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Immunogentic Study of Inflammatory Bowel Disease in Iraqi Patient

by
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من قبل
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تموز **٢٠٠٥**

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Dedication

All the success I had or will

have belongs to her

not to me

Whatever I say, is not as true

as she

To the one

Who backed me

To be

To my mother

Hamsa

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Summary

The present study was designed to investigate the role of HLA-class I (A and B) and class II (DR and DQ) antigens and blood group phenotypes in the aetiology of inflammatory bowel disease (IBD) in a sample of Iraqi patients. The patients were also evaluated for total and differential counts of leucocytes, subpopulations of lymphocytes (CD3+, CD4+, CD8+ and CD19+ cells) and phagocytosis.

Sixty–five patients with IBD were investigated during the period May-December, 2004. The disease was clinically diagnosed by the consultant medical staff at Al-kadhamyiah Teaching hospital and Gastrointestinal Tract (GIT) and liver disease Center in Baghdad. The diagnosis was based on a clinical evaluation using colonoscopy and a histopathological examination of a biopsy. According to the point view of consultants, the patients were clinically subdivided into ulcerative colitis (ULC; 50 patients) and Crohn's disease (CRD; 15 patients). A control sample of 67 individuals (apparently healthy), matched for age, sex and ethnic background (Arab Muslims), were further investigated.

At HLA-class I region, the total patients showed significant increased frequencies of A9 (52.3 *vs*. 17.9%) and B41 (66.1 *vs*. 5.9%), and a significant decreased frequency of A11 (3.1 *vs*. 22.4%) as compared to controls, and similar findings were outcome when the clinical types (ULC and CRD) were considered. While at HLA-class II region, DR8 was significantly increased in the total patients (30.8 *vs*. 8.9%), but neither ULC nor CRD maintained such association, and instead ULC was significantly associated with DQ1 (40 *vs*. 18%). However, comparing ULC with CRD revealed a significant difference in the antigen B16 (2 *vs*. 33.3%).

Blood group phenotypes (A, B, AB and O) were similarly distributed in the patients (total and subtypes) and controls, and no significant difference was observed as judged by Chi-square analyses. However, summing A, B, and AB

blood groups increased their frequency to 60% in CRD patients, while their frequency in the controls was 51.4%.

The total count of leucocytes showed no significant differences between patients (total and subtypes) and controls, while the differential count showed some variations. The neurophil count was significantly increased in total patients and ULC patients, while monocytes and eosinophils showed significant decreased counts in the patients (total and subtypes).

Lymphocytes positive for the markers CD3, CD4 and CD19 showed significant increased percentages in total, as well as, ULC patients, while CD8+ cells were significantly increased in the total and subtypes of IBD patients. However, the CD4/CD8 ratio was significantly decreased in the patients (total and subtypes).

The phagocytic index of patients (total and subtypes) was significantly increased, while the NBT index showed a significant elevated percentage in the total IBD and ULC patients.

List of Abbreviations

HLA	Human Leucocyte Antigen
α	Alpha
β_2M	Beta-2 microglobulin
C3	The third component of complement
C4	The fourth component of complement
CD	Cluster designation
CRD	Crohn's Disease
EF	Etiological fraction
IBD	Inflammatory Bowel Disease
Ig	Immunoglobulin
IL	Interleukin
Kb	Kilobase pair
MHC	Major Histocompatibility complex
ml	Milliliter
µl	Micro liter
NBT	Nitroblue Tetrazolium
P	Probability
Pc	Corrected probability
PCR	Polymerase chain reaction
PF	Perventive Fraction
RR	Relative Risk
TCR	T cell receptor
Th	T helper
TNF	Tumor necrosis factor
ULC	Ulcerative colitis

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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 (Table 2-2). These diseases are generally of unknown aetiology and pathogenesis, and are either autoimmune or have immunological abnormalities (Thomson, 1993).

Table 2-2: Some of HLA-Disease associations (Thomson, 1993).

