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Al-Nahrain University
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Investigating HLA Typing of Chronic Gastritis in a Sample of Iraqi Patients

A Thesis
Submitted to the College of Science / AL-Nahrain University
In Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

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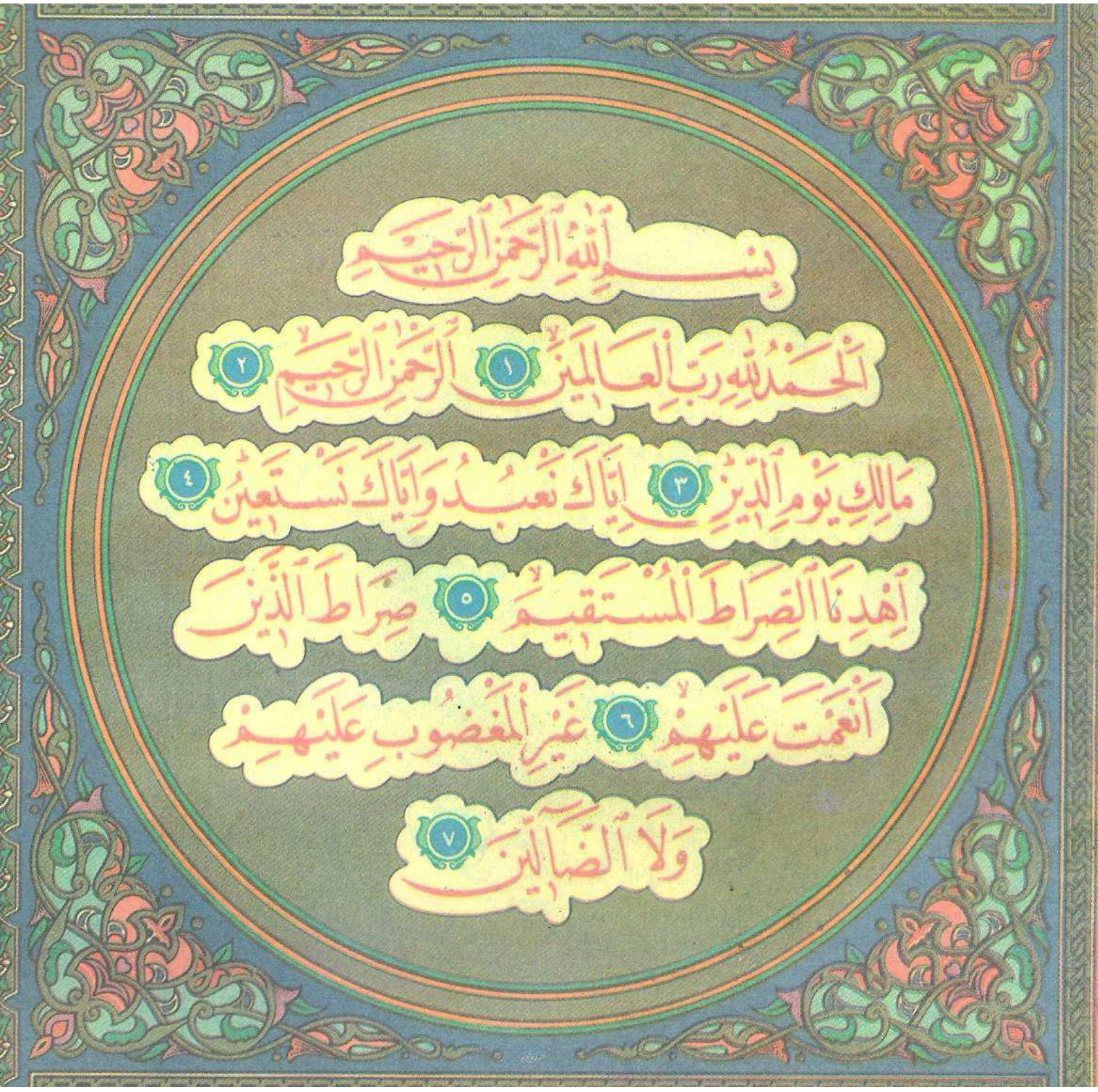
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وَلَا الضَّالِّينَ ٧

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DEDICATION

To the one who bears the sweetest name
And adds a luster to the same

Who shares my joys

Who cheers when sad
The greatest friend I ever had

Long life to her for there's no other
Can take the place of my dear mother

Ahmed

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Summary

The present study was proposed to investigate the role of Human Leucocyte Antigen HLA-class I (A and B) and class II (DR) antigens and blood group phenotypes in the aetiology of chronic atrophic gastritis in a sample of Iraqi patients. The patients were also evaluated for total and absolute counts of leucocytes, immunophenotypes of lymphocytes (CD3+, CD4+, CD8+ and CD19+ cells) and positivity of their sera for anti-*Helicobacter pylori* IgG antibody.

Forty-nine patients with the disease were investigated during the periods January-October, 2006. The disease was clinically diagnosed by the consultant medical staff at Al-Kadhmiyah Teaching hospital in the Gastrointestinal Tract (GIT) and Liver Diseases Center. The diagnosis was based on clinical evaluation using endoscopy and a histopathological examination of the stomach mucosal biopsy.

According to the point view of consultants, the patients were clinically subdivided into two groups according to the location of inflammation; corpus gastritis (31 patients) and antrum gastritis (18 patients). A control sample of 50 individuals (apparently healthy), matched for age, sex and ethnic background (Arab Muslims), were investigated.

The patients were also further divided into *Helicobacter pylori*-positive (HP+) and *H. pylori*-negative (HP-) according to the presence of IgG anti-*H. pylori* antibodies in their sera. There were 32 patients (65.3%) positive for IgG anti-*H. pylori* antibodies, while 17 patients (34.7%) were sero-negative for IgG anti-*H. pylori* antibodies.

At HLA-class I and class II regions, six antigens showed increased frequencies in the total patients or their subgroups as compared to controls. They were A3, A9, B17, DR2, DR3 and DR10. The A3 antigen showed a significant increase in the total patients and antrum gastritis patients, while DR10 was consistently increased in the patients, and the Relative Risk (RR) value showed a range from 8.21 in *H. pylori*-positive gastritis to 22.38 in *H. pylori*-negative

gastritis, and the Etiological Fraction (EF) value also showed variations (total gastritis: 0.39; corpus gastritis: 0.41; antrum gastritis: 0.34; *H. pylori*-positive gastritis: 0.30; *H. pylori*-negative gastritis: 0.56). In contrast, the antigen A19 was consistently decreased in the patients with a Preventive Fraction (PF) value more than 0.30 (total gastritis: 0.38; antrum gastritis: 0.33; *H. pylori*-positive gastritis: 0.38; *H. pylori*negative gastritis: 0.39). Also, this antigen was not recorded in corpus gastritis patients, moreover, this group of patients showed a further two positive associations, which were A9 and DR2, and the EF values of such associations were 0.42 and 0.38, respectively.

The immunogenetic predisposition to develop chronic atrophic gastritis was further explored with other immunogenetic markers; they were ABO blood group phenotypes. Two phenotypes were important in this regard; A and O. The blood group A was significantly increased in total patients (46.7 *vs*. 18.0%) and almost their subgroups, while a reverse outcome was observed for blood group O (22.2 *vs*. 48.0%) as compared to controls. Such two observations may suggest that the first blood group is involved in the aetiopathogenesis of chronic atrophic gastritis, while the second confers some resistance to develop the disease.

Investigating the CD profiles of peripheral lymphocytes revealed a significant decrease of CD3+ (55.3 *vs*. 79.4%) and CD4+ (25.7 *vs*. 39.5%) cells in total patients of chronic atrophic gastritis and their subgroups, while CD8+ lymphocytes did not show such variation. The CD4/CD8 ratio was also significantly decreased in patients (1.23 *vs*. 1.81%). In contrast CD19+ lymphocytes showed a significant increase in total patients (27.4 *vs*. 18.7%) and their subgroups. Such observations suggest the importance of these cells in the aetipathogenesis of the disease.

Finally, the total and absolute counts of leucocytes showed no significant differences between patients (total and subtypes) and controls.

List of Contents

List of Abbreviations

HLA	Human Leukocyte Antigen
MHC	Major Histocompatibility Complex
HP	*Helicobacter pylori*
IL	Interleukin
TNF	Tumor Necrosis Factor
Ig	Immunoglobulin
MALT	Mucosa Associated Lymphoid Tissue
ATP	Adenosine Tri-Phosphate
CD	Cluster Designation (Cluster of Differentiation)
ELISA	Enzyme Linked Immuno-Sorbant Assay
PBS	Phosphate Buffer Saline
HRP	Horseradish Peroxidase
CAL	Calibrator
NC	Negative Control
PC	Positive Control
EIU	Enzyme Immuno Units
RR	Relative Risk
EF	Etiological Fraction
PF	Preventive Fraction
P	Probability
Pc	Corrected Probability
SPSS	Statistical Package for Social Sciences
SE	Standard Errors
LSD	Least Significant Difference
NS	Not Significant
vs.	Versus

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Chapter One
Introduction

1-1: Introduction

Chronic gastritis is an inflammation of stomach lining, and recently, it has become clinically important to examine predisposition, immunological profile and aetiology of the disease, because it correlates not only with a functional disorder but also with organic diseases including peptic ulcer and gastric cancer. As considered in other organs, such as hepatitis, which is a basic disorder for hepatocellular carcinomas, continuous gastric inflammation can lead to the generation of gastric carcinoma (Zhang *et al.*, 1996; Suerbaum and Michetti, 2002). Therefore, the evaluation of gastric inflammation and its control are quite important.

Helicobacter pylori infection is, in addition to being the main aetiological agent for chronic gastritis, a major cause of peptic ulcer and gastric cancer (Feldman, 2001). In developing countries, the prevalence of *H. pylori* infection is around 80% among middle-aged adults, whereas in developed countries prevalence ranges from 20-50%, and accordingly, approximately 10-15% of infected individuals will develop a gastric disease (Parsonnet *et al.*, 1991). Therefore, *H. pylori* infection is a necessary but not a sufficient cause of severe forms of gastric disease. In this regard, host genetic constitution is also thought to play a role in chronic gastritis. Among genetic factors, individual differences in inflammatory responses may protect or predispose to malignant transformation of the gastric mucosa (Fan *et al.*, 1998). Such theme is best interpreted in terms of human leukocyte antigens (HLA) of the major histocompatibility complex (MHC), which are a group of highly polymorphic genes located in the short arm of chromosome 6 and are particularly important in controlling specific immune recognition (Rhodes

and Trosdate, 1999). HLA antigens are capable of binding foreign peptides, and T-cell recognition of a combination of HLA antigen may result in either induction of an effective immune response or suppression of such response (Bekker-Mendez *et al.*, 1998). Moreover, adherence of *H. pylori* to HLA class II molecules expressed in gastric epithelial cells has been demonstrated, and previous investigations have linked specific HLA-class II alleles to gastric diseases, although the results have been inconsistent (Herrera-Goepfert *et al*., 2006).

Currently, chronic gastritis is discussed not only by the static evaluation, but also by its predisposition aspect. Moreover, it is also important to speculate the functional or immunological factors that correlate with the morphological status. Because of these concepts, convenient and objective immunological markers are required for evaluating the clinical status of such disease (Suerbaum and Mitchetti, 2002). Therefore, there is a need to evaluate the immunological status that shows a correlation with chronic gastritis, and as a result may be useful to evaluate the disease from the point views of immunogenetics and immunity.

1-2: Aim of Study

Based on the forthcoming presentation, the present study was planned to investigate the association of HLA-A, -B and -DR antigens with chronic atrophic gastritis in a sample of Iraqi patients. Other parameters, such as blood group phenotypes, the CD profile of lymphocytes and total and absolute counts of leucocytes, were also inspected.

Chapter Two

Literature Review

2-1: Chronic Gastritis

Chronic gastritis is an inflammatory condition of the gastric mucosa characterized by elementary lesions whose extent and distribution are related to their aetiology and host responses. The two main features of the disease are infiltration of the lamina propria by inflammatory cells and atrophy of the glandular epithelium. Plasma cells and lymphocytes predominate among inflammatory cells, but eosinophils and neutrophils may also present (Rosai and Ackerman, 2004). Infection with *Helicobacter pylori* is by far the most common cause of chronic active gastritis worldwide; chemical agents and autoimmune phenomena account for a small proportion of chronic, usually non-active gastritis. Chronic gastritis is epidemiologically and biologically linked to the development of gastric cancer and *H. pylori* has been listed as a class I carcinogen. However, the assessment of cancer risk in individual patients is difficult in part because gastric carcinogenesis is modulated by poorly defined factors, including environment, bacterial strain, and host responses (Kashiwagi, 2005).

Gastritis can be viewed at two different levels: a basic level represented by the elementary lesions and a hierarchically higher level that defines the disease (i.e. the combination and topographical distribution of the different elementary lesions). Both the 1990 original Sydney System and its updated 2001 version (also known as the Houston-updated Sydney System) provided a structure to describe and quantify the elementary lesions, that is, the inflammatory cell populations, and the accompanying changes of the epithelial district (Stolte and Meining, 2001). Although the Houston-updated Sydney System's guidelines are now widely used, the interobserver agreement among pathologists has shown variable levels of

consistency, particularly with regards to atrophy. In an attempt to correct this problem, an international group of pathologists revisited the spectrum of gastric atrophy and intestinal metaplasia. A new definition of atrophy, which includes a metaplastic and a nonmetaplastic category, was proposed, and new criteria for the two main phenotypes of chronic gastritis (nonatrophic and atrophic) were established. Gastrointestinal pathologists who tested this classification were able to obtain a highly satisfactory level of interobserver variation (Rugge and Genta, 2005).

2-1-1: Phenotypes of Gastritis

Chronic gastritis can be atrophic or nonatrophic. Each of these two main categories encompasses several clinicopathological entities with different patterns of inflammatory and epithelial alterations.

2-1-1-1: Non-atrophic Gastritis

- **Antral-predominant non-atrophic gastritis:** This pattern (synonymous with hypersecretory, diffuse antral, or superficial antral gastritis) is the most common expression of *H. pylori* gastritis in the Western world (Correa, 1988). It is characterized by (1) absence of atrophy, (2) a moderately to severely inflamed antrum and (3) a normal to mildly inflamed corpus. This condition is associated with either normal or increased acid secretion. Most patients with antrum-predominant gastritis experience no symptoms; they do, however, have an estimated lifetime risk of duodenal ulcer of about 20%, and possibly a minimally increased risk of adenocarcinoma of the distal stomach when compared to the noninfected population (Graham, 1997).

- **Nonatrophic pangastritis:** In some subjects infected with *H. pylori*, marked inflammation is distributed throughout the stomach, with little or no difference

between antrum and corpus. Particularly, it is frequent in poorly sanitized areas where *H. pylori* is highly endemic. Pangastritis is widely believed to be the background on which atrophy eventually develops (Miehlke *et al*., 1998).

2-1-1-2: Atrophic Chronic Gastritis

Gastric mucosal atrophy is defined as the loss of appropriate glands. This loss occurs when glands damaged by inflammation are replaced either by connective tissue (scarring) or by glandular structures inappropriate for location (metaplasia) (Genta and Graham, 1997). Most often, the metaplastic transformation assumes the phenotype of the glands lined by intestinal-type epithelium, but in the oxyntic mucosa, it may also take the form of mucin-secreting antral glands (pseudopyloric metaplasia). The histological criteria for scoring atrophic-metaplastic changes in both the antral and oxyntic mucosa have been extensively described, and visual analogue scales have been proposed as a reference standard (Rugge *et al*., 2002).

- **Antrum-restricted atrophic gastritis:** In the Western world, when atrophy is detected in biopsy specimens from dyspeptic patients, it is most frequently located in the antral biopsy samples. In such patients, atrophic-metaplastic changes are the consequence of current (or past) *H. pylori* infection. The biopsy set will show (1) patchy metaplastic atrophy restricted to the distal mucin-secreting mucosa (including the incisura angularis) coexisting with moderate to severe inflammation and (2) a normal or mildly inflamed corpus, with no atrophic changes (Rugge and Genta, 2005).

- **Corpus-restricted (corpus-predominant) atrophic gastritis:** In the oxyntic mucosa, atrophic-metaplastic changes can be detected in the absence of any coexisting atrophic changes of the distal stomach or in association with atrophic foci of the antral mucosa. The former condition is considered

virtually pathognomonic of an autoimmune aetiology and it is associated with an increased cancer risk. Rarely, autoimmune atrophy (by definition, affecting only oxyntic glands) may coexist with antral atrophy resulting from a concurrent *H. pylori* infection. In these cases, pathogenetically different atrophic changes may topographically merge with each other (corpus-autoimmune and *H. pylori*-associated atrophic gastritis), resulting in a substantially increased cancer risk (Fox and Wang, 2007).

