Infectious diseases, particularly microbial diseases continue to be leading cause of morbidity and mortality worldwide. Epidemiological studies indicate that their incidence will increase as the world’s population continues to grow. The emergence of previously unknown pathogens and antibiotic resistant strains pose a major threat to the mankind. New vaccines and treatment regimens are thus desperately needed, which requires a better understanding of how pathogens can promote a disease.

The knowledge in the field of microbial pathogenesis; the study of molecular basis of microbial diseases, has increased to a greater extent in recent years. Cellular microbiology facilitates to understand the molecular interactions between bacterial factors and cellular components or signaling pathways in vitro (Cossart et al., 1996). This study opens up the field of microbial pathogenesis to understand the successive interactions at different stages during the infectious process, including microbial adherence to host cells, bacterial survival, molecular cross talk between host and pathogen, cell intoxication and death caused by bacterial products. Pathogens have evolved a variety of strategies to hijack the host cell signalling systems, membrane trafficking events as well as the cytoskeletal machinery, and the discovery of these strategies has contributed significantly to advances in cell biology. The elucidation of the molecules and mechanisms underlying bacterial pathogenesis in humans yields practical applications ranging from refined diagnostics to new
antibiotics and improved vaccines. In addition to the pursuit of these practical purposes, recent research on bacterial pathogenesis has allowed insight into the complex beauty of highly adapted interactions between pathogens and their hosts at the cellular and molecular levels and also has given rise to new model systems for probing host cell function (Neibuhr and Dramsi 1999).

New techniques have been developed that allow most bacterial pathogens to be studied at molecular and cellular levels. The major progress in the field of cell biology like the development of in vitro system, the ongoing development of fluorescence, confocal, video, and electron microscopy has contributed to such studies to a greater extent. Importantly, the availability of suitable animal models has allowed the assessment of the contribution of bacterial pathogenicity factors to be analysed in vitro to the ultimate outcome of disease. The use of in vitro coculture systems has also provided considerable information on the effects of neutrophils, eosinophils, monocytes, and lymphocytes on epithelial functions. Epithelial cells acts as a sentinel to summon inflammatory cells when it is invaded by pathogens. Studies over the past several years have clearly indicated the integral role that epithelial cells at mucosal surfaces play in generating and transmitting signals between both invasive and noninvasive microbial pathogens, and adjacent and underlying cells in the mucosa. This led to the concept of epithelial cells as an integral component of a communications network that involves interactions between epithelial cells, luminal microbes, and host immune and inflammatory cells (Kagnoff and Eckmann. 1997).

Definition of the signal transduction and regulatory mechanisms that govern interactions between epithelial cells, mucosal microbes, and inflammatory and immune cells through tissue culture studies may lead to new
therapeutic approaches for manipulating and regulating inflammatory and immune responses at the mucosal surfaces of the gastrointestinal tract, respiratory tract, and genitourinary tract. Moreover the relative ease of genetic and biochemical analysis of bacteria and widely used tissue culture models of bacterial infection have dramatically increased our knowledge about the molecular components involved in host-pathogen interactions. The expression of most known virulence factors is regulated by environmental conditions in vitro that presumably reflect similar cues present in host tissues. Also understanding this complex interplay between pathogen and host will help us determine the biological foundation of pathogenicity and the differences between pathogens and other bacteria.

4.1 DIFFERENCES IN THE DEGREE OF ADHERENCE AMONG CLINICAL ISOLATES OF H. PYLORI

The attachment of microorganisms to host cells is considered to be the initial event in the infectious process. Adhesion of pathogenic bacteria to target cells is important step in the pathogenesis of many bacterial diseases (Beachey, 1981). The first step in an infectious disease, requires specialised protein factors synthesised by the bacteria which allow its binding to the host cells. Adhesion to mucosal surfaces is important for the full virulence of many bacterial pathogens in the human host. Bacterial binding promotes the delivery of bacterial toxins (Zafiri et al., 1987) and initiates the penetration of target host cells in case of invasive pathogens (Finlay and Falkow, 1989). It has been well established that several bacteria, viruses and bacterial toxins recognise lipid-linked oligosaccharides present in the plasma membrane of the host cells. Such factors determine the severity of the disease and thus the outcome of the host-parasite interaction. Enteropathogenic E. coli attaches to intestinal
epithelial cells and causes destruction of the microvillus surface of the cells. Other organisms such as *Salmonella*, *Shigella* and *Yersinia* spp. attaches to the intestinal epithelial cells and subsequently become highly invasive.

Adherence to the gastric mucosa is an important part of the virulence mechanism in the case of *H. pylori* also. Ultrastructural analysis of gastric mucosa infected with *H. pylori* shows that this bacterium causes effacement of normal gastric epithelial microvilli and closely adheres to the apical cell membrane on cellular projections called adherence pedestals. Cell culture has been used to examine the invasiveness of many bacteria, including *E. coli* (Knutton, 1984), *Salmonella* (Mintz, 1983), and *Yersinia* spp (Lassen and Kapperud, 1986). Bacterial infection of cell culture is a useful tool since a uniform population of cells can be infected under defined conditions. Binding of *H. pylori* to HEp-2 and INT 407 epithelial cells in tissue culture is already reported (Simha et al., 1988). Adherence assay *in vitro* could be used to identify the adhesions expressed by *H. pylori* and could serve as a useful model to determine the mechanism of bacterial adherence to gastric epithelial cells.