Disease	Antigen	Race	Frequency (%)		Relative Risk
			Patients	Controls	
Narcolepsy	DR2	Caucasians	100	22	129.8
		Orientals	100	34	358.1
Ankylosing spondylitis	B27	Caucasians	89	9	69.1
		Orientals	81	1	354.4
		Negros	58	4	54.4
Type I diabetes mellitus	B15	Caucasians	22	14	2.1
	DR3	Caucasians	52	22	3.8
	DR4	Caucasians	74	24	9.0
	DR2	Caucasians	4	29	0.1
	DRB1*0301	Caucasians	54	27	3.2
	DRB1*0401	Caucasians	59	25	4.3
	DRB1*0301	Caucasians	25	9	3.5
	DQA1*0301	Caucasians	85	35	10.7
	DQB1*0302	Caucasians	81	23	14.1
Rheumatoid arthritis	DR4	Caucasians	68	25	3.8
		Orientals	66	39	2.8
		Negros	44	10	5.4
Hodgkin's disease	A1	Caucasians	40	32	1.4
	DRB1*1104	Caucasians	6	1	4.5
Psoriasis	Cw6	Caucasians	87	33	13.3
Celiac disease	DR3	Caucasians	79	26	15.4
Multiple sclerosis	DR2	Caucasians	59	26	4.1

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, also 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 *et al*., 1991). 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; Schlosstein *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, 1993). 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 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 are not well defined. However, several models

have been proposed, including the cross-reactivity of antibodies to microorganisms with particular HLA-molecules, and the molecular mimicry of T-cell responses to viral antigens for self antigens. Other investigators have speculated that each disease may have its own aetiopathogenic mechanism, and the HLA system exerts some role in this mechanism (Bains & Ebringer, 1992; Oldstone, 1997).

2-2: Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a group of disorders, defined by the presence of chronic gastrointestinal inflammation not due to a specific disease-producing organism. Two clinical forms of the disease exist, ulcerative colitis (ULC) and Crohn's disease (CRD), which in the main have different clinical and pathological features (Brown *et al*., 1995). Ulcerative colitis involves the colon as a diffuse mucosal disease with a distal predominance. The rectum is virtually always involved, and additional portions of colon may be involved extending proximally from the rectum in a continuous pattern. While, CRD can involve any part of the gastrointestinal tract, but most frequently involves the distal small bowel and colon. Inflammation is typically transmural and can produce wide clinical presentations from a small ulcer over a lymphoid follicle to a deep fissuring ulcer to transmural scaring and chronic inflammation (Jewell , 1998). Despite these differences between ULC and CRD, in some 5-10% of patients (those in whom there is a large bowel involvement only) the two diseases may be indistinguishable (Brown *et al*., 1995). A comparison between the two diseases is given in table 2.3.

Table 2.3: Comparison of ulcerative colitis and Crohn's disease.*****

Feature	Ulcerative Colitis	Crohn's Disease
Distribution	Diffuse, Distal Predominance	Segmental or Diffuse, often Proximal Predominance
Rectum	Always involved	Often Spared
Microscpic Distribution	Diffuse	Often Focal
Depth of Inflammation	Mucosal	Transmural
Sinus Tracts and Fistulae	Absent	Often Present
Strictures	Absent	Often Present
Granulomas	Absent	Often Present

*****: (Hanauer, 2001)

Both types of IBD are recognized as important causes of gastrointestinal disease in children and adults of various races and regions, however, the two diseases are more common in some regions (United States of America, United Kingdom and Scandinavia) than in others. Also, peoples living in colder climates have greater rate of IBD than peoples living in warmer climates and a similar conclusion can be drawn for urban areas versus rural areas. The general incidence rate of IBD is 4-10 cases/100000 per year, while the prevalence rate is 40-100/ 100000 persons (Hendrickson *et al*., 2002). These numbers may be affected by race, and in this regard it has been observed that Americans of Asian and Hispanic descent showed a lower prevalence rate than Americans of African or European descent, although the two later populations showed a similar estimated rate (Rowe, 2004).

The aetiology of IBD (ULC or CRD) is not well understood, however the forthcoming epidemiological approach, as well as, other clinical and laboratory based evidences suggest that the disease may be multifactorial, and genetic, immunological and environmental factors

have been of a great concern. But, what is the nature of these factors, and how they interact to produce a disease, is a matter of speculations (Parkes & Jewell, 2001).