2-1-2: Aetiology of Chronic Gastritis

The aetiology of chronic gastritis is not well defined, although genetic, immunological and environmental factors have been suspected, but the nature of these factors or how they interact to produce the disease is a matter of speculations. Among the genetic factors are alleles of the human major histocompatibility complex (Herrera-Goepfert *et al*., 2006) and blood groups (Oba-Shinjo *et al*., 2004). However, these alleles may confer some predisposition and other interacting factors are required. Among the latter is *H. pylori*, which is the main environmental factor that contribute to chronic gastritis and is also associated with enhanced risk of developing peptic ulcer and gastric cancer (Vorobjova, 2005). Moreover, the genetic and immunological aetiologies of chronic gastritis have been interpreted in the context of this bacterium (Quintero *et al*., 2005; Lee *et al*., 2006).

2-2: Pathology of Chronic Gastritis and *H. pylori*

When Warren and Marshall first identified spiral organisms closely applied to the gastric epithelium in active chronic gastritis, they brought to light an aetiological explanation for a whole series of pathological changes that had been long-recognized but not understood. It was widely appreciated that chronic gastritis was a common denominator linking peptic ulceration, gastric carcinoma, and

lymphoma and that the histological picture encompassed chronic inflammation, atrophy, and intestinal metaplasia (Warren and Marshall, 1983). But the state of ignorance concerning aetiology led to a plethora of possible causes including stress, excessive alcohol consumption, bile reflux, and even the ingestion of hot beverages and spicy food (Parfitt and Driman, 2007). The importance of *H. pylori* infection to the pathologist is three fold, the accumulated knowledge of bacterial pathogenicity and the host response now offers plausible explanations for the tissue changes observed. Finally, the ecology of the organism with regard to tissue colonization and local environment affords explanations for hitherto inexplicable differences in the patterns of inflammation seen in different clinical phenotypes (Kononov, 2006).

2-2-1: General Features of *H. pylori* Gastritis

The initial, acute phase of infection is subclinical in the great majority of subjects. Following ingestion, organisms penetrate through the viscid mucous layer and multiply in close proximity to the surface epithelial cells. The epithelium responds to infection by mucin depletion, cellular exfoliation, and compensatory regenerative changes. Polymorph infiltration into foveolar and surface epithelium, and lamina propria edema are conspicuous. Collections of polymorphs in the foveolae and adherent neutrophil exudate on the surface may also be present (Sobala *et al.*, 1992).This acute phase is accompanied by profound hypochlorhydria and a failure of ascorbic acid secretion into gastric juice. It may take several weeks for acid output to return to preinfection levels, and in a proportion of patients output remains low. However, ascorbic acid secretion remains lower than normal for the duration of chronic gastritis, indicating that low secretion is related to persisting inflammation rather than hypochlorhydria (Sobala *et al.*, 1993).

The acute response is mediated by release of bacterial lipopolysaccharide and a number of directly acting chemotactic moieties, which penetrate through the

damaged surface epithelium and induce polymorph emigration into the lamina propria and epithelium (Slomiany *et al.,* 1998). Bacterial products also activate mast cells, and a subsequent degranulation releases other acute inflammatory mediators that increase vascular permeability, up-regulate expression of leukocyte adhesion molecules on endothelial cells, and increase polymorph emigration (Graham, 1997). *H. pylori* stimulates the gastric epithelium to produce a potent neutrophil chemokine interleukin-8 (IL), whose production is up-regulated by tumor necrosis factor alpha (TNF-α) and IL-1 released by macrophages in response to bacterial lipopolysaccharide. Additional IL-8 is released by the polymorphs themselves in response to soluble *H. pylori* proteins (Kim *et al.,* 1998).

The acute phase is short lived. In a small minority of people, and particularly in childhood, the organisms may be spontaneously cleared, the polymorph infiltrate resolves, and appearances return to normal. In the majority, however, the host immune response fails to eliminate the infection and over the next 3 or 4 weeks there is a gradual accumulation of chronic inflammatory cells that come to dominate the histological picture. As a consequence, the diagnosis of an acute neutrophilic gastritis gives way to that of an active chronic gastritis (Sobala *et al.*, 1991).

The arrival of lymphocytes and plasma cells in the mucosa signals augmentation of the acute inflammatory response by the production of cytokines and specific anti-*H. pylori* antibodies. B-cell proliferation and subsequent plasma cell differentiation result in the synthesis of IgM-opsonizing and complement-fixing antibodies, which amplify the inflammatory reaction. However, this vigorous response fails to eliminate infection, and the continued presence of *H. pylori* leads to the development of a second arm of the immune response more specifically aimed at preventing the damaging effects of intraluminal pathogens (Beswick *et al.,* 2006). This second-line response involves the recruitment of primed B cells into lymphoid follicles, with the production of plasma cells largely committed to

the synthesis of "mucosally protective" IgA antibodies (Jevremovic *et al.*, 2006). The fact that, even when augmented by IgA, the response is insufficient to eradicate *H. pylori* in the great majority of cases means that antigenic stimulation persists and the formation of follicles becomes a consistent feature of chronic *H. pylori* gastritis. Indeed, it is claimed that if enough biopsies are examined, follicles will always be found in *H. pylori*-infected stomachs. This acquisition of "organized" lymphoid tissue in the gastric mucosa constitutes a mucosa-associated lymphoid tissue (MALT). As such it provides the background tissue in which gastric marginal zone (B-cell) lymphoma (so-called MALToma) arises, and this underlines the crucial role of *H. pylori* in lymphomagenesis in the stomach (Huang *et al.*, 2004).

The two arms of the reaction to *H. pylori*, the acute "inflammatory" and the chronic "immune," are thought to be directed by two subsets of T-helper cells, T_H1 cells that promote inflammation and by activating CD8+ T cells lead to autoantibody formation and cell-mediated epithelial damage, and T_H2 cells that are responsible for the secretory immune response, which has the potential to reduce the bacterial load (Mohammadi *et al.*, 1997). While these two arms are both interrelated and to a large degree inseparable, the response leans heavily toward the T_H1 arm, although its strength will be influenced by bacterial and host factors. The typical histological picture of active chronic gastritis with lymphoid follicles reflects the overlap of these two processes, but down-regulation of the acute inflammatory component can result in a picture of inactive chronic gastritis, i.e., one in which there is no polymorph component. In human *H. pylori* infection this disparity between T_H1 and T_H2 responses is most evident in children in whom the lymphofollicular pattern is dominant and is sufficient to give rise to a characteristic nodularity of the mucosa, yet polymorph activity is minimal or absent (Moss and Sood, 2003). The dissociation between inflammatory and immune (follicular) lymphoid infiltration reaches its ultimate expression in some animal models. For

example, in the BALB/c mouse infected with *Helicobacter felis*, no gastritis is observed for most of the animal's life span. However, aged infected mice may develop a pronounced follicular lymphoid infiltrate in the corpus mucosa, which in some animals progresses to lymphoma (Enno *et al.*, 1995). These findings indicate the importance of host factors in determining the balance between inflammation and immunity, but bacterial (strain) factors should not be overlooked. Thus, while genotypic differences in cagA and vacA appear to be important in determining inflammatory activity, follicle formation seems to be a universal response to *H. pylori* irrespective of the infecting strain (Warburton *et al.*, 1998).

2-2-2: Atrophy

Atrophy in the stomach is conventionally defined as loss of glandular tissue from repeated or continuing mucosal injury and is a common denominator in all pathological processes causing progressive mucosal damage, including long-standing *H. pylori* infection. Thus, loss of glands may follow erosion or ulceration of the mucosa, with destruction of the glandular layer, or as a result of a prolonged inflammatory process in which individual glands undergo destruction (Zhang *et al.*, 2005). When such loss occurs, it is followed by fibrous replacement. However, atrophy can also be thought of as simply "a loss of specialized or functional cells." Under this broader definition it is possible to include situations in which there is loss or replacement of parietal and chief cells without glandular destruction. Such partial or "preatrophy" has been described in human autoimmune gastritis and is frequently encountered in animal models of both autoimmune gastritis and chronic *Helicobacter* infection (Sakagami *et al.*, 1996). In these latter situations, specialized oxyntic cells within intact glandular tubules are replaced by mucous cells ("mucous metaplasia"). It is likely that downward proliferation of mucous neck cells is the explanation, but whether this gives rise to so-called pyloric gland metaplasia has yet to be determined (Arikan *et al.*, 2004).

The prevalence and severity of atrophy among patients with chronic *H. pylori* gastritis increase with time. However, atrophy is not an effect of aging per se; there is no evidence that atrophy occurs as a physiological aging phenomenon and elderly subjects without gastritis have a normal acid output (Maaroos *et al.*, 1999).

Atrophy in *H. pylori* gastritis could result from direct bacterial effects or alternatively as a consequence of the host inflammatory or immune response. Direct injury by cytotoxins and ammonia products can bring about epithelial cell destruction, but this is unlikely to be an important mechanism in the glandular layer as bacterial colonization is restricted to surface and foveolar epithelium (Beales *et al.*, 1996). While infection with more virulent strains of *H. pylori*, that is, cytotoxin-positive and CagA-positive strains, is more likely to be associated with atrophy (Ito *et al.*, 1996). This does not necessarily argue for a direct mechanism. Such strains are "proinflammatory" and could also lead to increased release of potentially autodestructive agents such as proteases and free radicals from inflammatory cells. Interestingly, active chronic *H. pylori* gastritis is characterized by accumulations of neutrophil polymorphs around and within the stem-cell compartment in the gastric pit, a phenomenon possibly related to the rich vascular supply of this segment. Release of injurious products at this site could destroy stem cells, arrest the renewal of glandular epithelial cells, and lead to complete loss of the pit-gland unit (Fox and Wang, 2007).

2-3: Human Leukocyte Antigen (HLA) System

The HLA system represents the human major histocompatibility complex (MHC), which is located on the short arm of chromosome 6 in the region 6p21.1-21.3. It spans a region of about four million base pairs, which is equivalent to 0.1% of the human genome. The HLA genes are divided into three classes; class I, II and III regions. The class I genes are located in the telomeric side of the chromosome and encode the α-chain of the antigen-presenting HLA-A, -B and -C molecules.

The centromeric class II genes, previously known as immune-response genes, encode both α and β antigen-presenting HLA-DR, -DP and -DQ molecules. The HLA class III region, which is located in the middle, contains many genes with varying functions, but the most important of these are components (C2, C4 and BF) of the complement system (Figure 2-1) (Mehra and Kaur, 2003).

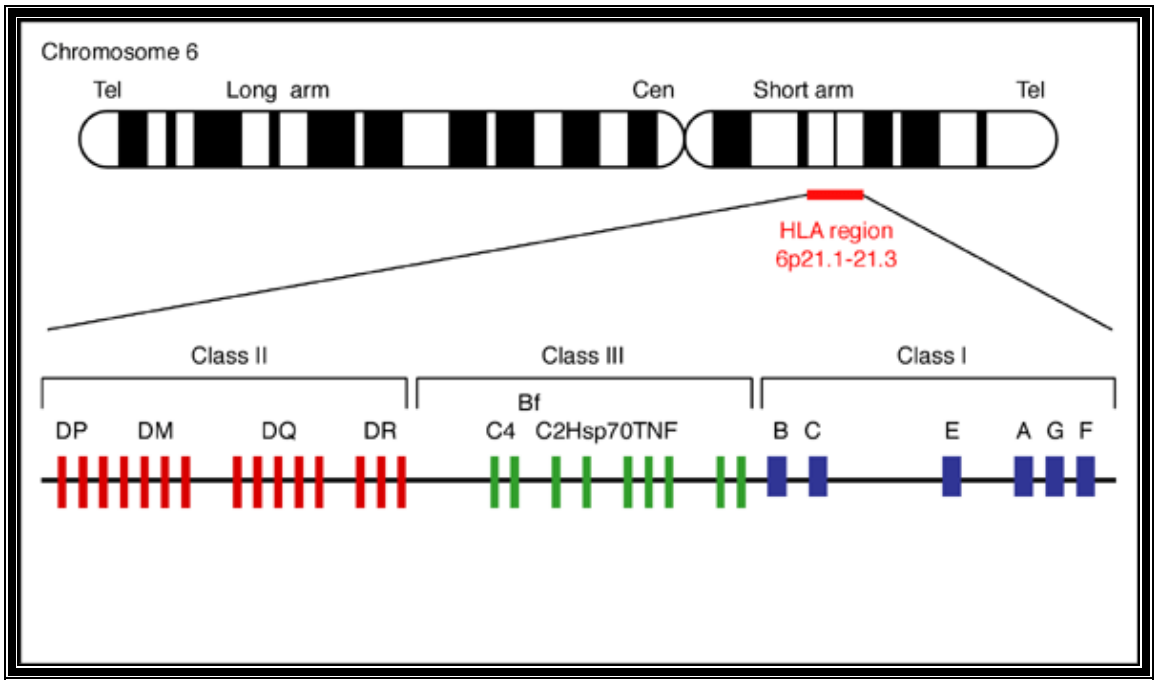

Figure 2-1: Gene map of HLA system (Adopted from Mehra and Kaur, 2003).

2-3-1: Genetics and Structure of HLA System

The HLA complex on chromosome 6 contains over 200 genes, more than 40 of which encode leukocyte antigens. The rest are an assortment of genes that are not evolutionarily related to the HLA genes themselves, although some are involved with them functionally (Schreuder *et al.*, 2005). The class I genes code for the alpha (α) polypeptide chain of the class I molecule, while the beta (β) chain is encoded by a gene on chromosome 15 (the beta-microglobulin gene). The α-chain has five domains: two peptide-binding domains (α1 and α2), one immunoglobulin-like domain (α3), the transmembrane region, and the cytoplasmic tail (Figure 2-2). There are some 20 class I genes in the HLA region; three of these, HLA-A, B, and

C, the so-called classical, are the main actors in the immunologic reactions, and others (HLA-E, F and G) has been classified as non-classical loci with gene products that supports other facets of the immune system.

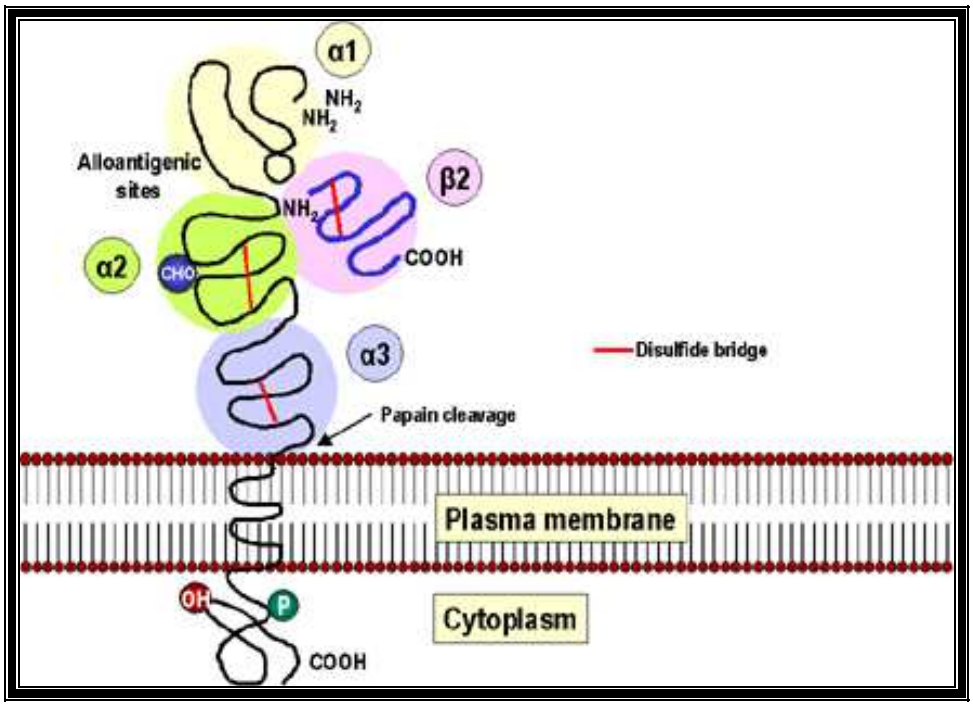

Figure 2-2: Structure of HLA-class I molecule (Belmares *et al.*, 2002).

