone. Very little proliferation was noted with BSA. ConA served as a positive control in all these experiments (Fig. 8). Detailed analysis of the culture supernatant of splenocytes from immunized mice, stimulated with Hsp-24 revealed that both the Th1 as well as Th2 type of cells are involved in the ongoing immune responses since IL-2, IFN-γ as well as IL-6 and IL-4 were detected in culture supernatant (Table 1).

The alternative pathway of activation, which involves the CD2 ligand expressed on the cell surface, plays an important role in T cell activation. In order to determine the involvement of CD2 receptors in activation of mucosal T cells,the cells were incubated with Hsp-24 and the change in rosette forming ability was assessed. It was observed that the T cells (NKG1) prestimulated with Hsp-24, showed

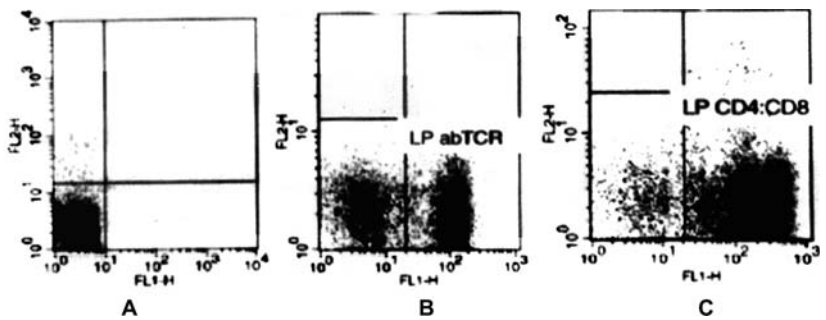

Fig. 6. Phenotypic analysis of the receptor expression in T cell clone (NKG1). (A) Control cells. (B) FL1-T cells incubated with FITC-labeled anti-TCR$\alpha\beta$ antibody, FL2-T cells incubated with PE-labeled anti-TCR$\gamma\delta$ antibody. (C) FL1-T cells incubated with FITC-labeled anti-CD4, FL2-T cells incubated with PE-labeled anti-CD8.

Table 1. Lymphokine Profile of The cells on stimulation with Hsp-24

Lymphokine	Cell type	Time 24 h	Time 48 h	Time 72 h
IL-2	T cell line	200 ± 1.02	375 ± 2.8	132 ± 3.3
	T cell clone	ND	ND	ND
	Splenocytes	120 ± 1.7	270 ± 2.5	88 ± 3.0
IFN-γ	T cell line	500 ± 2.2	485 ± 1.8	125 ± 1.1
	T cell clone	ND	ND	ND
	Splenocytes	68 ± 1.02	142 ± 2.3	45 ± 1.3
IL-4	T cell line	177 ± 4.1	285 ± 5.1	103 ± 1.3
	T cell clone	1250 ± 2.2	222 ± 3.1	750 ± 1.6
	Splenocytes	516 ± 2.2	769 ± 4.4	225 ± 2.7
IL-6	T cell line	ND	ND	ND
	T cell clone	1076 ± 5.5	1889 ± 4.6	666 ± 2.2
	Splenocytes	475 ± 7.6	694 ± 8.3	210 ± 5.7

Lymphokine levels expressed as pg/ml.
Data are represented as mean ± SD of triplicate experiments.
ND is not determined.

significantly higher number of rossettes (58.22 + 11.25%) as compared to unstimulated cells (38.32+ 8.51%). Similarly, the Hsp-24 stimulated splenocytes could form a higher number of rosettes (41.17 ± 4.92%) as compared to the unstimulated counterparts (32.25 ± 5.31%) although the number is less in comparison to the T cell clone.

Discussion

The *vibrios* get exposed to various stressful conditions during their transition to infective vegetative state in the human host and start synthesis of a battery of Hsps. These Hsps not only help the bacteria to overcome lethal temperature elevations by inducing thermotolerance but are also immunogenic in nature [12]. The present study was aimed to look at the the immunomodulatory role of an immunodominant Hsp on the gut immune responses through the generation of Hsp driven T cell clone and T cell line of mucosal origin in mice. It has been reported that mice are susceptible to *V. cholerae* 7–8 days after birth following, which they appear to become resistant to the bacteria [13]. It seems that the immature immune system in the young animals enables the bacteria to colonize the intestine and cause cholera [14]. Subjecting *V. cholerae 0139* to an incubation temperature of 42 °C, subsequently labelling with ^{35}S-Methionine and analysing the Hsps by SDS-PAGE followed by autoradiography showed that Hsps were more abundant on the membrane fraction of the bacteria and thus the membrane fraction was chosen for further immunological studies. In *E. coli*, it