In the present study the adherence pattern of the various clinical strains of *H. pylori* was compared to the standard strain 26695, which is *cag*+/*vacA*+. In this study Human epithelial cell line HEp-2, originally derived from a carcinoma of the human larynx was used as model system for characterising adherence pattern of these isolates. HEp-2 cells imitate the epithelial lining of the gut and therefore present an appropriate and useful model to characterise the clinical strains as virulent or non-virulent. Sixteen clinical isolates (8 duodenal ulcer, 5 Non-ulcer dyspepsia, 2 gastric ulcers and 1 gastric cancer) and 1 reference strain were studied with regard to their extent of adherence to host cell to characterise as virulent and nonvirulent. Microbial
adherence was assessed by microscopical evaluation of Giemsa-stained preparations. There was not much significant correlation between adherence pattern and the disease status of the isolates. Four isolates from different disease conditions did not exhibit any adherence at all, but formed clusters, which were found as nonspecifically binding to the HEp-2 cells. In the case of strains exhibiting good adherence, the bacteria attached to the HEp-2 cells with the greatest binding affinity in a diffuse manner (Fig. 3.1). Among the isolates from gastric ulcer, one did not show any binding while the other exhibited lesser extent of adherence (Fig. 3.1). No adherence was seen in two isolates from duodenal ulcer and one from non-ulcer dyspepsia. These adherence patterns did show a marginal correlation with the vacuolating activity of the isolates.

4.2 DIFFERENCES IN THE VACUOLATING ACTIVITY OF CLINICAL ISOLATES REFLECTS THEIR NATURE OF VIRULENCE

One important virulence factor of *H. pylori* is the vacuolating cytotoxin (*VacA*), which is a secreted toxin that induces eukaryotic cell vacuolation *in vitro* and epithelial erosion when administered orally in mice (Cover, 1990) (Cover et al., 1992; Marchetti et al., 1995). Although most clinical isolates of *H. pylori* have a functionally expressed *vacA* gene only approximately 50% of clinical isolates of *H. pylori* produce detectable cytotoxic activity. Toxicity has been associated with mosaicism in *vacA* genes in toxic and nontoxic isolates (Cover et al., 1994). Three different signal peptide sequences (s1a, s1b, and s2) and two variants of the midregion have been described (Atheron et al., 1995). Isolates with the s1-m1 forms are toxic, whereas the s2-m2 forms are essentially nontoxic or less toxic. The s1-m1
vacA alleles encode products with high level of cytotoxicity. One study on the vacuolating cytotoxin shows that type ml alleles were associated with more epithelial damage (Atherton, 1997). The vacuole caused by this cytotoxin is acidic and accumulate membrane-permeative weak bases such as neutral red, which provides a rapid and quantitative assay of the extent of vacuolisation (Catrenich et al., 1992; Cover et al., 1991).

In this study 16 isolates were collected and analysed for the vacuolating cytotoxic activity to classify as type I and type II strains. Out of these sixteen isolates four strains showed less apparent neutral red uptake in the vacuolation assay (Fig. 3.2). These four isolates were genotyped as type II by PCR and identified as s2 genotype. Between these, 2 isolates were negative for cag gene when analysed by PCR for the hydrophilic region of the cag gene. Although 10 of 16 strains (66%) could be classified as type I, 5 isolates showed intermediate phenotypes. One group (2 isolates) which have the gene coding for cagA, produce cagA as shown by western blot, which do not show very high vacuolating activity. The b group (3 isolates) includes strains, which have the cag gene but did not induce any significant increase in the neutral red uptake. The level of neutral red uptake treated with the culture supernatant of the type I strains was comparable to that induce by toxigenic reference strain 26695 (Fig. 3.2). Attempts to correlate clinical disease with infection with type I bacteria or type II bacteria did not show any strong correlation. The strains that showed markedly reduced cytotoxic activity can be correlated with their adherence pattern. The adherence pattern of these weakly vacuolating strains was lesser compared to that of the toxigenic reference strain.
4.3 OUTER MEMBRANE PROTEINS - MEDIATED CYTOTOXICITY OF *H. PYLORI* ON HEP-2 CELLS

Colonisation by *H. pylori* involves an interaction between the outer membrane of the bacterium and the gastric epithelium of the host. The outer membrane and composition of *H. pylori* is unique in its protein content and lipopolysaccharide structure, which are consistent with the persistence of *H. pylori* in a restricted niche. Compared to other bacteria, *H. pylori* devote a significantly higher percentage of its coding capacity to that of outer membrane proteins, further emphasising the importance of these proteins. A number of genes involved in determining the composition of outer membrane is differentially regulated by slipped-strand repair (Alm et al., 1999). This differential regulation and strain-specific outer membrane–related genes might play a role in the severity of *H. pylori*-related disease and the ability of the *H. pylori* to persist chronically in its host. Three adhesins (AlpA, AlpB and BabA) described in *H. pylori* belong to the large OMP family (Boren et al., 1993; Odenbreit et al., 1999). From this it appears that *H. pylori* use OMP and several other adherence mechanisms for successful attachment to epithelial cells. These OMP are merely characterised as adhesins, but their exact role in virulence mechanism has not been clearly elucidated. To answer this question the membrane fractions from *H. pylori* toxigenic strain was extracted and analysed for its cytopathic effect.