The class II genes code for the α and β polypeptide chains of the class II molecules. The designation of their loci consists of three letters: the first (D) indicates the class, the second (M, O, P, Q, or R) is the family, and the third (A or B) represents the chain (α or β, respectively). HLA-DRB, for example, stands for class II genes of the R family coding for the β chains. The individual genes of the HLA system are differentiated by Arabic numbers, and the notation for the numerous allelic variants of these genes is a number preceded by an asterisk. For example, HLA-DRB1*0401 stands for allelic variant 0401 of gene 1, which encodes the β chain of a class II molecule belonging to the R family (Hertz and Yanover, 2007). Each of the class II α and β chains has four domains: the peptide-

binding domain (α1 or β1), the immunoglobulin-like domain (α2 or β2), the transmembrane region, and the cytoplasmic tail (Figure 2-3).

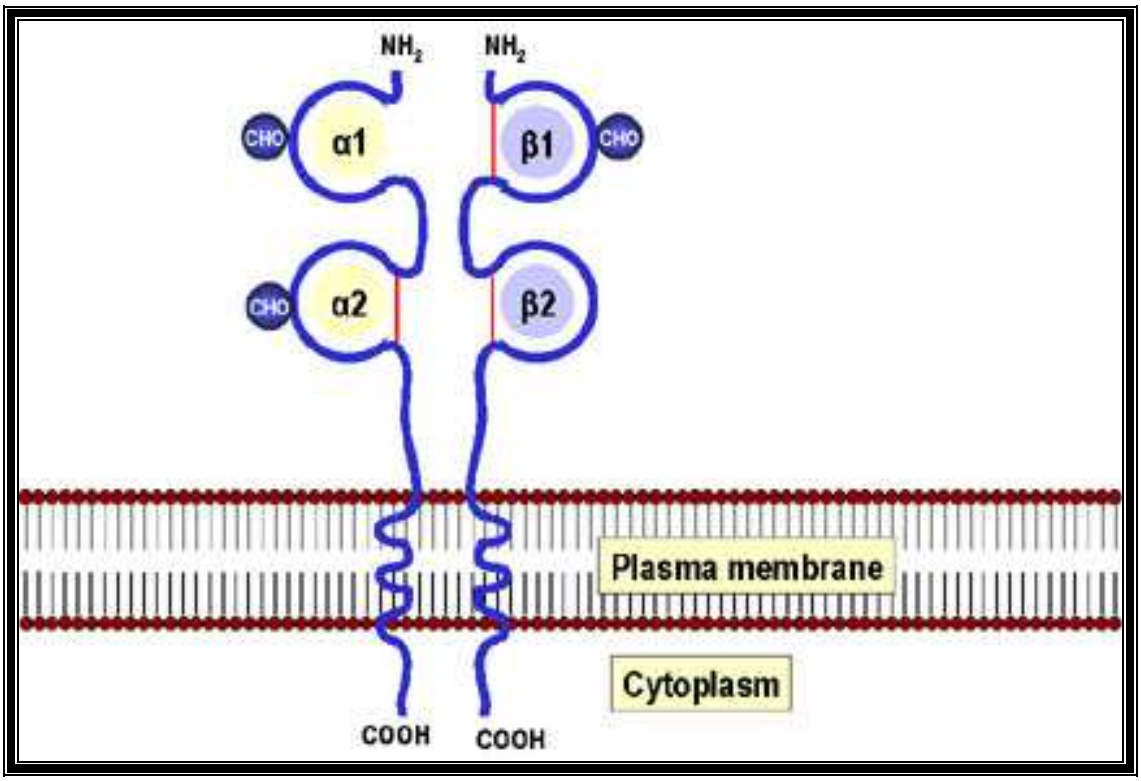

Figure 2-3: Structure of HLA-class II molecule (Belmares *et al.*, 2002).

HLA-class I genes are expressed by most somatic cells, although the level of expression varies depending on the tissue. By contrast, class II genes are normally expressed by a subgroup of immune cells that includes B lymphocytes, activated T lymphocytes, macrophages, dendritic cells and thymic epithelial cells. In the presence of interferon-γ, however, other types of cells can express class II HLA molecules (Busch *et al.*, 1998).

2-3-2: Function of HLA Antigens

The function of both class I and class II molecules is the presentation of pathogen-derived peptides to T cells, a process that initiates the adaptive immune

response. HLA class I molecules bind to peptides produced by the intracellular degradation of viral proteins and display them on the cell surface of antigen presenting cell for recognition by CD8+ T lymphocytes. The CD8 T lymphocytes bear receptors specific for the HLA class I antigens and route pathogens such as viruses. Surface expression of HLA I MHC molecules depends on the availability of peptides that bind HLA molecules in the endoplasmic reticulum. A peptide transporter, associated with antigen processing (TAP), plays an important role in maintaining adequate levels of peptide (Klein and Sato, 2000). The transporter is a heterodimer encoded by two genes, TAP1 and TAP2, located in the HLA class II region. TAP genes belong to the adenosine triphosphate (ATP) binding cassette super family of transport proteins, which have two ATP-binding cassette domains and two transmembrane domains. TAP genes are polymorphic (Sebzda *et al.*, 1999), and allelic HLA differences may be associated with disease by altering the peptides that bind class I HLA molecules. Class I, i.e., HLA-A, -B, and -Cw molecules, therefore play an important role in viral infections and lysis of target cells by cytotoxic killer T lymphocytes (Figure 3-4) (Petrovsky and Brusic, 2004).

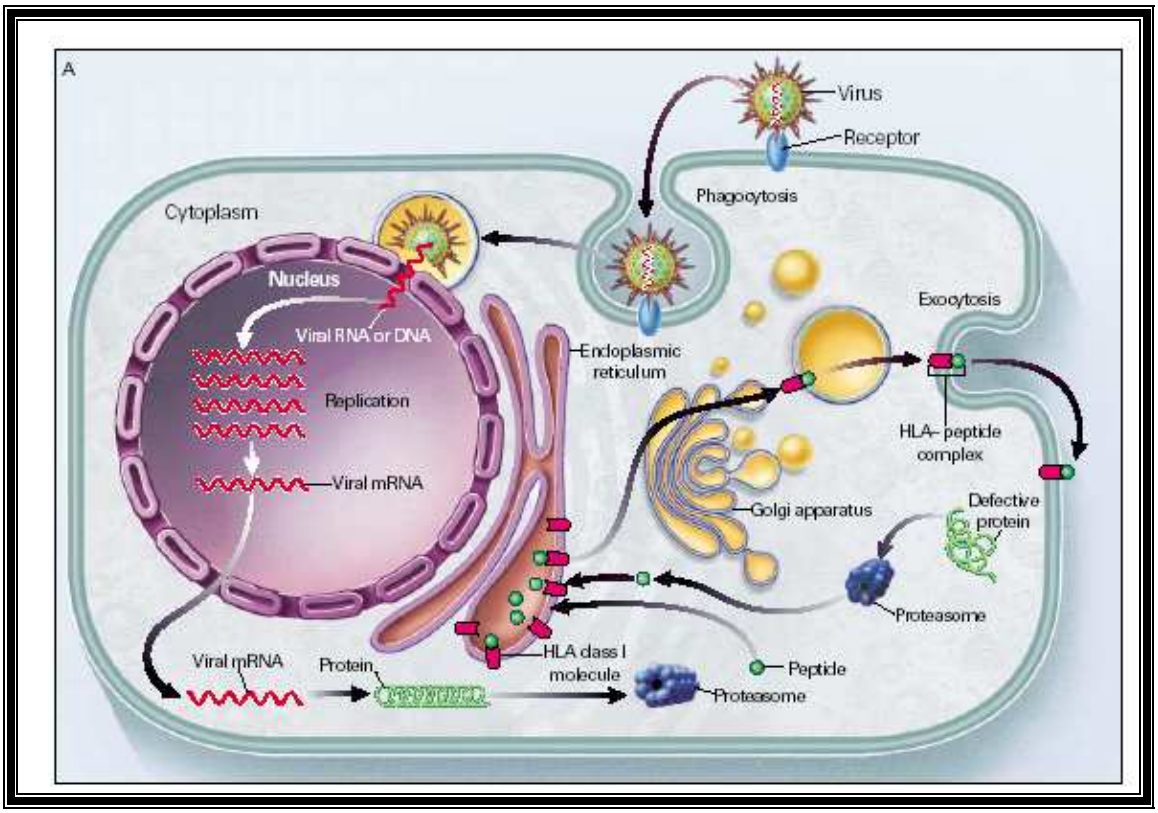

Figure 3-4: The principle pathway of generating viral peptides for loading onto HLA class I molecules on the surface of antigen presenting cell (Klein and Sato, 2000).

HLA class II molecules are highly polymorphic membrane glycoproteins that bind peptide fragments of proteins and display them for recognition by CD4+ T lymphocytes. The CD4+ T lymphocytes are of central importance in defeating the bacteria and other parasites that live within cells (Allen and Trowsdale, 2004). These cells are called helper T cells because they secrete substances that amplify and control virtually all aspects of immunity. The CD4+ T cells have receptor molecules that can recognize one particular peptide-HLA class II antigen combination. The binding capability of any given peptide to HLA class II molecules depends on the primary sequence of the peptide and allelic variation of the amino acid residues in the binding site of the HLA receptor. Anchor residues defining allele-specific peptide motifs have been identified in the class II binding

peptides. The proposed anchor residues combining with MHC pockets through their side chains seem to be a primary requirement for peptide-MHC interaction (Figure 3-5). The invariant chain (Ii) plays a critical role in the assembly, intracellular transport, and function of HLA class II molecules (Klein and Sato, 2000).

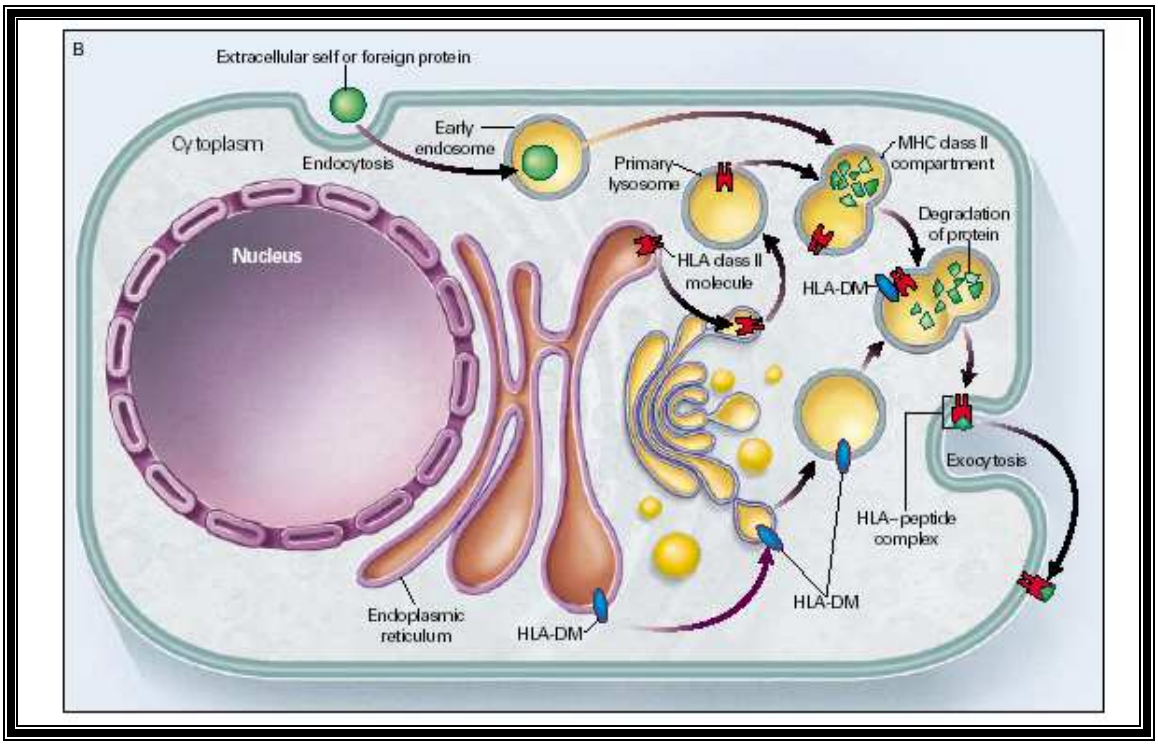

Figure 3-5: The principle pathway of generating bacterial peptides for loading onto HLA class II molecules on the surface of antigen presenting cell (Klein and Sato, 2000).

2-3-3: HLA Polymorphism

One of the main characteristics of the HLA system is its extreme polymorphism. Among the expressed loci, the MHC has the greatest degree of polymorphism in the human genome. The numbers of alleles recognized at the classical loci by December 2006 are presented in Table 2-1.

Table 2-1: Number of alleles at the classical HLA loci.*

Locus	DNA-level Alleles	Serological Equivalents
HLA-A	119	40
HLA-B	245	88
HLA-Cw	74	9
HLA-DRB1	201	80
HLA-DQB1	39	7
HLA-DPB1	84	(-)

*: Marsh (2007).

It is difficult to explain such extensive polymorphism, however, HLA molecules that have the task of presenting intra-cellular antigens on the surface of cells to cytotoxic and helper T cells, have adopted another strategy to increase their antigen presenting repertoire. This strategy included extensive duplication of genes with redundant function but subtle differences in the way such function is implemented (Dorak, 2006). All classical HLA molecules present small portions of antigenic proteins (epitopes) to T cells; however the selection of these epitopic determinants markedly varies across the HLA genes and their alleles. Thus, HLA molecules are generally conserved in domains of the protein responsible for interactions with conserved components of the T cell receptors and their co-receptors (like CD8 molecules) while displaying extensive polymorphism in domains responsible for antigen binding and interactions in variable regions of the T cell receptors. Therefore, a first lesson that the HLA system has taught us is that polymorphism can occur preferentially in functional domains of a given molecule with dramatic effects on epitope selection and presentation (Bettinotti *et al.*, 1998).

It is believed that the major benefit provided by the extensive HLA polymorphism is an increased likelihood that individuals of a given species will be heterozygous and consequently carry two different HLA alleles for each HLA locus. Since HLA polymorphism(s) occur in domains responsible for epitope binding, heterozygosity may double the antigen presenting potential of each individual within an ethnic group. Most importantly, since individuals within the same ethnic group are likely to express different HLA phenotypes, the overall repertoire of the group is exponentially broadened by the presence of extensive polymorphism enhancing the likelihood of the species of surviving a wide variety of pathogens (Dawkins *et al*., 1991).

2-3-4: Inheritance of HLA Antigens

A highly relevant feature of the HLA antigens is their co-dominant expression; therefore both alleles have an equal contribution to the phenotype. Also important is the fact that the HLA is inherited *en bloc* as a haplotype with the exception of the rare recombinational events. Recombination occurs at 1-3% frequency mostly at the HLA-A or HLA-DP ends. The large segment from HLA-B to HLA-DQB is almost always inherited as a whole. The co-dominant expression and haplotypical transmission have an important consequence: within a family, HLA-identical sibling frequency should be 25% according to Mendelian expectations. This has been, however, found to be higher than that in some diseases (Dorak, 2006).

Despite the enormous number of alleles at each expressed loci, the number of haplotypes observed in populations is much smaller than theoretical expectations. This is to say that certain alleles tend to occur together on the same haplotype rather than randomly segregating together. This is called linkage disequilibrium (LD) (Begovich *et al.*, 1992).

An interesting group of HLA haplotypes is the ancestral or extended haplotypes (also called supratypes). These are specific HLA-B, -DR, BF, C2, C4A and C4B combinations in significant linkage disequilibrium in chromosomes of unrelated individuals. They extend from HLA-B to DR and have been conserved *en bloc* (Gaudieri *et al.*, 1997). In some Caucasian populations, the extended haplotypes constitute 25-30% of all HLA haplotypes and together with recombinants between any two of them, they account for almost 75% of unselected haplotypes (Degli-Esposti *et al.*, 1992). In Caucasians, there are 10 to 12 common extended haplotypes that show significant linkage disequilibrium. They are relatively population-specific and are believed to represent the original HLA haplotypes of our ancestors which are still segregating unchanged, and they are easily recognized from their characteristic class III polymorphisms, which are called complotypes. Disease associations with extended haplotypes are generally stronger than allelic associations (Gaudieri *et al.*, 1997). The best examples of extended haplotype associations are those with rheumatoid arthritis, multiple sclerosis, insulin-dependent diabetes mellitus, and systemic lupus erythematosus(Fraser *et al.*, 1990, Christiansen, 1990).

2-3-5: HLA Antigens and Disease Associations

Probably, one of the most striking features of HLA antigens is their association with certain diseases. Both population and family studies have been employed to demonstrate the relationship between alleles encoded by HLA system and various disease states. Such association studies are helpful in understanding the disease, firstly by providing clues for its pathogenesis, and this information may in turn be valuable in relation to diagnosis, treatment and prevention, and secondly the association may give important knowledge about the biological significance of the immunogenetic system being studied (Ad'hiah, 1990).