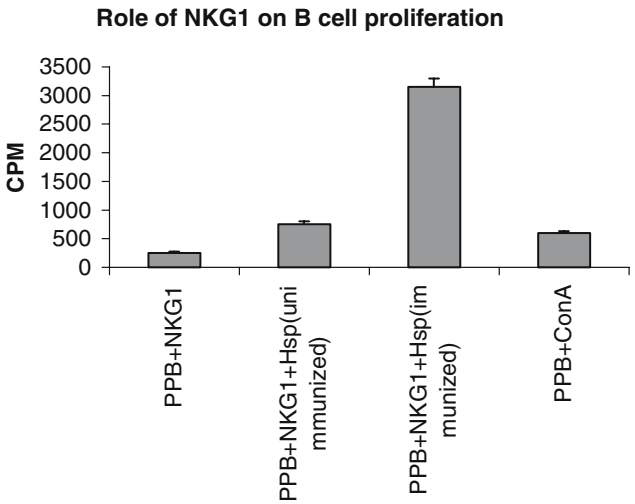

Fig. 7. Role of T cell clone (NKG1) on the proliferation of B cells. Results are mean ± SD of 3 set of experiments.

Table 2. Level of *in vitro* IgG/IgA generation during co-culture experiments

Antibody type	Days	Uninfected mice	Infected mice
IgA	5 (−RSS)	0.012 ± 0.01	0.272 ± 0.02*
	5 (+RSS)	0.016 ± 0.01	0.470 ± 0.01*
	7 (−RSS)	0.013 ± 0.03	0.317 ± 0.07*
	7 (+RSS)	0.018 ± 0.05	0.615 ± 0.21**
IgG	7 (−RSS)	0.020 ± 0.05	0.997 ± 0.01**
	7 (+RSS)	0.051 ± 0.03	1.150 ± 0.18**

Results are expressed as mean ± SD of OD values of triplicate wells from three sets of experiment. *$p < 0.05$, **$p < 0.01$ when compared with control.

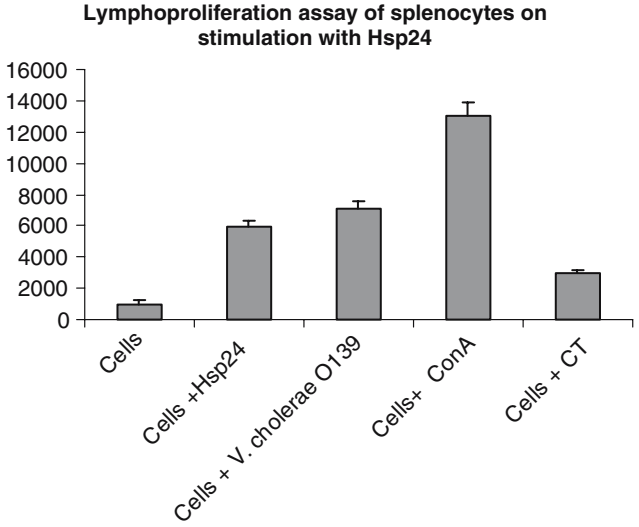

Fig. 8. Lymphoproliferation assay of the splenocytes on stimulation with *V. cholerae* Hsp. Results are mean ± SD of 3 set of experiments.

has been demonstrated that the synthesis of Hsps attains maximum level around 5 min after the temperature changes [15]. However, for the 0139 strain of *V. cholerae* used in the present study, the maximum expression of the Hsps occurred about 20 min after their transition to higher temperatures.

Several Hsps were found to interact with convalescent patient sera and among them the Hsp-24 seemed to be the most immunodominant as judged by the band intensity. Earlier work of Manning and Sahu [16] has shown the existence of a similar low molecular weight immunogenic band from *V. cholerae*. Thus, the presence of a highly immunogenic low molecular weight Hsp may play some role in modulation of enterobacterial infection. The presence of antibodies against this 24-kDa protein in the sera of patients at acute stage (day 1) of infection might be because of antigenic memory due to prior exposure to *V. cholerae* and subclinical infections due to antigenic cross reactivity. Since the band could not be detected when sera from neonatal individuals were used for immunoblotting the second alternative seems to be more attractive.