4.3.1 Dose response study and Time course analysis of the cytotoxicity

Gastric mucosal defence can be defined in terms of the ability to resist injury and to repair the damage by establishing epithelial continuity. Several studies hypothesise that *H. pylori* in addition to causing gastric mucosal damage
may also impair the process of mucosal healing. Significant inhibition of cell proliferation has been demonstrated by broth culture filtrates of Vac\textsuperscript{+}CagA\textsuperscript{+}. Such results indicate that VacA specifically inhibited cell proliferation whereas cagA exerted no similar effect (Ricci et al., 1996). In this study, outer membrane protein preparations exhibit the proliferation inhibiting property, which is demonstrated by $[^{3}\text{H}]$ incorporation assay.

Among the various fractions checked for the cytotoxic effect, OMP induced a significant cytotoxic effect in HEp-2 cells in a cell free system as seen by thymidine incorporation assay. The other fractions such as cytosolic proteins and inner membrane exhibited cytotoxic effect at very high protein concentrations. This study observed differences in the cytotoxicity levels between various clinical strains, which differ in their degree of adherence and compared with the toxigenic strain 26695. The highly adherent strains do exhibit a very high cytotoxicity even at lower concentrations of OMP. The dose response study over a period of exposure of 2 and 4 hours indicated the reduction in the amount of tritiated thymidine incorporated in treated HEp-2 cells occurred only between 40-50 $\mu$g of the protein indicating its minimum lethal dose. The non-adherent strain and the less-adhering strain showed a less thymidine uptake, indicating its dosage to be little higher than 50 $\mu$g to induce significant cytotoxicity (Fig. 3.3B). This may be due to a reason that adherent bacteria are able to promote bacterial toxins more effectively than free-floating microbes. The controls included in this analysis of cytotoxic effect like untreated HEp-2 cells and TE (Tris-EDTA) treated HEp-2 cells (Solubilising buffer for OMP) did not result in any cytotoxic effect. This OMP-mediated cytotoxicity could be analogous to the direct cytopathic effects by membrane bound protein of \textit{Naegleria fowleri} (Young et al., 1989) and outer membrane proteins of \textit{Salmonella} (Weinburg et al., 1983) and Enteropathogenic \textit{E. coli} (kumar et al., 1998).
A time course study was done to measure the amount of \([^{3}H]\)-thymidine incorporated in HEp-2 cells treated with OMP from the virulent and less virulent strains over specific time intervals. The kinetics of the OMP-mediated cytotoxicity shows that, at the end of four hours maximum cytotoxicity was observed with 50 \(\mu\)g of the OMP preparation from different clinical isolates. The time course analysis for the cytotoxic effect of the OMP was carried out till 8 hours, which showed that after 4 hours the HEp-2 cells are completely degenerated and begins to float from the tissue culture plates. The OMP of these strains exerted its effect from 2 hours and this was clear from the amount of \([^{3}H]\)-thymidine incorporated in HEp-2 cells exposed to OMP at a concentration of 100 \(\mu\)g (Fig. 3.3C). These results indicate the proteins of the outer membrane preparation do mediate cytotoxic effect, which could also be an important phenomenon in the disease process. The mechanisms by which OMP affects cell proliferation are unclear. Alterations in the expression of the epidermal growth factors and transforming growth factor, which play a main role in the maintenance of epithelial integrity, may also contribute such inhibition of proliferation. Also cell proliferation inhibition can be due to activation of genes that negatively regulate cell cycle or induce apoptosis in response to DNA damage.