Since 1973, it has been recognized that HLA antigens play an important role in the aetiology of a number of diseases, and over 40 diseases have well-established associations with HLA markers. These diseases are generally of unknown aetiology and pathogenesis, and are either autoimmune or have immunological abnormalities (Thomson, 1995).

The association can be positive or negative. In positive associations, some HLA alleles occur at highly frequencies in patients with particular diseases than in the general population, while in negative associations, the opposite picture is observed (Svejgaard *et al*., 1983). Most studies are fascinated by the positive associations, although the negative associations can not be ignored, especially if we consider that HLA-B53 allele has been considered as a protective factor against malaria in West Africa (Hill, 1998). The first documented positive association involved an allele of HLA-class I region. It was between HLA-B27 and ankylosing spondylitis, and so far this association is the strongest reported one in this region (Brewerton *et al*., 1973). However, later studies have revealed the importance of HLA-class II alleles in the aetiology of investigated diseases, for instances, HLA-DR4 and rheumatoid arthritis (Winchester, 1995), HLA-DR3 and systemic lupus erythematosus (Kotzin and O'Dell, 1995) and HLA-DR2 and narcolepsy (Thomson, 1995). The class II alleles are further implicated in the aetiopathogenesis of diseases, especially autoimmune disorders, when the methods of DNA sequencing were introduced (Margulies, 1999).

The HLA-disease association studies are based on patient samples randomly selected from the population (population approach). Other studies, and with the aim to map a predisposing gene for a disease, approached the HLA system in families, in which the linkage analyses are employed. However, these studies were less successful in establishing a positive linkage between the investigated diseases and HLA alleles, and a recall to employ the population approach has been supported (Blackwood *et al*., 1996).

The precise mechanism underlying the association of investigated diseases and HLA alleles is not well defined. However, several models have been proposed, including the cross-reactivity of antibodies to microorganisms with particular HLA-molecules and molecular mimicry of T-cell responses to viral antigens for self antigens. Other investigators have speculated that each disease may have its own aetiopathogenetic mechanism, and the HLA system exerts some role in this mechanism (Jones *et al.*, 2006).

2-3-6: HLA Antigens, Gastritis and *H. pylori*

Two approaches have been employed to investigate the role of HLA antigens in the aetiology of gastritis together with *H. pylori* infection. In the first, the association between these antigens and the disease has been investigated, while overexpression of HLA antigens in the gastric mucosa has been the subject of second approach (Naito and Cunha-Cruz, 2005).

Few studies have examined the association between HLA antigens and gastritis, and most of them have linked specific HLA-DQ alleles to gastric diseases, although some inconsistent observations have been reported. Azuma *et al*. (1994) found increased susceptibility for *H. pylori* infection in Japanese patients carrying the HLA-DQA1*0301 allele, whereas those displaying the HLA-DQA1*0102 allele were resistant to the infection; in other words, the HLA-DQA1*0102 allele has a lower frequency in *H. pylori*-positive patients with atrophic gastritis compared with those with superficial gastritis and normal controls (Azuma *et al*., 1998). Conversely, the HLA-DQB1*0401 allele was found to be associated with atrophic gastritis in *H. pylori*-infected patients (Sakai *et al*., 1999). On the other hand, the HLA-DQB1*0301 allele has been found more commonly in Caucasian patients with gastric adenocarcinoma (Lee *et al*., 1996). However, Yoshitake *et al*. (1999) have reported different associations between HLA-class II antigens and *H. pylori*-positive gastritis, and a negative association with HLA-DRB1*1501, -

DQA1*01021 and -DQB1*0602 alleles and a positive association with DPB1*0901 in gastritis have observed in the patients. More recently, Herrera-Goepfert *et al*. (2006) have reported a significant increased frequency of HLA-DQB1*0401 allele in *H. pylori*-positive patients with chronic gastritis.

In the second approach, several studies have demonstrated the over expression of HLA class II molecules in gastric mucosa (Ihan *et al.*, 1995 , Chiba *et al.*, 1995 , Archimandritis *et al.*, 2000) and circulating mononuclear cells (Ohara *et al.*, 2001) in patients with gastric inflammation. It has been reported that gastric epithelial cells, as well as, classical antigen presenting cells such as macrophages express HLA-DR, -DP, and –DQ antigens (Ihan *et al.*, 1995), and that the extent of epithelial HLA-DR expression is parallel with the degree of inflammation and activity (Archimandritis *et al.*, 2000). On the other hand, the expression of HLA-DR was not increased in the lamina propria of gastric mucosa infected with *H. pylori*. Correlation between epithelial HLA-DR expression and *H. pylori* density was also found (Chiba *et al.*, 1995). Combined with the finding that expression of HLA-DR was significantly decreased in *H. pylori*-eradicated patients (Archimandritis *et al.*, 2000), these results suggest that epithelial HLA-DR expression could be a marker of gastric inflammation with *H. pylori* infection. Costimulatory signals provided by antigen presenting cells are required for full T-cell activation. In *H. pylori*-associated gastritis, costimulatory molecules such as CD80 (B7-1), CD86 (B7-2), and intercellular adhesion molecule-1 are expressed on gastric epithelial cells, indicating that epithelial cells function as antigen presenting cells during *H. pylori* infection, and eradication therapy decreased the expression of these molecules (Naito and Cunha-Cruz, 2005).

2-4: Other Parameters Investigated

For a further understanding of chronic gastritis, other genetic and immunological parameters were employed, and theses were blood groups, total and absolute counts of leucocytes and lymphocyte immunophenotypes.

2-4-1: Blood Groups

The fact that people could be classified into groups by antigens on their erythrocytes was first demonstrated by Karl Landesteiner, who found that sera of some people are able to clump (agglutinate) erythrocytes from some, but not all, other people. This led to the discovery of the ABO blood group system, which was the first genetic polymorphism (Nora and Fraser, 1989). Four major phenotypes of ABO blood groups are recognized; A, B, AB and O, which are the products of a gene on chromosome 19. It has been hypothesized that these phenotypes are inherited as products of three alleles (I^A, I^B and I^O) at a single locus. The first two alleles are inherited in a codominant manner, but are both dominant to the allele I^O, which is in a homozygous status gives the phenotype O (Klug and Cummings, 1997).

Besides the practical applications of blood groups in the fields of blood transfusion and forensic medicine, they have played an important role in the development of basic genetics. Furthermore, several studies have shown that certain diseases are associated with particular blood groups more frequently than would be expected by chance. The best documented example of such association is that of duodenal ulcer and blood group O, which was demonstrated in Europeans, Japanese, North American Negroes and several other racial groups (Emery and Muller, 1988). Infective agents are also involved in associations with blood groups. In particular, blood group B is associated with infections of the mucus

membranes of the urinary tract, gastrointestinal tract and respiratory tract (Weir, 1988).

With respect to gastritis and *H. pylori* infection and their relationship to blood groups, most investigations have been concerned with Lewis blood groups and their secretor status. In this regard, Ikehara *et al*. (2001) have demonstrated that gastritis patients positive for anti-*H. pylori* IgG antibodies were positively associated with secretor alleles, while negative association was observed with Lewis alleles. Additionally, Heneghan *et al*. (2001) have examined the presence of anti-Lewis antibodies in patients with *H. pylori* infection and gastric cancer and the relationships between anti-Lewis antibody production, bacterial Lewis expression, gastric histopathology and host Lewis erythrocyte phenotype. Their data suggested that anti-Lewis antibodies are present in most patients with *H. pylori* infection, including those with gastric cancer, and such response was independent of the host Lewis phenotype but was related to the bacterial Lewis phenotype. A further examination of this subject suggests that the H type I structure, synthesized by the secretor enzyme in gastric foveolar cells, and its metabolite Lewis antigen, mediate the adhesion of *H. pylori* to the gastric epithelium, whereas *H. pylori* does not bind to modified forms of Lewis specific for blood types A and B. Such host factors (Lewis and secrtor genotypes and ABO blood type) may affect the establishment of *H. pylori* infection and, and therefore the risk of chronic atrophic gastritis can be increased (Shibata *et al*., 2003). These observations have been confirmed in *H. pylori* seropositive patients with and without gastric cancer (Oba-Shinjo *et al*., 2004; Lee *et al*., 2006).

2-4-2: Total and Absolute Counts of Leucocytes

Investigating the leucocytes numerically, totally and differentially, is still favoured by scientists seeking the understanding of the general immune response in healthy and diseased populations. Leucocytes are a collection of cells involved in

the immune defence mechanisms against non-self antigens that challenge the immune system in forms of viruses, bacteria, fungi and parasites (Barnett *et al.*, 1999). They are originated from the haemopoietic stem cell in the bone marrow, and differentiated into five types; neutrophils, lymphocytes, monocytes, eosinophiles and basophiles, and each is programmed to carry out an immune function. The neutrophiles are mainly involved in a non-specific immune function that is phagocytosis, which is also shared by monocytes, but the latter are involved in antigen presenting, and are known as macrophages or antigen presenting cells. The lymphocytes are the cellular elements in the specific cellular and humoral immune responses, and their counts are affected by the health status of an individual (Lydyard and Grossi, 1998).

2-4-3: Lymphocyte Immunophenotypes

Immunologists, and in their attempts to develop methods for the identification of functionally and developmentally distinct populations of lymphocytes, have recognized cell surface molecules by means of monoclonal antibodies (Abbas *et al.*, 2000). These molecules are specific antigens that mark different cell populations, some are specific for cells of a particular lineage or maturational pathway, and the expression of others varies according to the state of activation or differentiation of the same cells (Peakman and Vergani, 1997). Because several different antibodies (a cluster) could recognize the same surface protein, and because surface proteins indicated the differentiation of a cell, the monoclonal antibodies were assigned a number according to the cluster of differentiation (CD) to which they bound. Therefore, a CD is a surface molecule found on cells according to their lineage and differentiation and identifiable by one or more monoclonal antibodies (Lydyard and Grossi, 1998). Accordingly, several subpopulations of lymphocytes have been identified, and these were mainly CD3+ (pan T-lymphocytes), CD4+ (T-helper lymphocytes), CD8+ (T-cytotoxic

lymphocytes), CD19+ (B-lymphocytes) and CD56+ (natural killer cells) cells (Hyde, 2000). The value of CD markers in classifying lymphocytes is enormous, and has allowed immunologists to identify the cells participating in various immune responses, isolate them, and individually analyze their specificities, response patterns and effector functions (Roitt and Rabson, 2000).

Chapter Three

Subjects, Materials and Methods

3-1: Subjects

Forty-nine patients (27 males and 22 females) with chronic atrophic gastritis were investigated during the period January-October 2006. Their age range was 16-80 years. The disease was clinically diagnosed by the consultant medical staff at Al-Kadhemiyah Teaching Hospital, Department of Gastrointestinal Tract and Liver Diseases. The diagnosis was based on an endoscopy examination and two endoscopic gastric biopsy specimens (antral and corpus mucosa). Therefore, and according to the point of view of the consultants, the patients were clinically subdivided into two groups; antrum and corpus gastritis. Furthermore, the patients were also divided into *Helicobacter pylori*-positive (HP+) and *H. pylori*-negative (HP-) according to the presence of IgG anti-*H. pylori* antibodies in their sera.

Fifty apparently healthy subjects (25 males and 25 females) of blood donors were further investigated, and were considered as a control sample. Their age range was 18-52 years. They were matched with patients for age, sex and ethnic background (Arab Muslims)

3-2: Biological Materials and Kits

- HLA class I and class II antisera and control sera: Biotest, Germany.
- Mouse anti-human CD markers (CD3, CD4, CD8 and CD19): Serotec, U.K.
- Lyophilized rabbit complement: Duxted Rabbit, U.K.
- Blood group kit (anti-A and anti-B antibodies): Biotest, Germany.
- *Helicobacter pylori* IgG ELISA kit: Biohit, U.S.A.
- Foetal Calf Bovine Serum: BioWORLD, U.S.A.

3-3: Solutions

- Normal physiological saline: The solution was ready prepared (ADWIC, Egypt).
- Phosphate buffer saline (PBS): One tablet of PBS (Oxoid, U.K.) was dissolved in 100 ml distilled water. Then, the solution was autoclaved (121ºC., 15 pound/in^2, 20 minutes) and stored in the refrigerator (4ºC) until use.
- Leucocyte diluting solution: Two mls of glacial acetic acid were added to 98 ml of distilled water. Then, one drop of methylen blue was added as a colour indicator (Sood, 1986).
- Trypan blue stain solution (2%): Two grams of trypan blue stain powder were dissolved in 100 ml physiological saline. The stain solution was filtered before use (Ad'hiah, 1990).
- Eosin stain (5%): Five grams of eosin powder were dissolved in 100 ml distilled water. The stain solution was centrifuged (3000 rpm for 10 minutes) before use (Ad'hiah, 1990).
- Leishman's stain: The stain solution was ready-prepared (The Institutes of Sera and Vaccines, Baghdad).
- Fixative Solution: The following solutions were mixed: PBS (8 ml), formalin (33 ml) and acetone (60 ml). The solution was stored in the refrigerator (4ºC) until use (Serotec Data Sheet, 1999).
- Washing Medium: Terasaki medium was supplemented with 5% heat inactivated foetal calf serum (Ad'hiah, 1990).

3-4: Laboratory Methods

3-4-1: Collection of Blood Samples

From each subject, 10 ml of venous blood were collected and divided into two aliquots (2 and 8 ml). The first aliquot was pipetted in plain tube for the collection of serum, which was used in the assessment of IgG anti-*Helicobacter pylori* antibodies. The second aliquot was dispensed in heparinized tube, and used for total and absolute counts of leucocytes and blood group phenotypes, as well as, it was employed for the detection of HLA polymorphism and lymphocyte immunophenotypes. The blood was manipulated for laboratory investigation in less than two hours.

3-4-2: Total and Absolute Counts of Leucocytes

3-4-2-1: Total Count of Leucocytes

The conventional method of blood cell counting was employed, following the procedure of Sood (1986). A volume of 0.02 ml blood was dispensed in a test tube containing 0.38 ml of leucocyte diluting solution, and then the contents were mixed and the tube was left for three minutes. One drop of the diluted blood was applied to the surface of a counting chamber (Neubauer hemocytometer) under the cover slip. After that, the chamber was left for two minutes to settle the cells, and by then, the leucocytes were counted using the following equation:

$$\text{Total Count (cells/L x } 10^9) = \left(\frac{\text{Number of Cells Counted}}{4} \right) \text{x } 0.2$$

3-4-2-2: Absolute Count of Leucocytes

A blood smear was made on a clean slide and left for air drying. Then the slide was stained with Leishman's stain for 2 minutes and buffered for 10 minutes with Leishman's buffer. After that, the slide was rinsed with tap water and left for

air-drying (Sood, 1986). The stained smear was examined under oil immersion power (100x), and at least 200 leucocytes were randomly counted. Then, the percentage of each cell type was obtained. The absolute count of each type of leucocytes was calculated according to the following equation:

$$\text{Absolute Count (cells/L x } 10^9) = \left(\frac{\text{Percentage of Cells x Total Count}}{100} \right)$$

3-4-3: HLA Phenotyping

3-4-3-1: Principles

The test was performed in the Histocompatibility Laboratory of Al-Karama hospital in Baghdad. The most widely used procedure for a serological detection of HLA antigens is the microlymphocytotoxicity test, which was developed by Terasaki and McClelland (1964) and standardized in agreement with the National Institute of Allergy and Infectious Diseases. The test is a complement dependent reaction, in which antibodies recognize antigens on the surface of lymphocytes and form antigen-antibody complexes. The formed complexes thus are able to activate the added complement which results in death of reacted cells. Then, by a dye exclusion technique, it is possible to score the reactions and to determine the HLA phenotype (Ad'hiah, 1990).

3-4-3-2: Isolation of lymphocytes

By means of a density gradient centrifugation (Ad'hiah, 1990), the lymphocytes were isolated from the whole blood (section 2.4.1). The following steps were followed:

- The blood (8 ml) was centrifuged (1000 rpm) for 15 minutes.