It has been observed that following infection with *V. cholerae*, the patients after recovery are conferred with a strong protective immunity against the disease [17]. This immunity is independent of the biotype and serotype of the infecting strain [18] suggesting that a common antigen may be expressed in all pathogenic strains of *V. cholerae*. Since neither the bacteria nor its toxin penetrates beyond the intestinal epithelium during infection, protective immunity against cholera is thought to depend on preventive mechanisms operating against colonization or toxin action in the gut lumen and this is mediated primarily by locally secreted IgA. Thus, the immunology of cholera has centred in and around the humoral arm of the immune system and very little work has been done to elucidate the role of different T cells representing the mucosal immune system against cholera. Abbas et al. [19] have reported that the generation of an effective long lasting antibody mediated memory response might depend upon the presence of antigen specific CD4+ T cells in circulation, which underscores the need for analysing the role of T cells in cholera. It has been observed that T cells comprising both the T cell clone and the T cell line proliferated maximally against the specific Hsp. The low level of proliferation observed against membrane protein of *S. typhimurium* might be due to the presence of crossreactive antigen(s).

Phenotypic analysis revealed the T cell clone to be TCR $\alpha\beta^+$ $CD4^+$. The CD4:CD8 ratio of LP derived T cells was found to be similar to that of the peripheral organs and virtually all of them could express TCR $\alpha\beta$ [20]. Qadri et al. [21] have reported that natural cholera infection or cholera toxin could induce a Th2-type of immune profile and stimulate the humoral immune response, innate cells and mediators in the host. Thus, these cells and factors of the innate arm may be important in the host's defence against cholera. A large proportion of the LP TCR $\alpha\beta^+$ $CD4^+$ T cells were found to secrete Th2 type cytokines *in situ* [22] and because of the enormous amounts of IgA produced in the intestine, it was often assumed that LP TCR $\alpha\beta^+$ $CD4^+$ T cells belonging to the Th2 lineage could regulate the differentiation of IgA producing B cells [23]. The present study is in agreement with this hypothesis since the lymphokine profile indicated the T cell clone (NKG1) to be of the Th2 lineage.

When the T cell clone was co-cultured with syngenic B cells from the PP (inductive site of mucosal immune response [24]), the B cells were activated to proliferate and synthesize immunoglobulins. The true nature of this T cell help was evident from the experiments where no induction of B cell function was observed in absence of these T cells. Moreover, these T cells were antigen specific in nature since the antigen non-primed cells could not be triggered to the same extent in the control experiments.

Secretory immunoglobulins, specifically IgA appears to play a major role in antibody mediated protection of the mucosal surfaces although the precise mechanisms involved in this type of protection has not been completely characterized [25]. In the present study it was also observed that the amount of IgG produced by PP B cells prevailed over that of IgA at all time points. However, when RSS was added to the culture conditions a significant increase in the secretion of IgA was found, but its level always remained several fold lower than that of IgG. Hence, it can be said that the secretion of IgG rather IgA in the gut might be regulated locally for which the presence of T cells, their signals and products might be required. This T cell help could bring about the maturation of the B cells and induce their class switching. Thus, the T cell clone in this study probably acted as isotype switch regulators. T cell line specific for Hsp-24 (EMB1), also proliferated with *S. typhimurium* membrane proteins but to a lesser extent and this could have resulted due to the conserved nature of the enterobacterial antigens. However, this cross reactivity could also be a consequence of the oligoclonal or pauciclonal nature of the iIEL [26].

Phenotypic analysis revealed the T cell line to comprise mainly of CD8+ cells expressing the $\alpha\beta$ receptors. T cells with $CD4^+$ phenotype expressing $\gamma\delta$ receptors were also present but at lower level. This appears quite intriguing because the general evidence suggested the stress induced protein antigens to be the target of the $\gamma\delta$+ T cells. However, it has also been observed that with age and antigenic exposure, a preferential recruitment of TCR $\alpha\beta$+ cells and a concomitant reduction in the proportion of $\gamma\delta$+ iIEL could occur [20]. Moreover, it is now well established that the $\alpha\beta$ and $\gamma\delta$ bearing T lymphocytes cannot be activated in

identical fashion and the nature of the antigen along with antigen recognition requirements greatly influence the ultimate outcome of immune orchestration [27].