2-2-1: Genetics of IBD

Family and twin based evidences suggest that genetics plays an important role in predisposing an individual to develop ULC and CRD. A familial tendency has been clearly observed for IBD, and a 4% chance for the disease to occur in a child of a parent with IBD has been demonstrated, moreover, 10-15% of the patients are estimated to have a first degree relative with the disease (Tysk *et al*., 1994 ; Lindberg *et al* ., 1995; Amott *et al*., 2005). Recently, three groups of investigators have formalized a parameter (λs ratio) that estimates the risk of a disease within multiply affected families, and their result suggested ratios of 8-15 and 20-35 for ULC and CRD, respectively. Twin studies confirmed these findings, and a higher concordance rate of the disease was observed among monozygotic twins than dizygotic twins (ULC: 11 *vs*. 3%; CRD: 35 *vs*. 7%) (Farmer *et al*., 1980; Dally *et al., 2005* ; Annese *et al*., 2005).

A further evidence of a genetic predisposition came from HLA-IBD association studies, however, these associations have only been shown in homogeneous groups of populations. In Japanese patients, HLA-B5 has been recorded to be associated with ULC, while in Caucasian patients the disease has shown associations with two HLA-class I antigens (B27 and B44). Crohn's disease behaved in a similar manner, and the association has been reported with HLA-DR4 in Japanese patients and HLA-B44 in Caucasian patients (van Hell *et al*., 2001). Later studies, which were based on a molecular typing of HLA-class II region, have emphasized the importance of HLA-DR

polymorphism in the aetiology of IBD (Hendrickson *et al*., 2002). The results of HLA-IBD association studies are summarised in table 2.4.

Table 2-4: Some results of HLA-class I and -class II antigens in ULC and CRD patients of different populations (Zheng *et al*., 2003**)**

Population	Association with CRD	Association with ULC
American		DRB1*1601
Japanese	DRB1*0405,0410,DQA1 DQB1*0401,0402, DRB1*,DQB1*0602	
German	DRB1*07, DRB1*03,DPA1*02021 DPA1*02021	
French	DRB1*1302+,DRB1*04	DRB1*103, DRB1*12
French	DQB1*0501, DRB1*07, DQB1*0602/0603, DRB1*03	
French		DRB1*03
Dutch		DRB1*15, DRB1*13
Spanish		DRB1*1501, DRB1
American	DRB3*0301,DRB1*1302	
Dutch		DRB1*0103, DRB1*15
Japanese	DQB1*0402, DRB1*1502-	DRB1*1502,DRB5*, DQA1*0103,DQB1,DPA1*0201,DPB1, DRB4*0101,DQA1
Meta-analysis	DR7,DRB3*0301,DQ4, DR2,DR3	DR2, DR9, DRB1*0103, DR4
Estonians		DRB1*1501
Japanese		B52,DR2
Europe	B27,B58, DRB1*0103	

Fine examinations of HLA region have further impacted the genetic importance of this chromosomal region in the extra-intestinal manifestations of IBD. In an association study carried out on 348 Caucasian IBD patients and 472 ethnically matched controls, the haplotype HLA-DR3-DQ2 was significantly increased in extensive ULC, especially in females, and a further association of a severe disease with a rare HLA-DR allele (DRB1*0103) was also observed (Annese *et al., 2005*). The latter association was further confirmed in ULC patients requiring colectomy (Kallel *et al.,* 2005). The role of DRB1*0103 in the extra-intestinal manifestations of IBD was the subject of a further exploration, and accordingly, it was found that this allele is strongly associated in patients with a large-joint inflammatory oligo-arthropathy, whereas patients with a small-joint arthropathy did not show such association, and instead, they showed association with HLA-B44 (van Heel *et al., 2001*).

The evidence of agenetic contribution to the pathogenesis of IBD has stimulated efforts to identify susceptibility genes outside chromosome 6 (6p21) for both types of the disease. Genome scans for linkage have indicated multiple chromosomal regions of interest; these are 3p, 3q, 5q, 14q and 19p (Rowe, 2004).