- The plasma, buffy coat and the most upper layer of erythrocytes were collected in a 10 ml centrifuge tube, and the cell-suspension was diluted with physiological saline up to 10 ml.
- The diluted cell suspension (5 ml) was layered on 3 ml of Ficoll-isopaque separation fluid (lymphoprep; specific gravity = 1.077).
- The tubes were centrifuged (2100 rpm) for 30 minutes in a cooled centrifuge.
- After centrifugation, the lymphocytes were visible as cloudy band between the plasma and lymphoprep layers.
- The band was collected in a 10 ml test tube, and the cells were suspended in washing medium (5 ml).
- The tube was centrifuged (2000 rpm) for 5 minutes (first wash), then the supernatant was discarded, and the cells were re-suspended in 5 ml washing medium. This step was repeated (second wash).
- A third wash was also done but at a lower speed (1000 rpm) and for 10 minutes. This step is very necessary, because it helps to maintain must of the platelets in the supernatant.
- The obtained cells were suspended in 2 ml Terasaki medium supplemented with 10% heat inactivated (56ºC for 30 minutes) foetal calf serum.

3-4-3-3: Isolation of B Lymphocytes

HLA-class II antigens (HLA-DR) have a limited tissue distribution, being mainly expressed on the surface of B lymphocytes. This led to the consideration of B cells as the best in the serological detection of HLA-DR antigens. Therefore, there is a requirement to have a cell suspension rich in B cells and depleted from other populations of lymphocytes. One of the main methods that are employed is the adherence of B cells to nylon-wool (positive selection).The method is outlined in the following steps:

- About 0.15g of nylon-wool was loosely packed in the barrel of a 2ml disposable syringe. The nylon wool was rinsed with warm (37ºC) washing medium two times, and an avoidance of making air bubbles in the nylon wool was considered.
- The lymphocytes suspension (section 2.4.3.2) was transferred to the barrel of nylon-wool, and both ends of the syringe barrel were sealed with a parafilm. The syringe barrel was incubated for 30 minutes at 37ºC.
- After incubation, the parafilm was removed and the nylon-wool was rinsed several times with warm washing medium (about 10 ml).
- The washing medium was collected in a 10 ml centrifuge tube, which was centrifuged (2000 rpm) for 5 minutes to collect the T lymphocytes, which were used for phenotyping of HLA-class I antigens.
- The nylon-wool adherent B-lymphocytes were isolated by adding a warm washing medium (10 ml) to the syringe barrel, and the nylon-wool was squeezed by the syringe piston several times.
- The washing medium was collected in a 10ml centrifuge tube, which was centrifuged (2000 rpm) for 5 minutes to collect B-lymphocytes, which were used in the phenotyping of HLA-DR antigens.

3-4-3-4: Counting of lymphocytes and Assessment of Viability

An aliquot of 10 µl of cell suspension (section 2.4.3.3) was mixed with 90 µl of trypan blue for 3 minutes, and then the viability was determined according to the following equation:

$$\text{Viability (\%)} = \left(\frac{\text{Number of Living Cells}}{\text{Total Number of Cells}} \right) \text{x } 100$$

At the same time, the numbers of T- and B-lymphocytes were counted, and the cell concentration was adjusted to 2-3 x 10^6 cells/ml.

3-4-3-5: Preparation of Typing Plates

The plates were prepared in advance in batches of 50 plates each time. Terasaki plates (60 wells) were filled with neutral oil (liquid paraffin), and in each well, 1 µl of HLA antiserum was dispensed. Then the plates were covered with lids and stored in a freezer (-20ºC) until required. The wells contained antisera specific for HLA-class I (A and B) and -class II (DR) antigens, which were available in the Histocompability laboratory at Al-Karama hospital (Table 3-1).

Table 3-1: Listing of used HLA antisera in the present study.

HLA –A	HLA-B	HLA-DR
A1	B5	DR1
A2	B7	DR2
A3	B8	DR3
A9	B12	DR4
A10	B13	DR7
A11	B14	DR8
A19	B15	DR9
A28	B16	DR10
	B17	DR52
	B18	
	B21	
	B22	
	B27	
	B35	
	B37	
	B40	

3-4-3-6: Serological Typing

Before serological typing of HLA antigens, two typing plates (one for class I and the other for class II antigens) were obtained from the freezer and left for thawing at room temperature (25ºC) for 15 minutes. To each well in the plate, 1µl of cell suspension (2000-3000 cells) was added using Hamilton syringe. The plate was incubated (25ºC) for 30 minutes (HLA-class I antigens) or 60 minutes (HLA-class II antigens). After incubation, 5 µl of rabbit complement were added to each well, and a further incubation (25ºC) was carried out for 60 minutes (HLA-class I antigens) or 120 minutes (HLA-class II antigens). Then, 2 µl of eosin were added, and after 5 minutes, 4 µl of formaldehyde were added to each well. The plates were left to the next day for reading.

3-4-3-7: Reading the Plates

Each well in the plate was examined, using a phase-contrast inverted microscope, to score the percentage of cell death (percentage of eosin stained cells). The score ranges are given in table 3-2.

Table 3-2: The score ranges of reaction in the microlymphocytotoxity test.

Score	Reaction	Percentage of Cell Death
1	Negative	Same viability as negative control.
2	Doubtful negative	10-19% killed lymphocytes.
4	Weak positive	20-39% killed lymphocytes.
6	Positive	40-79% killed lymphocytes.
8	Strong positive	80-100% killed lymphocytes.
0	invalid	Reaction cannot be accurately read.

3-4-4: Blood Group Phenotyping

Blood group phenotypes (A, B, AB, and O) were determined by employing the conventional method presented by Sood (1986). The method is based on agglutination reactions between blood group specific antisera and blood group antigens which found on the cell surface of erythrocytes.

3-4-5: Lymphocyte Immunophenotyping for CD markers

3-4-5-1: Principles

Lymphocytes express a large number of different molecules on their surfaces, which can be used to distinguish cell subsets. Many of these cell markers can be identified by specific monoclonal antibodies. A systematic nomenclature has been developed in which the term CD (cluster designation) refers to groups of monoclonal antibodies that bind specifically to particular markers (Lydyard and Grossi, 1998). In the present study, four CD markers were investigated: CD3 (Pan T-lymphocytes), CD4 (T-helper lymphocytes), CD8 (T-cytotoxic lymphocytes) and CD19 (B-lymphocytes).

3-4-5-2: Procedure

The isolated lymphocytes prepared in section (2.4.3.2) were adjusted to a cell count of 1 x 10^6 cell /ml, and 10 µl of the cell suspension were dispensed in the well of a CD marker specific slide. Then, the slide was left for air-drying at room temperature for 30 minutes. After drying, 10 µl of the fixative solution were added to each well on the slides and left for air-drying (about 2 minutes) at room temperature .Then, the slide was covered with Aluminum foil and stored in the freezer (-20ºC).

Before carrying out the procedure of CD typing, the slide was taken out from the freezer and left for thawing at room temperature for 15 minutes. Then, 10 µl of a specific anti-CD marker antibody (Fluorescin Isothiocyanate labeled monoclonal

antibody) were added to the well, and the slide was incubated at room temperature in a humid chamber for 60 minutes. After incubation, the well was washed with phosphate buffer saline to remove any unreacted antibody. The slide was air-dried and examined under fluorescent microscope to score the percentage of fluorescent cells (Serotec Data Sheet, 1999).

3-4-6: Assessment of Serum IgG Antibodies to *Helicobacter pylori*

The *H. pylori* IgG antibodies kit (Biohit, Plc., Finland) is a microplate-based qualitative enzyme immunoassay (ELISA) for the determination of human *H. pylori* IgG antibodies from serum or plasma samples. This *H. pylori* test is based on an enzyme immunoassay technique with partially purified *H. pylori* bacterial antigen adsorbed on a microplate and a detection antibody labelled with horseradish peroxidase (HRP) (*Helicobacter pylori* IgG ELISA kit insert, 2003).

3-4-6-1: Kit Contents and Reagent Preparations

- Microplate precoated with partially purified *H. pylori* bacterial antigen.
- Washing Buffer: The phosphate buffer concentrate (10X), containing Tween 20 and preservative, was diluted with distilled water (100 ml phosphate buffer concentrate + 900 ml distilled water) to prepare the washing buffer.
- Diluent Buffer: It was phosphate buffer containing protein, Tween 20, preservative and red dye extract, and was ready to use.
- Calibrator: It was one vial containing 1.5 ml of human serum-based *H. pylori* IgG calibrator with preservative.
- Negative Control: It was one vial containing 1.5 ml of human serum-based *H. pylori* IgG negative control with preservative.
- Positive Control: It was one vial containing 1.5 ml of human serum-based *H. pylori* positive control with preservative.

- Conjugate Solution: It was 0.15 ml of HRP-conjugated monoclonal anti-human IgG in stabilizing buffer. The solution was diluted with diluent buffer (40 µl conjugate solution + 3960 µl diluent buffer) before use.
- Substrate Solution: It was 15 ml of tetramethylbenzidine in buffer containing preservative.
- Stop Solution: It was 15 ml of 0.1 mol/L sulphuric acid.
- Incubation Covers: They were four plastic sheets to cover the microplate during incubation.

3-4-6-2: Test Procedure

Before carrying out the test, the plate and all reagents were obtained from the refrigerator (4ºC) and allowed to reach the room temperature (20-25º). As recommended by the manufacturer, the calibrator, controls and samples were applied on the plate as duplicates; also it was necessary to use the calibrator and controls in each test run. The procedure included the following steps:

- Serum Sample Dilution: The serum sample was diluted with diluent buffer (5µl serum + 995 µl diluent buffer), and mixed well.
- Aliquots of 100 µl of the diluent buffer (Blank), the calibrator (CAL), the negative control (NC), positive control (PC) and diluted samples were pipetted into the wells as duplicates. The plate was covered with the incubation cover, and incubated for 30 minutes at 37°C. The samples were dispensed into the wells within 10 minutes to avoid assay drift within the plate.
- The wells were washed with the diluted washing buffer, and the plate was inverted and gently tapped on a clean paper towel.
- Aliquots of 100 µl of the substrate solution were pipetted into the wells, and the plate was incubated for 30 minutes at room temperature (20-25°C). A direct exposure to light during incubation was avoided.

- The washing step was repeated.
- Aliquots of 100 µl of the stop solution were pipetted into the wells.
- The absorbance of each well was measured at 450 nm using ELISA Reader.

3-4-6-3: Calculation and Interpretation of the Results

The mean absorbance (A) value for the calibrator, controls and patient samples assayed in duplicate was calculated. Then, the enzyme immuno units (EIU) were calculated using the following equation:

$$\text{Sample EIU} = [(\text{Sample A} - \text{Blank A}) \div (\text{Calibrator A} - \text{Blank A})] \times 100$$

Depending on the value of sample EIU, the serum was considered negative (sample EIU < 34) or positive (sample EIU > 42), while sample EIU of 34-42 was considered as a border line (cut-off value ~38 EIU).

3-5: Statistical Analysis

The statistical manipulations included two main analyses, which were evaluation of HLA and disease association and assessment of significance between means of the other parameters. These analyses are presented by Ad'hiah (1990).

3-5-1: HLA and Disease Associations

The association between a marker and a disease was expressed in terms of relative risk (RR), etiological fraction (EF) and preventive fraction (PF). The RR value can range from less than one (negative association) to more than one (positive association). If the association was positive, the EF was calculated, while if it was negative, the PF was calculated.The significance of such association (positive or negative) was assessed by Fisher's exact probability (P), which was

corrected for the number of antigens tested at each HLA locus (8, 16 and 8 for A, B and DR loci, respectively), therefore, only significant corrected probability (Pc) was considered. A similar approach was adopted for blood group analysis, in which the P correction factor was 4.The mathematical calculations of such parameters are as the following:

$$RR = \left(\frac{a \times d}{b \times c} \right)$$

$$EF = \left(\frac{RR - 1}{RR} \right) x \left(\frac{a}{a + b} \right)$$

$$PF = \left(\frac{1 - RR\left(\frac{a}{a+b} \right)}{RR\left(1 - \frac{a}{a+b} \right) + \left(\frac{a}{a+b} \right)} \right)$$

- a: number of patients positive for the antigen
- b: number of patients negative for the antigen.
- c: number of controls positive for the antigen
- d: number of controls negative for the antigen.

The Fisher's exact probability was calculated using the computer program; Statistical Package for Social Sciences (SPSS) version 10.0.

3-5-3: Differences between Means

For total and absolute counts of leucocytes and lymphocyte immunophenotypes, the means ± standard errors (S.E.) were given. Differences between means of patients and controls were assessed by ANOVA test and the Least Significant Difference (LSD), and accordingly three significant levels of probability were considered ($P \leq 0.05$, 0.01 and 0.001). These analyses were carried out using the computer program SPSS.

Chapter Four

Results

4-1: Subgroups of Patients

Based on endoscopic evaluations and histopathalogical examinations of stomach mucosa biopsies, the patients were divided into corpus and antrum gastritis. The corpus patients were 31 subjects (63.3%), while antrum patients included 18 subjects (36.7%). The patients were also further divided into *Helicobacter pylori*-positive (HP+) and *H. pylori*-negative (HP-) according to the presence of IgG anti-*H. pylori* antibodies in their sera. There were 32 patients (65.3%) positive for IgG anti-*H. pylori* antibodies, while 17 patients (34.7%) were sero-negative for IgG anti-*H. pylori* antibodies (Figure 4-1).

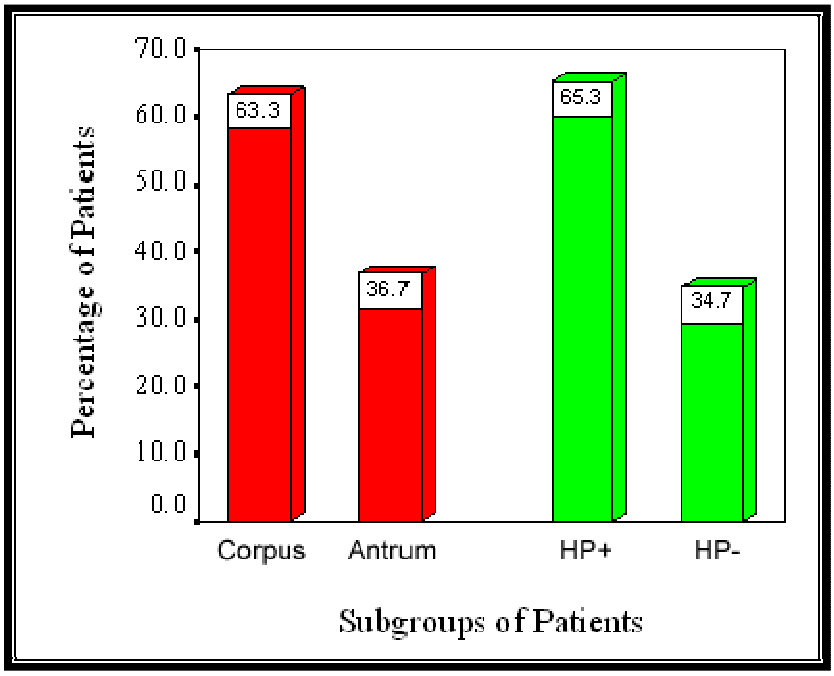

Figure 4-1: Percentages of patients in the clinical subgroups (corpus and antrum gastritis) and subgroups based on a serum IgG anti *H. pylori* antibody detection (HP+ and HP- gastritis).

4-2: HLA Antigens

4-2-1: HLA-A Locus

Observed numbers and percentage frequencies of HLA-A antigens in total gastritis patients, corpus gastritis, antrum gastritis, *H. pylori*-positive (HP+) gastritis and *H. pylori*-negative (HP-) gastritis are given in table 4-1, while antigens showing significant variations between patients and controls are summarized in table 4-4.

- **Total Gastritis Patients**: Two antigens (A3 and A19) showed differences between patients and controls. The antigen A3 was significantly (corrected probability; Pc = 0.04) increased in the patients (42.86 *vs*. 22.00%), and such difference was associated with a relative risk (RR) value of 2.66 and an etiological fraction (EF) value of 0.27. In contrast, the antigen A19 was significantly ($Pc = 4.02 \times 10^{-4}$) decreased in the patients (12.24 *vs*. 46.00%), and such variation was associated with a preventive fraction (PF) of 0.38.
- **Corpus Gastritis**: Two antigens (A9 and A19) showed differences between patients and controls. The antigen A9 was significantly (Pc = 0.03) increased in the patients (55.56 *vs*. 24.00%), and such difference was associated with RR value of 3.96 and EF value of 0.42. In contrast, none of the patients was positive for the antigen A19, while its frequency in the controls was 46.00%. Such difference was highly significant ($Pc = 2.69 \times 10^{-4}$)**.**
- **Antrum Gastritis**: Two antigens (A3 and A19) showed differences between patients and controls. The antigen A3 was significantly (Pc = 0.03) increased in the patients (48.39 *vs*. 22.00%), and such difference was associated with RR value of 3.36 and EF value of 0.34. In contrast, the antigen A19 was significantly (Pc = 0.03) decreased in the patients (19.35 *vs*. 46.00%). The PF value of such variation was 0.33.