From the present study, it seems that the Hsp may have immunodominant epitope(s), which might preferentially activate the $\alpha\beta$+ T cells. Analysis of the lymphokines secreted by T cell line demonstrated the presence of both helper and suppressor cells in them. The increased production of IFN-γ followed by IL-2 and IL-4 in Hsp specific cell line seems to result because of epitope hierarchy. However, one cannot exclude the possibility of this being unique with localized cellular gut immune responses, where iIELs while responding to a gut pathogen like *V. cholerae* or *V. cholerae* derived protein could exploit both arms of the acquired T cell responses. Enhanced production of IFN-γ and IL-2 suggested a more significant role of cell-mediated immunity at the epithelial surface while the other arm (humoral) arm being active at other anatomical locations like LP or PP.

Another interesting aspect that was observed in the present study was the lower level of proliferation in the iIEL T cell lines as compared to the T cell clone against Hsp-24. This reduced proliferative activity could reflect the possibility that iIEL might require Ag presentation and costimulatory activities or that iIEL might already be fully differentiated effector cells [20]. The latter idea appears to be true since in the present study, phenotypic analysis of the T cell line revealed the expression of the CD45RO isoform characteristic of memory/activated T cells.

It was also observed, that the clonal T cells underwent an increase in the expression of the CD2 receptors on stimulation with the Hsp-24. Previous studies have also emphasized the importance of the CD2 activation pathway in the induction of proliferation in lamina propria and intraepithelial T cells [28]. Targan et al. [29] have also shown the CD2 pathway in LPLs to be more potent inducer of IL2, IFN-γ, IL4 and TNFα secretion than CD3.

Following cholera infection, a significant amount of antibacterial as well as antitoxic antibodies could be detected in the serum of the infected individuals. In animal system, such antibodies could confer protection against experimental cholera [30]. Furthermore, it has been reported that antibodies, could act on mucosal surface after diffusing from the circulation [31]. All the evidences clearly indicate that there may exist a bi-directional transmigration of cells and messages between the systemic and mucosal arm of the immune response, which could serve as a basis to incorporate the possibility of any of peripheral immune responses against *V. cholerae* Hsp-24 in the present study.

Splenocytes from mice orally immunized with live *V. cholerae* when challenged *in vitro* with the Hsp-24 proliferated significantly. However, the extent of proliferation was much lower than that observed for the mucosal T lymphocytes. It is possible that the oral route, which is also the natural route of *V. cholerae* infection and the non invasive nature of the pathogen itself could be responsible for the ultimate low level responses at the peripheral or systemic level. The lymphokine profile of the splenocytes while responding to the Hsp-24 or *V. cholerae 0139* also could not identify the involvement of any particular cell population.

Another interesting observation obtained from the present study is the involvement of the alternative pathway of activation, which might have a more important role to play in modulating the cell mediated immunity in the distinct anatomical locations within the gut where the immune responses are tightly regulated. Hence, it can be concluded that the Hsp-24 of *V. cholerae 0139* is an important biomolecule, which can effectively immunomodulate the mucosal T cells, which in turn can provide help for B cell functioning at least at the level of LP. In the intestinal epithelium, the Hsp-24 can activate the effector arm of the T cell responses. Thus, the mucosal immune system appears to be highly compartmentalized with each anatomical location serving a particular function.

References

1. Albert MJ, Siddique AK, Islam MS, Faruque SM, Sack RB: Large outbreak of clinical cholera due to *Vibrio cholerae* non-01 in Bangladesh. Lancet 341: 704
2. Sack DA, Sack RB, Nair GB, Siddique AK: Cholera. The Lancet 363: 223–233, 2004
3. Chakraborty D, Ghosh A: Reversal of cyclic AMP of the urea induced inhibition of the synthesis of a catabolic repressible enzyme in *V. cholerae*. J Gen Microbiol 133: 3265–3270, 2004
4. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein dye binding. Anal Biochem 72: 248–254, 1976
5. Laemmli UK: Cleavage of ultrastructural protein during the assembly of the head of bacteriophage T4. Nature (London) 227: 680–685, 1970
6. Strahler JR, Kuick R, Hanash SM: Two dimensional polyacrylamide gel electrophoresis of proteins. In: TE Crighton (ed.) Protein Structure: A Practical Approach. Oxford: IRL Press, 1989, p. 65–67
7. Towbin H, Staachelin T, Gordon J: Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some application. Proc Natl Acad Sci USA 76: 4350–4354, 1979
8. Dass SV, Raja M, Verma RK, Mishra GC: Characterization of novel costimulatory molecules. J Biol Chem 270: 23429–23436, 1995
9. Waite JH, Wang CY: Spectrophotometric measurement of dodecyl sulfate with basic fuchsin. Anal Biochem 70: 279–280, 1976
10. Davies MDJ, Parrot DMV: Preparation and purification of lymphocytes from the epithelium and lamina propria of murine small intestine. Gut 22: 481–488, 1989
11. Wybran J, Fudenberg HH: Thymus-derived rosette forming cells. N Engl J Med 288: 1072–1073, 1973
12. Jeevanjyot Ghosh A: Induction of heat shock responses in *Vibrio cholerae*. Microbiology 141: 2101–2109, 1995
13. Neutra MR, Kraehenbuhl JP: Mucosal immunization via M cells for production of protective secretory IgA antibodies. Am J Trop Med 50: 10–13, 1994