4.4 MORPHOLOGICAL CHANGES INDUCED BY OMP-MEDIATED CYTOPATHIC EFFECTS VARY BETWEEN CLINICAL STRAINS

The cytopathic effects during *H. pylori* infection have been attributed to vacuolating cytotoxin (Leunk et al., 1988), cell lysates (Hupertz et al., 1988), Urease activity (Barer et al., 1988; Smoot et al., 1990). This study observed the cytopathic effects mediated by the outer membrane proteins of *H. pylori*. There are increasing evidences of outer membrane
proteins playing a role in cytopathic effects, forming the basis for the establishment of the disease (Fenno et al., 1998). Outer membrane preparations from the various clinical strains and the toxigenic reference strain 26695 were exposed to HEp-2 cells in a cell free system for the indicated periods of time. The whole cell sonicate, inner membrane treated and untreated HEp-2 cells were taken as control as the Inner membrane proteins did not induce any morphological changes in the host cells. Extensive morphological changes were observed in HEp-2 cells treated with OMP from toxigenic strains similar to that of the reference strain. The HEp-2 cells lose their spindle shape and degenerated cells begin to float. The cytopathic changes observed in HEp-2 cells induced by the OMP of these strains were similar to that of the induction of apoptosis. The less virulent strains as characterised by adherence and vacuolation assays, showed different cytopathic effect with the HEp-2 cells losing their cell-to-cell adhesion retaining their intact spindle shape. After the loss of cell-cell adhesion the cells increase in size. The viability and changes in the morphology of the cytopathic effects as observed by trypan blue exclusion assay showed that the cells become non-viable at the end of four hours (Fig. 3.4). The cytopathic effects mediated by the outer membrane protein preparations do signal as an important indicator of infection. Though these morphological responses to the OMP treatment are preliminary, such studies do have a role in understanding the disease progression. Cytopathic effects on eukaryotic cells due to translocation of bacterial porin-like molecules to the cell membrane have been reported for *Neisseria gonorrhoeae* (Haines et al., 1991), *Salmonella typhimurium* (Galdiero et al., 1993), *Porphyromonas gingivalis* (Novak et al., 1991), and *Eikenella corrodens* (Tufano et al., 1986).
4.5 OUTER MEMBRANE PROTEIN AS A CANDIDATE ANTIGEN IN \textit{H. pylori} DIAGNOSIS

The diagnosis of \textit{H. pylori} infection can be conducted by using direct (invasive) or indirect (noninvasive) methods (Megraud1996). Among the noninvasive methods serology is a valuable tool for seroepidemiological studies (van de Wouw et al., 1996) or for post treatment follow up studies (Wang et al., 1994). These serological assays, which are, basically enzyme immunoassays are hampered by cross-reactions with a variety of antigenic preparations. Therefore it would be of interest to understand the antigens of \textit{H. pylori} to make an ideal preparation for the serodiagnosis of \textit{H. pylori} infection. In this work the frequencies of the antibodies to various antigenic preparations was studied in the sera of 20 patients' clinically and histologically documented. This study determines the antibodies, which are the best markers of colonisation and the antibody patterns, associated with any particular disease. The whole cell sonicate from the standard strain 26695 was used as a antigenic preparation against a set of patients' sera (culture positive and urease positive). There was an extensive polymorphism in the serum antibody response against the sonicate. Among the immunoreactive bands, antibodies to \textit{cagA} and \textit{VacA}, Hsp proteins, urease and other low molecular weight proteins were observed. The reference strain chosen in this study, as the source of antigen appears to be representative of the strains isolated most frequently from these patients from Chennai. This polymorphism of the antibody response could be attributed to the host immune response or antigenic difference of the infecting strain. Also proteins \textit{cagA}, \textit{vacA}, 66, 54, 43, 35 and 29 kDa elicited antibodies more frequently (Fig. 3.5A). The presence of \textit{cagA}, \textit{vacA} and 29-kDa proteins showed immunoreactive bands in the case of duodenal ulcers and nonulcer dyspepsia. The reason for this frequency may be because vacuolating cytotoxin has been suspected to be
involved in the phenomenon of ulcerous lesions of the mucosa (Ricci et al., 1996). Despite this varied antibody response the whole cell sonicate possess the advantage of exposing a maximum number of antigens; but there is also a risk of non-specific binding of immunoglobulins. There are reports of false positive results in case of ELISA’s with the whole cell antigen (Schaber et al., 1989), but the partially purified antigens such as Acid-glycine extract offer a better specificity compared to the whole cell sonicate. There was a strong antibody response to the high molecular proteins such as \textit{cagA} and \textit{vacA} in the partially purified acid-glycine extract. Of the 10 culture positive patients’ sera, 9 showed strong immune response to 29 kDa and between 66 and 43 kDa proteins (Fig. 3.5A). The immunoreactivity with the 29-kDa antigen has already been reported by Andersen et al., (1995). There was no cross reactivity with the healthy control sera. There was a good relation between positive culture results and immunoblot positive sera in both cases of whole cell sonicate and partially purified acid-glycine extract antigen.

In this study, an immunoblot assay has been designed in which a crude \textit{H. pylori} outer membrane protein preparation is used as an antigen. Among the major proteins considered as vaccine candidates against \textit{H. pylori} are urease, heat shock protein (Ferrero et al., 1995) and purified \textit{vacA} cytotoxin (Marchetti et al., 1995). All these proteins have a common factor of being associated with the outer membrane of \textit{H. pylori} (Dunn et al., 1990; Phadnis et al., 1996; Keenan et al., 2000). Outer membrane as a continuous structure on the gram-negative bacterial pathogens has particular significance as a potential target for protective immunity. Outer membrane vaccines have been successfully used against a number of organisms, including \textit{Neisseria meningitides} (Bjune et al., 1991), \textit{Pasteurella multocida} (Lu et al., 1991), \textit{Porphyromonas gingivalis} (Kesavalu et al., 1992), and \textit{Moraxella cattarrhalis}.
(Maciver et al., 1993). Crude outer membrane preparation extracted by sarkosyl insoluble method from the reference strain 26695 was used in this study against a set of culture positive and urease positive patients' sera. Immunoblotting with the crude OMP preparation demonstrated antibody response against the urease subunit B (UreB), UreA, heat shock proteins, and also a high molecular weight protein corresponding to cagA. Apart from this known proteins/antigens a 29 kDa and 18 kDa immunoreactive bands was also observed as a strong antibody response in the tested sera (Fig. 3.5B). Earlier reports have indicated the 29 kDa as a major antigen capable of inducing strong antibody response (Nishizono et al., 1998). Immunoblotting demonstrated specific serum IgG immunoreactivity to an OMP component with an apparent molecular mass of 18 kDa. These immunoreactive species-specific 18-kDa \textit{H. pylori} OMP was described in an earlier study as a lipoprotein (Drouet et al., 1991; Kostrzynska et al., 1994). The results with the OMP as an antigen in the serodiagnosis by immunoblot assay showed significant sensitivity and specificity. The major outcome of this immunoblot assay is the strong antibody response to 29 and 18kDa protein, which can be included as a novel antigen to make an ideal preparation of antigen in the immunoblot assay. It is important to identify the \textit{H. pylori} proteins, which elicit a strong immune response in humans in order toanalyse their capability to confer protective immunity. This property can be attributed to the OMP preparation, which will contribute to the improvement of serological tests for detecting and monitoring \textit{H. pylori} infections.