- ***H. pylori*-positive Gastritis**: The antigen A19 showed a decreased frequency in the patients (12.50 *vs.* 46.00%). Such difference was significant and associated with a Pc value of 2.65×10^{-3}, moreover the PF value was 0.38.
- ***H. pylori*-negative Gastritis**: As in *H. pylori*-positive gastritis, the antigen A19 showed a decreased frequency in the patients (11.76 *vs.* 46.00%), but a less significant level of corrected probability (Pc = 0.02) was recorded. However, the EF value was similar (EF = 0.39).

4-2-2: HLA-B Locus

Observed numbers and percentage frequencies of HLA-B antigens in total gastritis patients, corpus gastritis, antrum gastritis, *H. pylori*-positive (HP+) gastritis and *H. pylori*-negative (HP-) gastritis are given in table 4-2, while antigens showing significant variations between patients and controls are summarized in table 4-4.

- **Total Gastritis Patients**: The antigen B17 was significantly (Pc = 0.05) increased in the patients (14.29 *vs.* 2.00%). Such deviation was associated with RR value of 8.17 and EF value of 0.13.
- **Corpus Gastritis**: There were no significant variations in HLA-B antigen frequencies between patients with corpus gastritis and controls.
- **Antrum Gastritis**: The antigen B17 was significantly (Pc = 0.02) increased in the patients (19.35 *vs.* 2.00%). Such deviation was associated with RR value of 11.76 and EF value of 0.18.
- ***H. pylori*-positive Gastritis**: As in antrum gastritis patients, the antigen B17 was significantly (Pc = 0.03) increased in the patients (18.75 *vs.* 2.00%). Such deviation was associated with RR value of 11.31 and EF value of 0.17.

- ***H. pylori*-negative Gastritis**: There were no significant variations in HLA-B antigen frequencies between patients with corpus *H. pylori*-negative gastritis and controls.

4-2-3: HLA-DR Locus

Observed numbers and percentage frequencies of HLA-DR antigens in total gastritis patients, corpus gastritis, antrum gastritis, *H. pylori*-positive (HP+) gastritis and *H. pylori*-negative (HP-) gastritis are given in table 4-3, while antigens showing significant variations between patients and controls are summarized in table 4-4.

- **Total Gastritis Patients**: Two antigens (DR3 and DR10) showed significant (Pc = 0.02 and 2.72 x 10^{-5}, respectively) variations between patients and controls. Both antigens showed increased frequencies in the patients (DR3: 38.78 *vs*. 16.00%; DR10: 42.86 *vs*. 6.00%). The associated RR values of such variations were 3.33 and 11.75, respectively, while EF values were 0.27 and 0.39, respectively.
- **Corpus Gastritis**: Two antigens (DR2 and DR10) showed differences between patients and controls. The antigen DR2 was significantly (Pc = 0.04) increased in the patients (50.00 *vs*. 20.00%), and such difference was associated with RR value of 4.00 and EF value of 0.38. Similarly the antigen DR10 was significantly (Pc = 1.2 x 10^{-3}) increased in the patients (44.44 *vs*. 6.00%), but such difference was associated with higher values of RR and EF (12.53 and 0.41, respectively).
- **Antrum Gastritis**: The patients of antrum gastritis shared the theme of total gastritis patients, in which two antigens (DR3 and DR10) showed significant (Pc = 0.01 and 2.61 x 10^{-4}, respectively) variations between patients and controls. Both antigens showed increased frequencies in the patients (DR3:

45.16 *vs*. 16.00%; DR10: 41.94 *vs*. 6.00%). The associated RR values of such variations were 4.32 and 11.31, respectively, while EF values were 0.35 and 0.38, respectively.

- ***H. pylori*-positive Gastritis**: As in antrum gastritis patients, two antigens (DR3 and DR10) showed differences between patients and controls. The antigen DR3 was significantly (Pc = 0.03) increased in the patients (40.63 *vs.* 16.00%), and such difference was associated with RR value of 3.59 and EF value of 0.29, similarly the antigen DR10 was significantly (Pc = 2.57×10^{-3}) increased in the patients (34.38 *vs*. 6.00%), but such difference was associated with higher values of RR and EF (8.21 and 0.30, respectively).
- ***H. pylori*-negative Gastritis**: The variation in patients with *H. pylori*-negative gastritis was restricted to the antigen DR3. Such antigen was significantly (Pc = 3.12×10^{-5}) increased in the patients (58.82 *vs*. 6.00%). The RR value of such variation was 22.38, which contributed in EF value of 0.56.

4-2-4: Immunogenetic Heterogeneity of Gastritis

In order assess the immunogenetic heterogeneity of gastritis, the antigen frequencies of HLA-A, -B and -DR alleles were compared between the subgroups of gastritis patients.

- **Corpus Gastritis *vs.* Antrum Gastritis**: Ten antigens (A3, A9, A10, A11, A19, B12, B17, B35, DR2 and DR3) showed variations between the two subgroups of gastritis (Tables 4-1, 4-2 and 4-3), but a significant corrected probability (Pc = 0.04) was recorded for A9 only. Such antigen was observed in 55.66% of corpus gastritis patients, while its frequency in antrum gastritis patients was 22.58.
- ***H. pylori*-positive Gastritis *vs.* *H. pylori*-negative Gastritis**: Six antigens (A1, A2, B7, A11, B17, DR8 and DR10) showed variations between the two

subgroups of gastritis (Tables 4-1, 4-2 and 4-3). However, none of these variations reached a significant corrected probability ($Pc > 0.05$).

Table 4-1: Observed numbers and percentage frequencies of HLA-A antigens in gastritis patients (total and sub groups) and controls.

HLA-A Antigens	Controls (No. = 50)		Gastritis Patients									
			Total (No. = 49)		Corpus (No. = 31)		Antrum (No. = 18)		HP+ (N0. = 32)		HP- (No. = 17)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
A1	8	16.00	14	28.57	6	33.33	8	25.81	8	25.00	6	35.29
A2	15	30.00	15	30.61	6	33.33	9	29.03	11	34.38	4	23.53
A3	11	22.00	21	42.86	6	33.33	15	48.39	13	40.63	8	47.06
A9	12	24.00	17	34.69	10	55.56	7	22.58	12	37.50	5	29.41
A10	4	8.00	7	14.29	1	5.56	6	19.35	4	12.50	3	17.65
A11	11	22.00	8	16.33	4	22.22	4	12.90	5	15.63	3	17.65
A19	23	46.00	6	12.24	0	0.00	6	19.35	4	12.50	2	11.76
A28	2	4.00	1	2.04	0	0.00	1	3.23	0	0.00	1	5.88

HP+: *H. pylori* positive; HP-: *H. pylori* negative

Table 4-2: Observed numbers and percentage frequencies of HLA-B antigens in gastritis patients (total and sub groups) and controls.

HLA-B Antigens	Controls (No. = 50)		Gastritis Patients									
			Total (No. = 49)		Corpus (No. = 31)		Antrum (No. = 18)		HP+ (N0. = 32)		HP- (No. = 17)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
B5	17	34.00	9	18.37	4	22.22	5	16.13	6	18.75	3	17.65
B7	2	4.00	3	6.12	1	5.56	2	6.45	1	3.13	2	11.76
B8	4	8.00	1	2.04	0	0.00	1	3.23	0	0.00	1	5.88
B12	8	16.00	11	22.45	7	38.89	4	12.90	7	21.88	4	23.53
B13	3	6.00	3	6.12	1	5.56	2	6.45	2	6.25	1	5.88
B14	5	10.00	2	4.08	0	0.00	2	6.45	1	3.13	1	5.88
B15	5	10.00	2	4.08	1	5.56	1	3.23	1	3.13	1	5.88
B16	1	2.00	6	12.24	2	11.11	4	12.90	4	12.50	2	11.76
B17	1	2.00	7	14.29	1	5.56	6	19.35	6	18.75	1	5.88
B18	2	4.00	3	6.12	0	0.00	3	9.68	2	6.25	1	5.88
B21	2	4.00	5	10.20	1	5.56	4	12.90	4	12.50	1	5.88
B22	0	0.00	1	2.04	0	0.00	1	3.23	0	0.00	1	5.88
B27	1	2.00	2	4.08	2	11.11	0	0.00	1	3.13	1	5.88
B35	10	20.00	15	30.61	8	44.44	7	22.58	10	31.25	5	29.41
B37	1	2.00	3	6.12	1	5.56	2	6.45	2	6.25	1	5.88
B40	1	2.00	1	2.04	0	0.00	1	3.23	1	3.13	0	0.00

HP+: *H. pylori* positive; HP-: *H. pylori* negative

Table 4-3: Observed numbers and percentage frequencies of HLA-DR antigens in gastritis patients (total and sub groups) and controls.

HLA-DR Antigens	Controls (No. = 50)		Gastritis Patients									
			Total (No. = 49)		Corpus (No. = 31)		Antrum (No. = 18)		HP+ (N0. = 32)		HP- (No. = 17)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
DR1	7	14.00	7	14.29	2	11.11	5	16.13	4	12.50	3	17.65
DR2	10	20.00	17	34.69	9	50.00	8	25.81	12	37.50	5	29.41
DR3	8	16.00	19	38.78	5	27.78	14	45.16	13	40.63	6	35.29
DR4	6	12.00	5	10.20	2	11.11	3	9.68	3	9.38	2	11.76
DR7	7	14.00	1	2.04	0	0.00	1	3.23	1	3.13	0	0.00
DR8	5	10.00	3	6.12	1	5.56	2	6.45	1	3.13	2	11.76
DR9	0	0.00	1	2.04	0	0.00	1	3.23	1	3.13	0	0.00
DR10	3	6.00	21	42.86	8	44.44	13	41.94	11	34.38	10	58.82

HP+: *H. pylori* positive; HP-: *H. pylori* negative

Table 4-4: HLA-A, -B and -DR antigens showing significant variations between gastritis patients (total and subgroups) and controls.

HLA Antigens	Patients		Controls		Relative Risk	EF or PF	Probability	Corrected Probability
	No.	%	No.	%				
	Total Gastritis (No. = 49) *vs.* Controls (No. = 50)							
A3	21	42.86	11	22.00	2.66	0.27	5.50×10^{-3}	0.04
A19	6	12.24	23	46.00	0.16	0.38	5.03×10^{-5}	4.02×10^{-4}
B17	7	14.29	1	2.00	8.17	0.13	3.44×10^{-3}	0.05
DR3	19	38.78	8	16.00	3.33	0.27	2.50×10^{-3}	0.02
DR10	21	42.86	3	6.00	11.75	0.39	3.43×10^{-6}	2.72×10^{-5}
	Corpus Gastritis (No. = 31) *vs.* Controls (No. = 50)							
A9	10	55.56	12	24.00	3.96	0.42	4.13×10^{-3}	0.03
A19	0	0.00	23	46.00	-	-	3.36×10^{-5}	2.69×10^{-4}
DR2	9	50.00	10	20.00	4.00	0.38	4.63×10^{-3}	0.04
DR10	8	44.44	3	6.00	12.53	0.41	1.50×10^{-4}	1.20×10^{-3}
	Antrum Gastritis (No. = 18) *vs.* Controls (No. = 50)							
A3	15	48.39	11	22.00	3.36	0.34	3.38×10^{-3}	0.03
A19	6	19.35	23	46.00	0.28	0.33	3.25×10^{-3}	0.03
B17	6	19.35	1	2.00	11.76	0.18	1.44×10^{-3}	0.02
DR3	14	45.16	8	16.00	4.32	0.35	1.20×10^{-3}	0.01
DR10	13	41.94	3	6.00	11.31	0.38	3.26×10^{-5}	2.61×10^{-4}
	***H. pylori* Positive Gastritis (No. = 32) *vs.* Controls (No. = 50)**							
A19	4	12.50	23	46.00	0.17	0.38	3.31×10^{-4}	2.65×10^{-3}
B17	6	18.75	1	2.00	11.31	0.17	1.63×10^{-3}	0.03
DR3	13	40.63	8	16.00	3.59	0.29	3.38×10^{-3}	0.03
DR10	11	34.38	3	6.00	8.21	0.30	3.21×10^{-4}	2.57×10^{-3}
	***H. pylori* Negative Gastritis (No. = 17) *vs.* Controls (No. = 50)**							
A19	2	11.76	23	46.00	0.16	0.39	2.50×10^{-3}	0.02
DR10	10	58.82	3	6.00	22.38	0.56	3.91×10^{-6}	3.12×10^{-5}

4-3: Blood Groups

The observed numbers and percentage frequencies of blood group phenotypes in gastritis patients (total and subgroups) and controls are given in table 4-5, while phenotypes showing variations between patients and controls are given in table 4-6.

As shown in the tables a significant increased frequency of blood group A was observed in total gastritis patients as compared to controls (46.7 *vs*. 18%). Such difference was significant (Pc = 0.02) and associated with RR and EF values of 3.98 and 0.34, respectively. In contrast, the blood group O was decreased in patients (22.2 *vs*. 48%) and the difference was also significant (Pc = 0.03). A similar picture was drawn in patients with corpus gastritis, but neither differences were significant. However, the patients with antrum gastritis restored the observation of total gastritis patients, in which blood groups A and O were significantly (Pc = 0.02 and 0.01, respectively) increased (50 *vs*. 18%) and decreased (13.3 *vs*. 48%), respectively. Moreover, the increased frequency of blood group A was associated with higher values of RR and EF (4.55 and 0.39, respectively), while the PF value of blood group O was 0.40.

The *H. pylori* positive patients also showed a significant (Pc = 0.03) increased frequency of blood group A (46.4 *vs*. 18%) as compared to controls. Such deviation was associated with RR and EF values of 3.94 and 0.34, respectively. In contrast, the blood group O was significantly decreased in patients (25 *vs*. 48%), and the difference reached a PF value of 0.36, however, the probability was significant before correction (P = 0.024; Pc > 0.05). The *H. pylori* negative patients shared the theme of *H. pylori* positive patients, and an increased frequency of blood group A (47.1 *vs*. 18%) and a decreased frequency of blood group O were observed [17.6 *vs*. 48%] in the patients. The RR value of blood group A was 4.04, while the PF value of blood group O was 0.36. However, both variations were significant before correction (P = 0.045 and 0.025, respectively).

Table 4-5: Observed numbers and percentage frequencies of blood group phenotypes in gastritis patients (total and sub groups) and controls.

Blood Groups	Controls (No. = 50)		Gastritis Patients									
			Total (No. = 4٥)		Corpus (No. = 1٥)		Antrum (No. = 3٠)		HP+ (No. = ٢٨)		HP- (No. = 17)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
A	9	18.0	٢١	46.7	٦	40.0	١٥	50.0	١٣	46.4	٨	47.1
B	11	22.0	١١	24.4	٢	13.3	٩	30.0	٦	21.4	٥	29.4
AB	6	12.0	٣	6.7	١	6.7	٢	6.7	٢	7.2	١	5.9
O	24	48.0	١٠	22.2	٦	40.0	٤	13.3	٧	25.0	٣	17.6

HP+: *H. pylori* positive; HP-: *H. pylori* negative

Table 4-6: Blood group phenotypes showing variations between gastritis patients (total and subgroups) and controls.

Blood Groups	Patients		Controls		Relative Risk	EF or PF	Probability	Corrected Probability
	No.	%	No.	%				
	Total Gastritis (No. = 45) *vs.* Controls (No. = 50)							
A	٢١	46.7	9	18.0	3.98	0.34	5.14×10^{-3}	0.02
O	١٠	22.2	24	48.0	0.31	0.33	7.71×10^{-3}	0.03
	Antrum Gastritis (No. = 15) *vs.* Controls (No. = 50)							
A	٦	40.0	9	18.0	3.03	0.26	N.S.	N.S.
O	٦	40.0	24	48.0	0.72	0.13	N.S.	N.S.
	Corpus Gastritis (No. = 30) *vs.* Controls (No. = 50)							
A	١٥	50.0	9	18.0	4.55	0.39	5.92×10^{-3}	0.02
O	٤	13.3	24	48.0	0.16	0.40	2.68×10^{-3}	0.01
	***H. pylori* Positive Gastritis (No. = 28) *vs.* Controls (No. = 50)**							
A	١٣	46.4	9	18.0	3.94	0.34	8.44×10^{-3}	0.03
O	٧	25.0	24	48.0	0.36	0.30	0.024	N.S.
	***H. pylori* Negative Gastritis (No. = 17) *vs.* Controls (No. = 50)**							
A	٨	47.1	9	18.0	4.04	0.35	0.045	N.S.
O	٣	17.6	24	48.0	0.23	0.36	0.025	N.S.