2-2-2: Immunology of IBD

Immunology is one of the most actively studied profiles in IBD, and disturbances of immune reactions have often been observed in the patients, for instances, increased number of immune cells in the lamina propria of the intestinal mucosa and humoral and cellular immune reactions both in blood and inflammed areas. Furthermore, association with other atuoimmune diseases, and effective treatment with steroids and immunosuppressive agents may further support the subject. Accordingly,

abnormalities of the intestinal mucosa immune function have been upgraded to explain some of the clinical and histopathological findings in IBD (Xia *et al.*, 1998). One of the important immunoregulatory abnormalities in IBD is related to T cell function (Sator, 1997). It has been demonstrated that the intestinal lamina propria of IBD patients contains CD4+ and CD8+ cells in a proportion of about 3:1, and it was shown that circulating T cell populations in IBD contained decreased mitogen-induced suppressor T cell activity (Elson and Mccabe, 1995). Histopathological examination of inflammed areas in IBD patients have demonstrated that the lymphocytes are increased in the lamina propria, and such variation was associated with increase expression of some activation marker including 1L-2 receptor and transferrin receptor on intestinal B and T cells (CD4+ and CD8+) in both ULC and CRD (Montelone *et al.*, 2002). Another important immunoregulory abnormality in IBD is related to the impalance of cytokine regulation (Sarto, 1997). Although cytokines consist of a wide range of structurally distinct peptides, the majority are categorized into three major groups: i. pro-inflammatory cytokines, so called Th1 type (1L-1, 1L-2, 1L-6, 1L-12 Interferon and tumor necrosis factor Beta), ii- Th2 type that secretes the immunomoulatory cytokines; 1L-4, 1L-5, 1L-10 and 1L-13, and finally iii- Growth factors and regulatory cytokines, including the colony-stimulating factors and fibroblast growth factors (Mosmann and Sad, 1996). An imbalance between positive and negative factors in favour of pro-inflammatory response can initiate an inflammatory cascade. One of the most interesting immunoregulatory balances is the IL-1/IL-1ra ratio. The former is a pro-inflammatory cytokine, whereas the latter is an anti-inflammatory cytokine, which inhibits the action of IL-1 by binding to IL-1 receptors without agonistic effects. A decreased IL-1/1L-1ra ratio has been observed in the mucosa of IBD patients when compared with

ratio in healthy individuals (Casini-Raggi *et al.*, 1995). Other important immunological abnormalities are related to the increased spontaneous IgG secretion in ULC patients where the intestinal B-lymphocytes secrete predominantly IgG1 and IgG3, while in CRD, the intestinal B-lymphocyte cells also exhibit increase of IgG1 but secrete IgG2 rather than IgG3. This increase in IgG secretion may be due to an increase in number and ratios of intestinal plasma cell populations in IBD patients compared with controls (Fusungan and Sanderson, 1998).

A further subject of IBD immunology has focused on the possibility to consider the disease as an autoimmune disorder. In 1983, Roche and colleagues identified anti-epithelial cell antibodies in the sera of IBD patients, and such observation was confirmed in the sera of IBD patient relatives. Such antibodies were also cross-reacting with some polysaccharide antigens of enteric bacteria (i.e. *Echerichia coli*) (reviewed by Brown *et al*., 1995). Such findings may suggest that both immunological and environmental factors are of concern in establishing the disease.

2-2-3: Enviromental Factor

Since ULC and CRD were first described much effort has been made to search for infectious agents that may cause the disease (Miskovitz and Rochwarger, 1993). Several agents have been suspected including viruses (e.g. herpes simplex, cytomegalovirus, retrovirus, Norwalk agent and influenza), bacteria (*Campylobacter jejuni*, *Shigella*, *Salmonella* and *Echerichia*) and parasites (*Entamoeba* spp. and *Schistosoma mansoni*) (Wakefield *et at.*, 1993), however, no definite evidence has been provided. Most recently, measles virus has also been proposed as a causative agent for CRD (Konstantinos *et al.*, 1999). Other factor including smoking, use of oral contraceptives, nutrition and dietary

habits, blood transfusion and prenatal infections, have been suggested as risk factors in IBD (Russel and Stockbrugger, 1996). None of these factors has been confirmed, but smoking may highlight some interest, especially when the disease clinical types (CRD and ULC) are considered. Smoking seems to have an opposite effect in ULC and CRD. Non-smokers are associated with ULC and ex-smokers are at even higher risk of developing ULC than never smokers (Nakmura and Labarthe, 1994). The protective effect of smoking in ULC seems to be related to nicotine, but clinical trials have shown variable results (Sartor, 1997). In contrast to ULC, smoking has been suggested to be associated with the development of CRD (Lindberg *et al.,* 1998).

In conclusion, no single mechanism is acting in the development of IBD, and interactions are required between different factors (genetics, environment and immunity) in order to develop a full-blown disease. The nature of these factors may be different in each group of patients due to the differences in genetic backgrounds and environmental profiles.