4.6 OUTER MEMBRANE PROTEINS INDUCES ELEVATED CYTOKINE EXPRESSION IN HEP-2 CELLS

Cytokines play a critical role in the host defence systems and also involved in the pathogenesis and development of symptoms in infection.
Invasion of the host cell by the bacteria or uptake of bacterial products into the cell may be required to induce a cytokine response (Eckmann et al., 1993). A number of in vitro and in vivo model systems have been used to characterise host epithelial cell responses to mucosal infections, with bacterial and protozoan pathogens that range from highly invasive to minimally invasive to noninvasive, as well as to colonisation with commensal bacteria. Bacterial adherence has been shown to enhance the cytokine expression in the case of uropathogenic Escherichia coli (Svensson et al., 1994). Infections with pathogens like Salmonella, Shigella, Yersinia and Listeria results in the increased production of proinflammatory cytokines (Eckmann et al., 1993; Jung et al., 1995).

H. pylori-associated gastroduodenal diseases are characterised by the severe infiltration of neutrophils, lymphocytes, monocytes and plasma cells in the gastric mucosa. Cytokines are proposed to play an important role in the pathogenesis of H. pylori-associated gastroduodenal diseases. Several potential virulence factors derived from H. pylori are considered to stimulate the cytokine induction in the gastric mucosa, thereby attracting and activating neutrophils and mononuclear cells (Huang et al., 1995; Reider et al., 1997). The gastric mucosal levels of the proinflammatory cytokines IL-1, IL-6, IL-8 and TNF- have been reported to be increased in H. pylori infected subjects (Moss et al., 1994; Crabtree et al., 1991; Ando et al., 1998; Noach et al., 1994). In this investigation the cytokine response of the HEP-2 cells infected with clinical samples of H. pylori was studied by RT-PCR. The time period of the infection was estimated to be four hours as maximum adherence of the bacteria to the host cells was seen. The cytokine expression was shown to be elevated by 4 hours for both IL-6 and IL-8. Four strains of H. pylori that are altered in their adhesive phenotypes were employed to examine the possible role of cell-cell
contact in the stimulation of IL-6 and IL-8. Clinical isolates, which showed lesser degree of adherence or no adherence, were both stimulatory for IL-6 and IL-8 production (Fig. 3.6A). Increased secretion of proinflammatory cytokines by human intestinal epithelial cells follow a parallel but delayed course with maximal secretion usually seen within 4–6 h after stimulation with return to baseline by 12 hour (Yang et al., 1997). Thus, proinflammatory signals provided by intestinal epithelial cells are rapidly upregulated but transient in nature, suggesting an important role in signalling the onset of the inflammatory response in the early period after infection.

In addition to examining the role of adhesion with live bacteria in stimulation of cytokine response, the effects of OMP on the induction of cytokines in HEp-2 cells was analysed. The cytotoxicity of the crude preparation of OMP on HEp-2 cells was clear by the trypan blue exclusion assay and therefore analysed for its role in cytokine induction. The kinetics of the stimulation of IL-6 and IL-8 in HEp-2 cells showed expression of IL-6 and IL-8 at half an hour, which reached a peak at 1 hour (Fig.3.6B) and returned to baseline and the end of 4 hours no expression was observed indicating its transient signaling triggering the host inflammatory response. The isolates showing differences in the cytopathic effect as such did not demonstrate any significant changes in the levels of IL-6 expression. In the case of IL-8 marginal levels of increase in the case of isolates that showed higher degree of adherence was observed. This phenomenon may be attributed to the highly adherent nature of those strains, because toxins or other active molecules are presented to host cells more efficiently by the attached microbes than by the free-floating microbes. On considering the genotype of these strains, all these strains were \textit{cag}^+ by PCR for the hydrophilic region of the \textit{cag} gene. Earlier studies on IL-8 expression indicate its production by gastric epithelial cells is greatly associated
with cagA+ strains (Crabtree et al., 1994). From this study, it is clear that outer membrane protein preparation may be an important virulence factor in relation to the risk of clinically significant outcomes of H. pylori infection.