N.S.: Not significant

4-4: Total and Absolute Counts of Leucocytes

The total leucocyte counts showed approximated means (range: 6.4-6.8 x 10^9 cell/L) in gastritis patients (total and subgroups) and controls, and therefore no significant ($P > 0.05$) difference was observed (Table 4-7). The absolute counts of neutrophils (Table 4-8), lymphocytes (Table 4-9), monocytes (Table 4-10) and

eosinophils (Table 4-11) did not show significant differences between patients and controls, or between the subgroups of patients. In this regard two exceptions were encountered, in which a decreased count of lymphocytes (1.70 *vs*. 2.10 x 10^9 cell/L) and an increased count of monocytes (0.36 *vs*. 0.27 x 10^9 cell/L) were observed in corpus gastritis as compared to antrum gastritis.

Table 4-7: Total leucocyte count in gastritis patients (total and subgroups) and controls.

Groups		Number	x 10^9 cell/L (Mean ± S.E.)	Probability * I ≤	Probability ** II ≤
Controls		50	6.6 ± 0.2		
Gastritis Patients	Total	45	6.7 ± 1.5	N.S.	
	Corpus	17	6.8 ± 2.1	N.S.	N.S.
	Antrum	28	6.5 ± 1.6	N.S.	
	H. pylori-positive	30	6.8 ± 1.7	N.S.	N.S.
	H. pylori-negative	15	6.4 ± 1.9	N.S.	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs. H. pylori*-negative).

N.S.: Not significant.

Table 4-8: Total neutrophil count in gastritis patients (total and subgroups) and controls.

Groups		Number	x 10^9 cell/L (Mean ± S.E.)	Probability * I ≤	Probability ** II ≤
Controls		50	4.1 ± 0.1		
Gastritis Patients	Total	45	4.2 ± 0.2	N.S.	
	Corpus	17	4.5 ± 0.4	N.S.	N.S.
	Antrum	28	4.0 ± 0.2	N.S.	
	H. pylori-positive	30	4.3 ± 0.3	N.S.	N.S.
	H. pylori-negative	15	4.0 ± 0.4	N.S.	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs. H. pylori*-negative).

N.S.: Not significant.

Table 4-9: Total lymphocyte count in gastritis patients (total and subgroups) and controls.

Groups		Number	x 10^9 cell/L (Mean ± S.E.)	Probability * I ≤	Probability ** II ≤
Controls		50	2.06 ± 0.77		
Gastritis Patients	Total	45	1.96 ± 0.11	N.S.	
	Corpus	17	1.75 ± 0.11	N.S.	0.01
	Antrum	28	2.10 ± 0.13	N.S.	
	H. pylori-positive	30	1.97 ± 0.12	N.S.	N.S.
	H. pylori-negative	15	1.95 ± 0.14	N.S.	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs. H. pylori*-negative).

N.S.: Not significant.

Table 4-10: Total monocyte count in gastritis patients (total and subgroups) and controls.

Groups		Number	x 10^9 cell/L (Mean ± S.E.)	Probability * I ≤	Probability ** II ≤
Controls		50	0.32 ± 0.19		
Gastritis Patients	Total	45	0.30 ± 0.02	N.S.	
	Corpus	17	0.36 ± 0.04	N.S.	0.04
	Antrum	28	0.27 ± 0.03	N.S.	
	H. pylori-positive	30	0.30 ± 0.03	N.S.	N.S.
	H. pylori-negative	15	0.30 ± 0.04	N.S.	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs. H. pylori*-negative).

N.S.: Not significant.

Table 4-11: Total eosinophil count in gastritis patients (total and subgroups) and controls.

Groups		Number	x 10^9 cell/L (Mean ± S.E.)	Probability * I ≤	Probability ** II ≤
Controls		50	0.18 ± 0.14		
Gastritis Patients	Total	45	0.15 ± 0.03	N.S.	
	Corpus	17	0.14 ± 0.02	N.S.	N.S.
	Antrum	28	0.16 ± 0.03	N.S.	
	H. pylori-positive	30	0.17 ± 0.03	N.S.	N.S.
	H. pylori-negative	15	0.12 ± 0.03	N.S.	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs. H. pylori*-negative).

N.S.: Not significant.

4-5: Lymphocyte Immunophenotypes

The lymphocytes of peripheral blood were immunophenotyped for the expression of four CD markers (CD3+, CD4+, CD8+ and CD19+ cells) in gastritis patients and controls.

4-5-1: CD3+ Lymphocytes

The mean percentage of CD3+ lymphocytes in the control sample was 79.4%, but such mean was significantly ($P \leq 0.001$) reduced in total gastritis patients (55.3%), corpus gastritis (53.9%), antrum gastritis (56%), *H. pylori*-positive gastritis (51.9%) and *H. pylori*-negative gastritis (62.6%). The highest reduction was observed in *H. pylori*-positive gastritis, in which the difference reached a significant level ($P \leq 0.001$) when such patients were compared to *H. pylori*-negative gastritis, while corpus and antrum gastritis showed approximated means (Table 4-12).

Table 4-12: Percentage of CD3+ lymphocytes (mean ± S.E.) in gastritis patients (total and subgroups) and controls.

Groups		Number	Mean ± S.E (%)	Probability * I ≤	Probability ** II ≤
Controls		20	79.4 ± 1.1		
Gastritis Patients	Total	44	55.3 ± 1.5	0.001	
	Corpus	16	53.9 ± 2.4	0.001	N.S.
	Antrum	28	56.0 ± 1.8	0.001	
	H. pylori-positive	30	51.9 ± 1.6	0.001	0.001
	H. pylori-negative	14	62.6 ± 1.8	0.001	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs*. *H. pylori*-negative).

N.S.: Not significant.

4-5-2: CD4+ Lymphocytes

The mean percentage of CD4+ lymphocytes in the control sample was 39.5%, but such mean was significantly ($P \leq 0.001$) reduced in total gastritis patients (25.7%), corpus gastritis (26%), antrum gastritis (25.5%), *H. pylori*-positive gastritis (25.9%) and *H. pylori*-negative gastritis (25.2%). The groups of gastritis patients when compared to each others showed no significant ($P > 0.05$) variations between means (Table 4-13).

Table 4-13: Percentage of CD4+ lymphocytes (mean ± S.E.) in gastritis patients (total and subgroups) and controls.

Groups		Number	Mean ± S.E (%)	Probability * I ≤	Probability ** II ≤
Controls		20	39.5 ± 0.6		
Gastritis Patients	Total	44	25.7 ± 0.6	0.001	
	Corpus	16	26.0 ± 1.0	0.001	N.S.
	Antrum	28	25.5 ± 0.7	0.001	
	H. pylori-positive	30	25.9 ± 0.7	0.001	N.S.
	H. pylori-negative	14	25.2 ± 0.8	0.001	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs*. *H. pylori*-negative).

N.S.: Not significant.

4-5-3: CD8+ Lymphocytes

The means of CD8+ lymphocytes showed similar distributions in controls (22%), total gastritis patients (21.5%), corpus gastritis (22.8%), antrum gastritis (20.8%), *H. pylori*-positive gastritis (21.7%) and *H. pylori*-negative gastritis (21.1%) (Table 4-14). Therefore, there were no significant differences ($P > 0.05$).

Table 4-14: Percentage of CD8+ lymphocytes (mean ± S.E.) in gastritis patients (total and subgroups) and controls.

Groups		Number	Mean ± S.E (%)	Probability * I ≤	Probability ** II ≤
Controls		20	22.0 ± 0.6		
Gastritis Patients	Total	44	21.5 ± 0.7	N.S.	
	Corpus	16	22.8 ± 1.2	N.S.	N.S.
	Antrum	28	20.8 ± 0.7	N.S.	
	H. pylori-positive	30	21.7 ± 0.8	N.S.	N.S.
	H. pylori-negative	14	21.1 ± 1.2	N.S.	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs. H. pylori*-negative).

N.S.: Not significant.

4-5-4: CD4/CD8 Ratio

As a consequence of decreased percentage of CD4+ lymphocytes and a normal percentage of CD8+ lymphocytes, the CD4/CD8 ratios were also decreased in total gastritis patients (1.23), corpus gastritis (1.18), antrum gastritis (1.27), *H. pylori*-positive gastritis (1.23) and *H. pylori*-negative gastritis (1.25) as compared to controls (1.81). These deviations were highly significant ($P \leq 0.001$). The means of such ratio in the subgroups of gastritis maintained approximated values, and therefore, no significant difference was recorded in this regard (Table 4-15).

Table 4-15: CD4/CD8 ratio of lymphocytes (mean ± S.E.) in gastritis patients (total and subgroups) and controls.

Groups		Number	Mean ± S.E	Probability * I ≤	Probability ** II ≤
Controls		20	1.81 ± 0.05		
Gastritis Patients	Total	44	1.23 ± 0.04	0.001	
	Corpus	16	1.18 ± 0.07	0.001	N.S.
	Antrum	28	1.27 ± 0.05	0.001	
	H. pylori-positive	30	1.23 ± 0.04	0.001	N.S.
	H. pylori-negative	14	1.25 ± 0.09	0.001	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs. H. pylori*-negative).

N.S.: Not significant.

4-5-5: CD19+ Lymphocytes

In contrast to CD3+ lymphocytes, the mean percentage of CD19+ lymphocytes were significantly increased in total gastritis patients (27.4%), corpus gastritis (26.6%), antrum gastritis (27.8%), *H. pylori*-positive gastritis (27.3%) and *H. pylori*-negative gastritis (27.6%) as compared to the mean of controls (18.7%). The groups of gastritis patients when compared to each others showed no significant ($P > 0.05$) variations between means (Table 4-16).

Table 4-16: Percentage of CD19+ lymphocytes (mean ± S.E.) in gastritis patients (total and subgroups) and controls.

Groups		Number	Mean ± S.E (%)	Probability * I ≤	Probability ** II ≤
Controls		20	18.7 ± 0.7		
Gastritis Patients	Total	44	27.4 ± 0.5	0.001	
	Corpus	16	26.6 ± 0.9	0.001	N.S.
	Antrum	28	27.8 ± 0.7	0.001	
	H. pylori-positive	30	27.3 ± 0.7	0.001	N.S.
	H. pylori-negative	14	27.6 ± 0.9	0.001	

* The comparison is between patients and controls.

** The comparison is between the subgroups of patients (Corpus *vs*. antrum, and *H. pylori*-positive *vs. H. pylori*-negative).

N.S.: Not significant.

Chapter Five

Discussion

The present study demonstrated that immunogenetic predisposition may be considered as an important requirement for the development of gastritis, and HLA antigens, as well as, blood groups are in favour of such generalization, in which several markers of human MHC and ABO systems showed different distributions in patients and controls, moreover, patients divided on the basis of their clinical types (antrum and corpus) or sero-positivity for anti-*H. pylori* IgG antibodies also showed some variations. These markers are coded by genes on two chromosomes; 6 and 19, respectively (Klug and Cummings, 1997; Mehra and Kaur, 2003), therefore what is the significance of these genes or their products (antigens) in conferring predisposition or resistance to develop chronic atrophic gastritis.

With respect to immunogenetic predisposition, six antigens showed increased frequencies in the total patients or their subgroups, they were A3, A9, B17, DR2, DR3 and DR10. The A3 antigen showed a significant increase in the total patients and antrum gastritis patients, while DR10 was consistently increased in the patients, and the RR value showed a range from 8.21 in *H. pylori*-positive gastritis to 22.38 in *H. pylori*-negative gastritis, and the EF value also showed variations (total gastritis: 0.39; corpus gastritis: 0.41; antrum gastritis: 0.34; *H. pylori*-positive gastritis: 0.30; *H. pylori*-negative gastritis: 0.56). In contrast, the antigen A19 was consistently decreased in the patients with a PF value more than 0.30 (total gastritis: 0.38; antrum gastritis: 0.33; *H. pylori*-positive gastritis: 0.38; *H. pylori*-negative gastritis: 0.39). Also, this antigen was not recorded in corpus gastritis patients, moreover, this group of patients showed a further two positive associations, which were with A9 and DR2, and the EF values of such associations were 0.42 and 0.38, respectively.

When the literatures were reviewed, the forthcoming observations have not been recorded in chronic atrophic gastritis or other related diseases. Indeed, reviewing the literature revealed only 13 publications in this regard (Mawhinney *et al*., 1975; Mielants *et al*., 1991; Ishii *et al*., 1992; Azuma *et al*., 1994; Azuma *et al*., 1995; Azuma *et al*., 1998; Yoshitake *et al*., 1999; Sakai *et al*., 1999; Kulcsarova *et al*., 2001; Azuma *et al*., 2002; Herrera-Goepfert *et al*., 2004; Watanabe *et al*., 2006; Herrera-Goepfert *et al*., 2006), which reported associations with different DQ antigens, as well as, A3 and Cw6 antigens. The latter two associations were reported in pernicious anaemia and autoimmune atrophic gastritis and dermatological disorders associated with *H. pylori* infection, respectively. Unfortunately, neither DQ nor Cw loci were investigated in the present study, because the antisera were not available.

Such discrepancy may be explained in the ground of racial differences, especially if we consider that HLA antigens show different frequencies in different populations including Iraqis (Ad'hiah, 1990; Ad'hiah *et al*., 1996; Mahdi *et al*., 2005). Risk for gastric diseases among ethnic groups with different HLA allele expression reflects several polymorphisms of this and other loci, as genes related to mucosa protection (i.e. mucins and trefoil peptides), inflammatory responses (i.e. interleukin-1b; interleukin-1 receptor antagonist, and tumor necrosis factor), and metabolic detoxifying enzymes (phase I enzymes like cytochrome P450 superfamily, and phase II enzymes like glutathione S- and N-acetyl transferases) (Gonzalez, 2002). The subtle mechanism by which such polymorphisms may drive the immune response and host susceptibility related with particular stimuli is unclear; nevertheless, in this case, the participation of a unknown and as yet uncharacterized neighboring HLA antigen could not be ruled out (Herrera-Goepfert *et al*., 2006).

However, looking at the subject from a different angle may help to bridge the discrepancy, and the estimated EF values may establish the theme. As suggested by the statisticians, the EF value can range from 0 (no association) to 1 (maximum association). In other term, a value of 1 for an antigen is interpreted that this antigen is fully responsible for the development of the disease, otherwise, if the value is in between 0 and 1, the interpretation is that this marker is partially involved in the disease development, and other factors (i.e. environmental pathogens) are operative (Svejgaard *et al.*, 1983). The EF values of A3 (0.27) and B17 (0.13) support the forthcoming statement, but what are these factors?

The answer may be augmented if we consider the immunological roles of HLA-class I and -class II antigens. These antigens are virtually involved in antigen presentation carried out by macrophages, and a default in such mechanism may render an individual vulnerable to any immunologically-mediated morbidity (Owen, 1997). In this respect, *H. pylori* has been suspected in different gastritis populations, and is linked to the aetiology of chronic atrophic gastritis (Du and Isaccson, 2002).

The host-immune response to *H. pylori* is initiated by the contact of this bacterium with antigen-presenting cells, which have the ability to process and to present *H. pylori* peptides on their surface. Foreign antigenic peptides are presented to their corresponding T-cell receptors by HLA class II molecules, resulting in the activation of $CD4^+$ T cells. Several studies have demonstrated the overexpression of HLA class II molecules in gastric mucosa (Papadimitriou *et al.*, 1988, Valnes *et al.*, 1990, Ishii *et al.*, 1992, Ihan *et al.*, 1995, Seifarth *et al.*, 1995, Chiba *et al.*, 1995, Archimandritis *et al.*, 2000) and circulating mononuclear cells (Ohara *et al.*, 2001) in patients with gastric inflammation. It has been reported that gastric epithelial cells, as well as, classical antigen presenting cells such as macrophages express HLA-DR, -DP, and -DQ molecules (Papadimitriou *et al.*, 1988, Valnes *et al.*, 1990, Ishii *et al.*, 1992, Seifarth *et al.*, 1995), and that the extent of epithelial

HLA-DR expression is parallel with the degree of inflammation (Papadimitriou *et al.*, 1988); and activity (Archimandritis *et al.*, 2000). On the other hand, the expression of HLA-DR was not increased in the lamina propria of gastric mucosa infected with *H. pylori* (Seifarth *et al.*, 1995). Correlation between epithelial HLA-DR expression and *H. pylori* density was also found (Chiba *et al.*, 1995). Combined with the finding that expression of HLA-DR was significantly decreased in *H. pylori*-eradicated patients (Archimandritis *et al.*, 2000), these results suggest that epithelial HLA-DR expression could be a marker of gastric inflammation with *H. pylori* infection. Costimulatory signals provided by antigen presenting cells are required for full T-cell activation. In *H. pylori*-associated gastritis, costimulatory molecules such as CD80 (B7-1), CD86 (B7-2), and intercellular adhesion molecule-1 are expressed on gastric epithelial cells (Archimandritis *et al.*, 2000), indicating that epithelial cells function as antigen presenting cells during *H. pylori* infection.