2-3: Other Parameters Investigated

For a further understanding of IBD, other genetic and immunological parameters were employed, and theses were blood groups, total and differential counts of leucocytes, lymphocyte subpopulations and phagocytosis.

2-3-1: Blood Groups

The fact that people could be classified into groups by the 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 ABO blood group system, which was the first genetic polymorphism (Nora & 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 & 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 an 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 & 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). Recently, Al-Sheikh (2004) has demonstrated that blood group phenotypes are significantly distributed in Iraqi aborted females with toxoplasmosis. These observations suggest a casual relationship between a particular disease and the blood group with which it is associated.

2-3-2: Total and Differential 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. 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 a specific 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: Lowell, 2001).

2-3-3: Lymphocyte Subpopulations

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 .These molecules are specific antigens that mark different cell populations, some are specific for cells of a particular lineage or maturational pathway, while 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 & Rabson, 2000).

2-3-4: Phagocytosis

The first line of innate cellular immune defence mechanism is phagocytosis, by which viruses, bacteria, fungi and other infectious agents are engulfed inside professional cells that are called phagocytes. The cells are of two types, polyomrphonuclear (PMN) cells and monocytes. The main functional cells of the former type are neutrophils that are found in peripheral blood, and can migrate to the site of infections in tissues. Monocytes share a similar function, however, these cells can be found in the blood stream, as well as, in tissues where they are named as macrophages. The latter cells are important in linking the innate and adaptive immune systems through presenting engulfed antigens to the lypmhocytes in the secondary lymphoid organs, in which the foreign invaders are specifically recognized (Weir, 1988).

The initial steps in successful phagocytosis require that phagocytes recognize and migrate to sites of pathogen invasion. The migration is initiated by chemical attraction of some humoral factors (i.e. C3a and C5a). Later on, the cells have to adhere to pathogens, and such process is facilitated and enhanced by opsonins (IgG and C3b), because phagocytes have cell surface receptors for these molecules. The pathogens, then can be engulfed and processed (digested) in the phagolysosomes inside the

phagocytes by means of chemical reaction pathoways, some are oxygen-dependent, while others are oxgen-independent (Jones *et al*., 1999).

The clinical significance of phagocytosis is augumented if we consider the consequences of phagocyte defects, which are observed in some inherited human diseases, for instances, cyclic neutropenia, Chédiak-Higashi syndrome, myloperoxidase deficiency and chronic granulomatous. These diseases are characterized by recurrent bacterial infections (Holland & Gallin, 1995; Hyde, 2000).

Chapter One: Introduction

1-1: Introduction

A man dies because his body has rejected heart transplantation; a woman is crippled by rheumatoid arthritis; a child goes into a comma that is brought on by cerebral malaria; another child dies of an infection because of an immunodeficiency; an elderly man has advanced hepatic cirrhosis caused by iron overload. These five clinical situations are as divers as can be, yet all have one thing in common: the cause of all of them involves the human leukocytes antigen (HLA) system, the human version of the major histocompatibility complex (MHC). Malfunction of the HLA system, which is at the root of these and many other clinical disorders, has such wide-ranging effects not only because of the system's role in the adaptive immune response, but also because of its genetic complexity.

Klein & Sato (2000)

This quotation was written after 27 years from the first discovery of an association between an allele of HLA system and a human disease (HLA-B27 and ankylosing spondylitis), which was demonstrated independently by two groups of investigators; Brewerton *et al.* (1973) and Schlosstein *et al.* (1973). Such demonstration paved the way for a considerable impact to understand the fabulous profile of the field human immunogenetics, which appreciates the close fundamental relationship between the disciplines of immunology and genetics. The field focuses on genes involved in the adaptive immune response. These genes are grouped under the heading MHC, which was discovered in mouse by Gorer in the thirties of the last century, and two decades later in human

by Dausset, but under different names: H-2 system and HLA system, respectively (Klein, *et al.*, 1983).