4.7 NF-κB ACTIVATION BY OUTER MEMBRANE PROTEINS OF H. PYLORI

NF-κB, an important transcription factor plays an integral role in regulating the human immune response. NF-κB is activated upon by stimulation by a large variety of pathogenic agents (Baeuerle and Henkel 1994). Activation occurs via phosphorylation, ubiquitinilation, and proteolytic degradation of IκB, the inhibitory subunit (Brown et al., 1995; Henkel et al., 1993; Sun et al., 1994). The activated NF-κB after translocation to the nucleus activates the transcription of genes including those encoding IL-1, IL-6, IL-8 and TNF. NF-κB activity has also been reported by Shigella flexneri in HeLa cells (Dyer et al., 1993), and by Staphylococcus aureus (Busam et al., 1992) and Listeria monocytogenes (Hauf et al., 1994) in macrophages. Exposure of gastric epithelial cell lines to H. pylori potentially activates NF-κB through a gene located within the pathogenicity island cagE (Munzenmaier et al., 1998). Knockouts of the certain PAI genes (CagA, CagF and CagN), which are not necessary for the functional integrity of the type IV secretion apparatus, suppress or reduce the activation of NF-κB. (Glocker et al., 1998). The signaling pathways for the activation of NF-κB has also been elucidated indicating the role of p21-activated kinase (PAK1) participating in a unique pathway that connects H. pylori-dependent effector molecules to the activation of NF-κB and the induction of innate immune response (Ludwig et al., 2000).
In the present study, the OMP preparation was considered and analysed for its role in NF-κB activation. OMP preparation was shown to induce IL-8 expression when treated on HEp-2 cells in a cell-free system. Therefore HEp-2 cells were treated with OMP at different time periods to reveal the kinetics of NF-κB activation. The OMP treated HEp-2 cell extracts were assayed for NF-κB DNA binding by an electrophoretic mobility shift assay (EMSA). Also the OMP from different clinical strains from different disease conditions were taken for this study. Maximum activation of NF-κB induced by OMPs was observed at 1-hour post treatment and was not detectable further (Fig.3.7AandB). There were no significant differences observed between activation induced by different OMPs. Earlier reports on the activation of NF-κB by toxigenic strain and mutant with no expression of type IV secretion machinery indicate the maximum activation between 90 and 180 minutes post infection (Ludwig et al., 2000). In this study OMP in a cell free system has shown to be cytotoxic to HEp-2 cells within 4 hours in a time course study, therefore the signaling pathways towards the activation of NF-κB may be initiated within 30 minutes after treatment. Also the time kinetics of the IL-8 expression shows the mRNA transcripts of IL-8 at 30 min. This indicates that OMP treatment at earlier time point below 30 min can induce NF-κB activation. The proteins encoded in the cag pathogenicity island may form a multimeric structure on the H. pylori surface, which is capable of eliciting a signal transduction cascade which leads to activation of transcription factor NF-κB.

4.8 CYTOSKELETAL REARRANGEMENTS INDUCED BY H. PYLORI OMP'S

The host cell cytoskeleton is known to play a vital role as target in the pathogenic mechanisms of several microorganisms by providing the basis
for a successful infection. Transmission electron microscopic studies show actin polymerisation in primary human gastric epithelial cells and AGS cells. The actin filament accumulation though similar to the EPEC association was less dense (Smoot et al., 1993). Cell proliferation and migration, both essential for mucosal healing are dependent on the cell cytoskeleton. \textit{VacA}-induced cell vacuolation strongly correlated with inhibition of wound re-epithelialisation by altering cytoskeleton-dependent cell functions and signalling (Pai et al., 2000). The actin polymerisation is followed by attachment of the bacteria to the host cells. The predominant role of the bacterial adherence is through the bacterial surface components, which also may interact with other virulence factors towards actin rearrangement. Therefore the outer membrane preparation was exposed to the HEp-2 cells and cytoskeletal rearrangements were observed by fluorescence microscopy after staining the cell actin with fluorescein-conjugated phalloidin. The actin accumulation was seen in the HEp-2 cells as bright spots with intense fluorescence (Fig. 3.8A). This occurred essentially when the strains were cytotoxin producers and with higher degree of adherence. The cytoskeletal rearrangement mediated by the outer membrane proteins has also been reported in other pathogenic organisms (Uitto et al., 1995). There are several reports on the modifications of the cytoskeleton induced by the bacterial factors such as cytotoxic necrotising factor (CNF) of \textit{E. coli} (Oswald et al., 1994), the dermonecrotizing toxin of \textit{Bordetella bronchiseptica} (Horiguchi et al., 1995), and by \textit{Pasteurella multocida} toxin (Lacerda et al., 1996).