The immunogenetic predisposition to develop chronic atrophic gastritis was further explored with other immunogenetic markers; they were ABO blood group phenotypes. Two phenotypes were important in this regard; A and O. The blood group A was significantly increased in total patients and almost their subgroups, while a reverse outcome was observed for blood group O. Such two observations may suggest that the first blood group is involved in the aetiopathogenesis of chronic atrophic gastritis, while the second confers some resistance to develop the disease. It is difficult to explain the role of these antigens in the pathogenesis of gastritis, but because the disease is limited to the mucosa of the stomach and it is well known fact that the general population can be divided into two groups according to the secretor status of blood group antigens (Daniels, 1999), therefore, can these antigens protect the mucosa or predispose it to inflammatory responses, the question waits an answer, and further investigations are required. However, reviewing the literature revealed that about 11 papers were published about the role

of blood group phenotypes and/or genotypes in the aetiopathogenesis of gastritis or their causative agents (Thompson *et al*., 1968; Johnston *et al*., 1973; Berndt, 1975; Al'tshuler, 1983; Lam, 1993; Umlauft *et al*., 1996; Lin *et al*., 1998; Shibata *et al*., 2003; Sheu *et al*., 2003; Kikuchi, 2004; Lee *et al*., 2006). The results of these studies were inconsistent, although they were concerned mainly with two types of blood groups; secretor status and Lewis phenotype. However, their conclusions suggest that blood group polymorphisms are valuable in identifying patients at high risk of developing duodenal ulcer, gastritis, gastric ulcer, adenocarcinomas of the distal stomach, and low-grade B-cell lymphoma of mucosa-associated lymphoid tissues.

Investigating the CD profiles of peripheral lymphocytes revealed a significant decrease of CD3+ and CD4+ cells in total patients of chronic atrophic gastritis and their subgroups, while CD8+ lymphocytes did not show such variation. Such observation suggests the importance of these cells in the aetipathogenesis of the disease. It has been demonstrated that both CD4 and CD8 cells are increased in the gastric mucosa during *H. pylori* infection but not in the peripheral blood, and activated by *H. pylori* antigens (Smagina, 2000). The expression of activation markers for T cells such as CD25 (IL-2 receptor), CD71 (transferrin receptor), CD45RO, and CD103 has been analyzed by immunohistochemistry (Seifarth *et al.*, 1995, Hatz *et al.*, 1996) and flow cytometry (Ihan *et al.*, 1995, Fan *et al.*, 1998). In these studies, T cells expressing these activation markers were increased in *H. pylori*-associated gastric inflammation. Furthermore, Hatz *et al.* (1996) showed a significant increase in $CD45RO^{+}$ activated lamina propria lymphocytes in *H. pylori*-associated inflammation, which was correlated with the degree of atrophy, inflammation, and *H. pylori* density, indicating that T-cell activation is a marker of gastric inflammation in *H. pylori* infections (Naito and Cunha-Cruz, 2005).

The subject *H. pylori* and immune response has been further explored, and it has been suggested that *H. pylori*-infected individuals develop cellular and humoral

immune responses that are ineffective in clearing the infection (Blanchard *et al.*, 1999). Infected individuals develop a predominantly inflammatory T helper 1 (T_H1) response in the gastric mucosa, the extent of which is linked to the severity of gastritis in humans (D'Elios *et al.*, 1997; Lindholm *et al.*, 1998; Sommer *et al.*, 2001). The dominance of T_H1 gastric T cells in *H. pylori* infection may explain the failure of infected individuals to induce immunity to *H. pylori*, and the production of gamma interferon (IFN-γ) is thought to contribute to the pathology of disease (Mohammadi *et al.*, 1996, Bamford *et al.*, 1998). The infiltration of IFN-γ-producing cells in the infected mucosa is accompanied by increased numbers of cells producing transforming growth factor ß (TGF-ß), suggesting that *H. pylori*-induced inflammatory responses may be partially regulated by $CD25^+$ T regulatory cells (Lindholm *et al.*, 1998). These cells constitute 5 to 10% of all peripheral $CD4^+$ T cells in normal naive mice and healthy humans (Sakagami *et al.*, 1997) and possess potent regulatory activity both *in vitro* (Thornton and shevach, 1998) and *in vivo* (Read *et al.*, 2000). $CD25^+$ T regulatory cells are thought to mediate their immunosuppressive activity through the release of soluble factors, including TGF-ß and interleukin-10 (IL-10) (Wahl and Chen, 2003) and/or directly by a cell contact-dependent mechanism (Takahashi *et al*., 2000). Also these cells not only play a key role in the maintenance of self-tolerance and protection from autoimmune diseases, such as autoimmune gastritis, but also influence the nature of the immune responses to a range of infectious organisms (Aseffa *et al*., 2002, Belkaid *et al*., 2002). More recently, the role of $CD4^+$ and $CD25^+$ T cells in regulation of *Helicobacter*-driven immune responses and autoimmune gastritis was examined in a BALB/c mouse model, using a monoclonal anti-CD25 antibody to deplete $CD25^+$ T regulatory cells during or after *Helicobacter* infection of BALB/c mice (Kaparakis *et al*., 2006), their results demonstrated that a depletion of these cells was associated with increased *Helicobacter*-specific antibody levels and an altered isotype distribution.

Based in the forthcoming presentation, chronic gastritis has been related to *H. pylori* infection, which may cause immunological reactions in peripheral mononuclear cells. The activity and characteristics of peripheral mononuclear cells may differ in ulcer and non-ulcer patients infected with *H. pylori* (Ohara *et al.*, 2001). It has been reported that CD4 cells are sensitized *in vivo* and migrate to gastric mucosa where they induce gastritis in response to *H. pylori* antigens, suggesting that CD4-dependent *H. pylori* gastritis could lead to epithelial damage with proliferative and metaplastic responses (Riedel *et al.*, 2001; Peterson *et al.*, 2003). Yuceyar *et al.* (2002) found that there was no obvious alteration in total T and B lymphocytes, CD4+ T, CD8+ T lymphocytes and natural killer cells in chronic antral gastritis patients compared to normal persons, and suggested that there is no systemic alteration in the specific immune system in response to *H. pylori* in patients with chronic antral gastritis, however, these findings may contradict the present results. Such discrepancy may be explained by the findings of Itoh *et al*. (1999), who have demonstrated that gastric T cells are differentiated to produce a large amount of IFN-γ by a mechanism unrelated to *H. pylori* infection. *H. pylori* infection appeared to activate T cells to secrete even more IFN-γ, which might contribute to maintaining a perpetual inflammation in *H. pylori* -infected stomach. Furthermore, recently Ai-Ping *et al*., (2005) showed that CD4 cells in gastric mucosa were much more in patients with *H. pylori* infection, while CD8 cells were similar in patients with or without *H. pylori* infection.

The percentage of CD8+ lymphocytes showed no significant variations between gastritis patients (total and subgroups) and controls; however, most investigations are concerned with the role of these cells in *H. pylori* infection, although their role in such bacterial infection is not clear. Thus, there have been conflicting results whether or not *H. pylori* infection leads to increased numbers of CD8+ T cells in the gastric mucosa, and also whether activation of CD8+ T cells by *H. pylori* antigens is increased or decreased in *H. pylori*-infected individuals.

Reports in favour of an involvement of CD8+ T cells in the immune response to *H. pylori* infection have shown that *H. pylori* colonization is associated with increased numbers of CD8+ lymphocytes in the epithelium of the crypts (Hood and Lesna, 1993), and it has also been shown that the CD8+ T cells in the gastric mucosa of infected subjects express increased levels of the activation marker HLA-DR (Ihan *et al.*, 1995). A recent study also reported increased numbers of CD8+ T cells in the gastric mucosa of volunteers subjected to experimental *H. pylori* infection (Nurgalieva *et al.*, 2005).

Furthermore, although oral immunization with *H. pylori* preparations in MHC class I knockout mice showed that CD8+ T cells were not involved in vaccine-induced protection against the infection (Ermak *et al.*, 1998, Pappo *et al.*, 1999), there were increased numbers of bacteria in the gastric mucosa of unvaccinated knockout mice, indicating that CD8+ T cells can regulate the extent of *H. pylori* infection *in vivo* (Pappo *et al.*, 1999). More recently, the CD8+ T cell response to *H. pylori* has been studied in more details by Azem *et al*. (2006), they have evaluated efficient conditions for activation of CD8+ T cells *in vitro*, and showed that *H. pylori*-reactive CD8+ T cells can be activated most efficiently by B cells or dendritic cells pulsed with *H. pylori* antigens, they also showed that the majority of CD8+ T cells in *H. pylori*-infected gastric mucosa are memory cells, and that memory CD8+ T cells sorted from peripheral blood of *H. pylori*-infected individuals respond 15-fold more than *H. pylori* urease compared to memory cells from uninfected subjects, their conclusion was that CD8+ T cells do participate in the immune response to *H. pylori*, and this may have implications for the development of more severe disease outcomes in *H. pylori*-infected subjects.

Finally, the conclusion of this discussion can reach that the aetiolgy of chronic atrophic gastritis involve the HLA antigens, as well as, blood group antigens. They are predisposing factors having the ability to react with an environmental factor(s),

H. pylori being the most important, and such reaction leads to an immune dysregulation and a disease manifestation.

Conclusions and Recommendations

1- Conclusions

- HLA system, genes and/or gene products, are important immunogenetic factors that can confer predisposition or resistance to develop chronic atrophic gastritis. In this regard HLA DR10 is important predisposing marker.
- Blood group phenotypes are also important in the predisposition or resistance to develop chronic atrophic gastritis.
- *H. pylori* infection is important environmental agent in inducing chronic atrophic gastritis.
- Lymphocytes and their immunophenotypes play a significant role in the aetiopathogenesis of chronic atrophic gastritis.

2- Recommendations

- Studying the HLA system by means of molecular technologies to shed light on the role of this system at the genotypic level.
- Studying the profile of secondary blood group phenotypes and their secretor status.
- Immunohistochemical examinations of stomach mucosa biopsies to determine their expression of HLA antigens and the infiltrating cells.
- The polarization of T_H1 and T_H2 in the patients requires detailed investigations.

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الخلاصة

اقترحت الدراسة الحالية لبحث دور خلايا الدم البيض البشرية – الصنف الاول (A , B) والصنف الثاني (DR) والطرز المظهرية لمجاميع الدم في الاصابة بمرض إلتهاب المعدة في عينة من مرضى عراقيين. كما درست أيضاً معايير مناعية أخرى شملت العد الكلي والمطلق لخلايا الدم البيض والمجاميع الثانوية للخلايا اللمفية (خلايا CD3+ , CD19+ , CD8+ , CD4+) وإيجابية أو سلبية أمصالهم لضد بكتيريا الـ*Helicobacter pylori* من الصنف IgG.

فحص تسعة وأربعون شخص مصاب بإلتهاب المعدة للفترة كانون الثاني- تشرين الأول من عام ٢٠٠٦، وشخص المرض سريريا من قبل الكادر الطبي الإستشاري في مستشفى الكاظمية التعليمي – مركز الجهاز الهضمي وأمراض الكبد. وكان التشخيص معقداً على التقييم بجهاز الناظور الداخلي والدراسة النسجية لخزع نسجية من بطانة المعدة. وبحسب وجهة نظر الإستشاريين تم تقسيم المرضى سريريا الى قسمين وبحسب موقع الإلتهاب إلى corpus gastritis (٣١ مريض) و antrum gastritis (١٨ مريض) كما تم فحص خمسون عينة سيطرة من أفراد أصحاء ظاهريا ومتطابقين من جهة العمر والجنس والعرق (مسلمين عرب). كما قسم المرضى أيضاً في ضوء إيجابية وسلبية أمصالهم لضد بكتيريا الـ*H. pylori* وإتضح بأن ٣٢ مريض (٦٥,٣%) موجبين لضد IgG و١٧ مريض (٣٤,٧%) سالبين لهذا الضد.

أظهرت ستة مستضدات من منطقتي الصنف الأول والصنف الثاني لنظام خلايا الدم البيض البشرية تكرارات مرتفعة في المرضى أو مجاميعهم الثانوية مقارنة بأفراد السيطرة وكانت هذه المستضدات (A3، A9، B17، DR2، DR3، DR10). أظهر المستضد A3 إرتفاعاً معنوياً ضد العدد الكلي من المرضى وفي مرضى antrum gastritis في حين كان المستضد DR10 مرتفعاً في كل المرضى وقد تراوحت قيمة الخطر النسبي مابين ٨,٢١ في المرضى الموجبين لضد بكتيريا الـ*H. pylori* إلى ٢٢,٣٨ في المرضى السالبين لهذا الضد. كما كانت قيمة العامل المسبب (العدد الكلي للمرضى = ٠,٣٩، corpus gastritis = ٠,٤١، antrum gastritis = ٠,٣٤، المرضى الموجبين لضد بكتيريا الـ*H. pylori* = ٠,٣٠، المرضى السالبين لضد بكتيريا الـ*H. pylori* = ٠,٥٦). وعلى العكس من ذلك فقد أظهر المستضد A19 إنخفاضاً واضحاً في جميع المرضى بحيث كانت قيمة عامل الحماية اكبر من ٠,٣٠ (العدد الكلي للمرضى = ٠,٣٨، antrum gastritis = ٠,٣٣، المرضى الموجبين لضد بكتيريا الـ*H. pylori* = ٠,٣٨، المرضى السالبين لضد بكتيريا الـ*H. pylori* = ٠,٣٩). بالإضافة إلى ذلك فأن هذا المستضد لم يسجل في مرضى الـcorpus gastritis وعلاوه على ذلك فأن هذه المجموعة من المرضى أظهرت مصاحبتين إيجابيتين والتي كانت مع A9، DR2 وإن قيمة العامل المسبب لهذين العاملين المصاحبين كانت مساوية ٠,٠٢ و ٠,٣٨ على التوالي.

إن الإستعداد الوراثي المناعي للإصابة بمرض إلتهاب المعدة المزمن قد بحث مرة أخرى من خلال عوامل وراثية مناعية أخرى وهي الطرز المظهرية لمجاميع الدم ABO. وفي هذا الصدد اظهر نمطان مظهريان اهمية في هذا المرض وهما O، A. حيث إرتفع تكرار مجموعة الدم A معنوياً في العدد الكلي للمرضى (٤٦,٧ مقابل ١٨,٠%) وتقريباً في جميع المجاميع الثانوية، وعلى العكس من ذلك فقد إنخفضت مجموعة الدم O (٢٢,٢ مقابل ٤٨,٠%) بالمقارنة مع أفراد السيطرة. تقترح هاتين الملاحظتين بأن مجموعة الدم الأولى لها أهمية في تطور المرض في حين تكمن الثانية في مقاومة تطور المرض.

وعند دراسة الخلايا اللمفية في ضوء واسماتها السطحية إتضح بان الخلايا الموجبة للواسم CD3 (٥٥,٣ مقابل ٧٩,٤%) والواسم CD4 (٢٥,٧ مقابل ٣٩,٥%) إذ قد إنخفضت نسبتها المئوية معنوياً في العدد الكلي للمرضى، وفي المجاميع الثانوية مقارنة بأفراد السيطرة. في حين لم تظهر الخلايا الموجبة للواسم CD8 مثل هذا التغاير، وفي ضوء ذلك فقد إنخفضت نسبة خلايا CD4/CD8 معنوياً في المرضى (١,٢٣ مقابل ١,٨١%). وعلى العكس من ذلك فأن الخلايا الموجبة للواسم CD19 قد إرتفعت نسبتها المئوية معنوياً في العدد الكلي للمرضى (٢٧,٤ مقابل ١٨,٧%) وفي المجاميع الثانوية. تقترح هذه الملاحظات اهمية تلك الخلايا في إمراضية المرض.

وأخيراً، فإن العدد الكلي والمطلق لخلايا الدم البيض لم يظهر فروقاً معنوياً مابين المرضى وأفراد السيطرة.

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البحث في مستضدات خلايا الدم البيض البشرية لمرض إلتهاب المعدة المزمن في عينة من مرضى عراقيين.

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

أحمد حسن أحمد

بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠٣

ربيع الثاني ١٤٢٨

آيار ٢٠٠٧

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