The HLA system is mainly recognized by the highly polymorphic status of its loci, and such polymorphism was the tool in the establishment of HLA-disease association studies, which yielded considerable information on the mode of inheritance, disease heterogeneity, aetiology, prognosis and pathogenesis of autoimmunity (Ad'hiah, 1990). In this regard, inflammatory bowel disease (IBD) is a further subject of HLA and diseases, and of a great importance is the demonstration that colonic epithelium is HLA-DR positive in IBD. Furthermore, HLA-DR positivty is related to the disease activity, and is not present in uninvolved mucosa (Brown *et al.*, 1995). The same authors further highlighted the importance of HLA polymorphism in the aetiopathogenesis of IBD, and considerations of disease heterogeneity and racial variations were augmented. Accordingly, the present study was designed to investigate the role of HLA polymorphisms in the aetiopathogenesis of IBD. The disease heterogeneity was also considered, and therefore, two clinical entities of the disease were studied. These were Crohn's disease (CRD) and ulcerative colitis (ULC).

1-2: Aim of Study

The forthcoming theme was evaluated by employing some immunogenetic and immunological parameters. These were:

1- HLA-class I and HLA-class II polymorphisms.
2- Blood group phenotypes.
3- Total and Differential counts of leucocytes.
4- Lymphocyte subpopulations (CD3+, CD4+, CD8+, and CD19+ cells).
5- Phagocytosis.

Chapter Two: Review of Literature

2-1: The HLA System

The term HLA refers to the human leucocyte antigen, which represents the human major histocompatibility complex (MHC). It is controlled by a set of linked genes located on the short arm of chromosome 6 between bands 6p21.31 and 6p21.32, and spans a DNA segment of about 4000 kbp (Salazar & Yunis, 1995). The number of genes has not precisely defined, however, Newell and colleagues (1996) tabulated a count of 212 genes, but only a fifth of that number encodes the leucocyte antigens (Forbes & Trowsdale, 1999).

The HLA genes are organized in three chromosomal regions: HLA-class I, II and III. Class I and class II regions occupy the telomeric and centromeric sides of the short arm of chromosome 6, respectively, and their gene products are cell surface glycoproteins, which are structurally and functionally related. HLA-class III region is located between class I and class II regions, and its gene products are serum proteins (Roitt & Rabson, 2000). A schematic presentation of HLA gene map is given in figure 2-1.

A characteristic feature of HLA genes and gene products is their extreme genetic polymorphism. The polymorphism can be defined as the occurrence in a population of two or more genetically determined forms (alleles) of a gene in such frequencies that the rarest of them could not be maintained by a mutation alone (Emery & Muller, 1988). Such definition is overwhelmed by the HLA system, in which, the expressed loci show a high degree of polymorphism at each locus (Dorak, 2002). The HLA polymorphism can be explained in the ground of immunological recognition between self and non-self, and in such theme, the HLA

antigens fit well the concept. Therefore, the polymorphism may have evolved to encounter the different invading microorganisms and antigens that challenge the immune system (Klein & Sato, 2000). A list of serologically recognized HLA antigens is presented in table 2-1.