Microtubules are polymers that are essential for, among other functions, cell transport, skeletal support and cell division in all eukaryotes. The regulation of the microtubule system results in the precise temporal and spatial pattern of microtubules that is observed throughout the cell cycle. The breakdown of microtubule network has been observed during bacterial, viral
and parasitic infection (Maloroni et al., 1990; Pereira et al., 1993; Giron et al., 1996). Since the infection of *H. pylori* leads to cell vacuolation and significant cytopathic changes in HEp-2 cells, the changes in the tubulin network of HEp-2 cells treated with OMP from various clinical isolates was observed by fluorescence microscopy with FITC-conjugated antibodies to tubulin. Examination of OMP treated cells by immunofluorescence showed increased numbers of cells blocked at prometaphase, with short microtubules extending from the spindle pole to the kinetochores (Fig. 3.8B). The microtubules appeared to be reduced and curled, particularly toward the periphery of the cells, which may be due to inhibition of microtubule assembly by the OMP. Rearrangement of tubulin network mediated by the cytopathic effects of OMP resulted in the loss of cell adhesion and the collapse of the cell cytoskeleton. The spatial distribution of tubulin was also rearranged with peripheral accumulation of tubulin filaments in HEp-2 cells treated with virulent strains (Fig. 3.8C). The differences in the morphological changes of HEp-2 cells induced by the OMP from virulent and less virulent strains can be correlated in the tubulin disruption pattern. This study shows that the microtubules are also the target for the OMP of *H. pylori*, apart from the actin filaments, and may be the OMP affects the subcellular signaling component that affects the regulation of tubulin distribution.

4.9 OUTER MEMBRANE PROTEIN OF *H. PYLORI*: ROLE IN APOPTOSIS

Apoptosis, a genetically programmed form of cell death plays an important role in the regulation of epithelial cell numbers in the gastrointestinal tract (Jones and Gores, 1997). Microbes have developed mechanisms to stimulate the apoptotic signal transduction cascade, which likely play a role in
the pathogenesis. *H. pylori* infection is also associated with elevated levels of mucosal apoptosis (Moss et al., 1996; Wagner et al., 1997). In *H. pylori* associated apoptosis concomitant Fas antigen expression has been reported (Houghton et al., 1999). Some results implicate LPS in the induction of NOS-2 expression, leading to the activation of caspase-3 in the process of gastric epithelial cells apoptosis (Slomiany et al., 1998). This study also confirms the role of *H. pylori* in apoptosis. The various clinical isolates, which differ in their property of adherence and vacuolation, infected on to HEp-2 cells, do induce apoptosis after 18 hours of exposure. A difference in the number of apoptotic cells induced by OMP from various clinical strains was observed by microscopic observation of the propidium iodide stained cells. The highly adherent and vacuolating strain showed significant induction of apoptosis of HEp-2 cells than the lesser adhering strains. Apoptotic cells surrounded by adherent bacteria were observed when infected with virulent clinical strains. Existing reports propose that Fas-mediated apoptosis in the malignant cell lines is not directly by *H. pylori* itself, but only through the addition of IFNγ enhancing the Fas expression (Houghton et al., 2000). The observations in this study on HEp-2 cells, malignant cell line show apoptosis can be mediated directly. HEp-2 cells were treated with the OMP for 4 hours after which the cells begin to detach from the culture plates as floaters. The treatment of OMP from *H. pylori* in a cell free system to HEp-2 cells resulted in apoptosis, with characteristic features of chromatin condensation, nuclear fragmentation and membrane blebbing as seen by propidium iodide staining. The OMP from virulent strains exhibiting greater degree of adherence and strongly vacuolating did exhibit the same extent of apoptosis in HEp-2 cells. These results indicate the direct involvement of the pathogen itself mediated by OMP alone or in combination with other virulence factors with the association of inflammatory cytokines.
Vacuolating cytotoxin, which damages the epithelial cells by vacuolation is encoded by *VacA* gene (Cover et al., 1992; Cover et al., 1994). *VacA* is present in all *H. pylori* isolates and it contains 2 variable parts in the signal sequence and middle region of the gene, which contributes to the differences in the genotype, and the expression of functional cytotoxin (Atherton et al., 1995). The s region encoding the signal peptide exists as s1 or s2 allelic types. Among the signal sequence region s1, variants as s1a and s1b have also been identified. The middle region occurs as m1 or m2 allelic types. There is a close association between s1 strains and *cagA* and particular combination of s and m genotype is a marker of the pathogenicity of the individual strain, since *in vitro* production of the cytotoxin, *in vivo* epithelial damage, and peptic ulcer disease are all related to the *vacA* genotype (Atherton et al., 1997).

The present study gives a clear picture about the variability of the s and m regions of the cytotoxin-encoding *vacA* gene among *H. pylori* strains from Chennai, South India. A PCR based assay to define the *vacA* genotypes of twenty four *H. pylori* isolates for the analysis of *vacA* allelic variation was carried out. The polymerase chain reaction was done with specific oligonucleotide primers for *vacA* and *cagA* that has been already reported (Rudi et al., 1998). Using these primers the types s1 and s2 were identified in all strains. *H. pylori* strains with the *vacA* signal sequence type s1 (70%) were predominant in this study and the type s2 frequency observed among the south Indian isolates was 29% (Fig.3.10.1). The low frequency of s2 type prevalence
may be due to the collection of strains representing dyspeptic and ulcer patients in this study. Cytotoxic *H. pylori* strains with vacA genotype s1 may occur frequently in such patients. Furthermore the increased cytotoxin activity was observed by vacuolation assay (neutral red uptake assay) in almost all s1 strains and the five isolates, which showed reduced cytotoxin activity might be the type s2. By using the primers for middle region, the genotyping was possible in all *H. pylori* isolates. The genotyping of the vacA middle region revealed m1 frequency as 79% (Fig. 3.11) and the m2 frequency as 20.83% (Fig. 3.12). But there were discrepancies in the classification of clinical strains as m1 or m2 as few strains yielded PCR products for both specific primers for middle region m1 and m2. The reason for these results can be due to the isolation of more than one isolate from one patient.