14. Richardson SH: Host susceptibility. In: IK Wachsmuth, PA Blake and O Olsvik (eds.) Vibrio cholerae and Cholera: Molecular to Global Perspectives. Washington: ASM press, 1994, p. 273–289
15. Lemaux PG, Herendeen SL, Bloch PL, Neidhardt FC: Transient rates of synthesis of individual polypeptides in *E. coli* following temperature shifts. Cell 131: 427–434, 1978
16. Manning PA: Involvement of cell envelope components with pathogenesis of *Vibrio cholerae*; targets for cholera vaccine development. Vaccine 5: 83–87, 1987
17. Cash RA, Music SI, Libonati JP, Snyder MJ, Wenzel RP, Hornick RB: Response of man to infection with *V. cholerae*: clinical, serological and bacteriologic responses to known inoculum. J Infect Dis 130: 325–333, 1974
18. Sciortino CV: Human immune response to the 18 KDa outer membrane antigen of *Vibrio cholerae*. J Med Microbiol 39: 135–140, 1993
19. Abbas AK, Murphy KM, Sher A: Functional diversity of helper T lymphocytes. Nature (London) 383: 787–793, 1996
20. Mowat AMI, Viney JL: The anatomical and cellular basis of immunity and tolerance in intestine. J Pediatr Gastroenterol Nutr 39: 5723–5724, 2004
21. Qadri F, Bhuiyan TR, Dutta KK, Raqib R, Alam MS, Alam NH, Svennerholm AM, Mathan MM: Acute dehydrating disease caused by *Vibrio cholerae* serogroups O1 and O139 induce increases in innate cells and inflammatory mediators at the mucosal surface of the gut. Gut 53: 62–69, 2004
22. Abreu-Martin MT, Torgon SR: Regulation of immune responses of the intestinal mucosa. Crit Rev Immunol 16: 277–309, 1996
23. Bailey M, Hall L, Bland PW, Stokes CR: Production of cytokines by lymphocytes from spleen, mesenteric lymph nodes and intestinal lamina propria of pigs. Immunology 82: 577–583, 1994
24. Staats HF, Jackson RJ, Marinaro M, Takahashi I, Kiyono H, McGhee JR: Mucosal immunity to infection with implication for vaccine development. Curr Opin Immunol 6: 572–583, 1994
25. Quiding M, Nordstrom I, Kilander A, Anderson G, Hanson LA, Holmgren J, Czerkinsky C: Intestinal immune responses in humans. Oral cholera vaccination induces strong intestinal antibody responses,gamma interferon productions and evokes local immunological memory. J Clin Invest 88: 143–148, 1991
26. Regnault A, Cumano A, Vassali P, Guy Grand D, Kourilsky P: Oligoclonal repertoire of the CD8 $\alpha\alpha$ and the CD8 $\alpha\beta$ TCR $\alpha\beta$ murine intestinal intraepithelial T lymphocytes: evidence for random emergence of T cells. J Exp Med 180: 1345–1358, 1994
27. Sim GK: Intraepithelial lymphocytes and the immune system. Adv Immunol 58: 297–343, 1995
28. Pirzer UC, Schurman G, Post S, Betzler M, Muer SC: Differential responsiveness to CD3 Ti Vs CD2 dependent activation of human intestinal T lymphocytes. Eur J Immunol 20: 2339–2342, 1990
29. Targan S, Deam RL, Liu M, Wang S, Nel A: Defination of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated the CD2 pathway. J Immunol 154: 664–675, 1995
30. Svernnerholm AM, Jonson G, Holmgren J: Immunity to *Vibrio cholerae* infection. In: IK Wachsmuth, PA Blake and O Olsvik (eds.) Vibrio cholerae and Cholera. Molecular to Global Perspectives. Washington: ASM press, 1994, p. 257–271
31. Holmgren J: Mucosal immunity and vaccination. FEMS Microbiol Immunol 4: 1–9, 1991