A number of genotyping for *H. pylori* epidemiological analysis like Random amplification of polymorphic DNA analysis (RAPD) (Akopyanz et al., 1992), oligofingerprinting (Marshall et al., 1996) and repetitive element-PCR (Rep-PCR) (Go et al., 1995) can easily distinguish unrelated isolates by the fingerprint profiles they generate. The use of the above techniques on isolates from multiple biopsies recovered from colonised individuals has revealed data on the proportion of individuals colonised by more than a single strain. For example, Taylor et al., (1995), using RAPD, found more than one colonising strain in only three out of 15 patients tested, and Shortridge et al., (1997), using PCR-RFLP of *ureC* genes, found multiple strains in only two out of 81 patients examined. In comparison, in developing countries, where colonisation with *H. pylori* is more common, up to 34% of individuals tested have been found to be colonised with multiple strains (Berg et al., 1997).
Subgroup analysis of the signal sequence and middle sequence combination demonstrated that all four combinations in this test population, s1-m1 combinations were observed at a frequency of 60% and s1-m2 with a frequency of 20%. The other two combinations seemed to be less prevalent in this small population of strains. Genotyping of a larger group of isolates with a varied disease conditions in equality will give a detailed picture of the disease association and the vacA status. When these isolates were checked for the presence of cagA with specific primers for the hydrophilic region and region of internal duplication, 20 (83.3%) isolates was \( cagA^+ \). The \( cagA^- \) strains were isolated from the three duodenal ulcer cases and one gastric cancer case. Strains lacking the \( cag \) are recovered from persons with benign infections (Atherton, 1998). Vac s2 lacking the \( cag \) gene were observed with duodenal ulcer condition. But the majority of the duodenal ulcers and peptic ulcer disease were infected with \( cag^+ \) strains. Size variations of the \( cagA \) gene by PCR within \( H. pylori \) isolates may reflect the size heterogeneity of \( cagA \) detected by immunoblot (Fig.3.5C). This size variation could affect its antigenic properties and thereby alter the host's immune systems ability to recognise the antigen. The reports from this study proved to be identical to the existing reports on Indian isolates of Calcutta, which also reports the high frequency of \( \text{vacAs}1 \) and cagPAI (Mukkhopadhyay et al., 2000). Such an abundance of \( \text{vacAs}1\text{cag}A^+ \) may be in accord with the high overall risk of \( H. pylori \) infection in India through increasing transmission rates favouring the emergence of more-virulent strains of a pathogen.

4.11 CONCLUSION

In conclusion, this work highlights a new finding in \( H. pylori \) pathogenesis, the involvement of outer membrane proteins in the pathogenic
mechanism, which is capable of inducing morphological and cytopathic changes in the host epithelial cells in cell free systems. The possible involvement of the outer membrane in the disease process of *H. pylori* infection is focussed and the existing heterogeneity of this organism is confirmed. The highlights of this observations in this study is as given below:

- The adherence patterns of the clinical isolates of *H. pylori* and their extent of adherence varied. Vacuolation activity of the clinical isolates showed the presence of toxigenic, vacuolating strains in the analysed population to be significantly more.

- Among the various fractions of the oxygenic reference strain that were analysed, outer membrane fraction exhibited significant cytotoxic effect. The dosage of the OMP inducing this lethal cytotoxic effect was elucidated and the kinetics of the OMP-mediated cytotoxicity was established.

- Marked difference in the cytopathic effects mediated by OMP’s differing in their adherence and vacuolating properties were observed by the trypan blue exclusion assay. The role of OMP in cytoskeletal rearrangements during *H. pylori* infection was shown by immunofluorescence studies.

- Among the various antigenic preparation analysed by immunoblot assay, outer membrane protein preparation emerged out to be an ideal candidate antigen when tested against patients’ sera from different disease status.

- Activation of transcriptional factor NF-κB was observed in OMP treated HEp-2 cells. Production of proinflammatory cytokines IL-6 and IL-8 was observed both in the bacterial and
OMP stimulated HEp-2 cells indicating the immunomodulatory role of OMP.

- It was shown that *H. pylori* could directly mediate apoptosis through the possible involvement of OMP, apart from fas-mediated signaling pathway for apoptosis.

- The allelic variations of the *vacA* gene in *H. pylori* in the South India, Chennai was shown by genotyping the *vacA* gene by polymerase chain reaction and correlated with the presence of *cagA* gene. This study showed the presence of *vacAs1* and *cagA* strains in high frequency, which represents the overall risk of high infection rates.