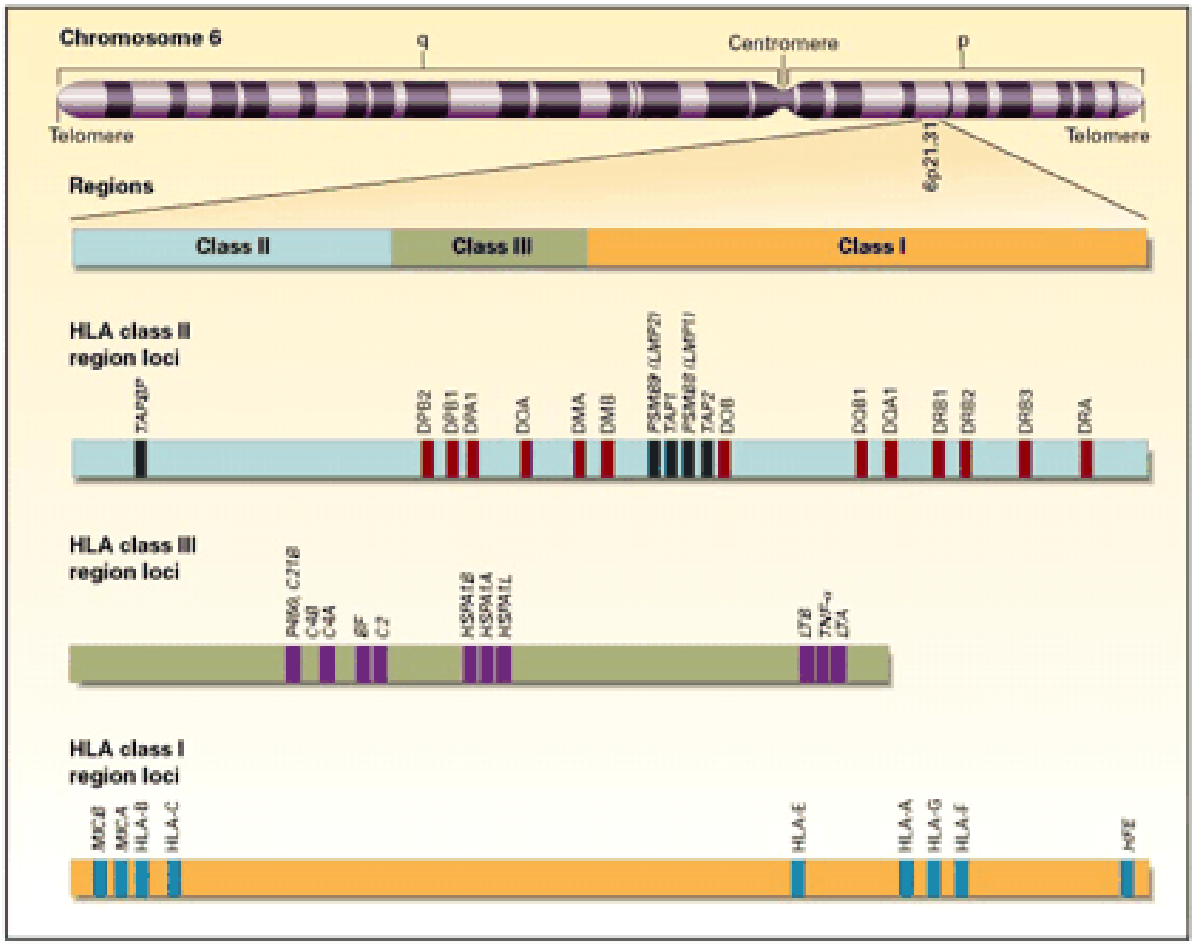

Figure 2-1: HLA gene map (Klein & Sato, 2000).

Table 2.1: Serologically recognized HLA alleles*.

HLA Alleles					
A Locus	**B Locus**		**C Locus**	**DR Locus**	**DQ Locus**
A1	B5	B49(21)	Cw1	DR1	DQ1
A2	B7	B50(21)	Cw2	DR103	DQ2
A203	B703	B51(5)	Cw3	DR2	DQ3
A210	B8	B5102	Cw4	DR3	DQ4
A3	B12	B5103	Cw5	DR4	DQ5(1)
A9	B13	B52(5)	Cw6	DR5	DQ6(1)
A10	B14	B53	Cw7	DR6	DQ7(3)
A11	B15	B54(22)	Cw8	DR7	DQ8(3)
A19	B16	B55(22)	Cw9(w3)	DR8	DQ9(3)
A23(9)	B17	B56(22)	Cw10(w3)	DR9	
A24(9)	B18	B57(17)		DR10	
A2403	B21	B58(17)		DR11(5)	
A25(10)	B22	B59		DR12(5)	
A26(10)	B27	B60(40)		DR13(6)	
A28	B2708	B61(40)		DR14(6)	
A29(19)	B35	B62(15)		DR1403	
A30(19)	B37	B63(15)		DR1404	
A31(19)	B38(16)	B64(14)		DR15(2)	
A32(19)	B39(16)	B65(14)		DR16(2)	
A33(19)	B3901	B67		DR17(3)	
A34(10)	B3902	B70		DR18(3)	
A36	B40	B71(70)			
A43	B4005	B72(70)		DR51	
A66(10)	B41	B73		DR52	
A68(28)	B42	B75(15)		DR53	
A69(28)	B44(12)	B76(15)			
A74(19)	B45(12)	B77(15)			
A80	B46	B78			
	B47	B81			
	B48				

*: (Klein and Sato , 2000)

2-1-1: HLA-class I Region

As shown in figure 2-1, class I region occupies the telomeric side of the short arm chromosome 6, and spans about 1600-2000 Kbps of the

DNA in this location. The region contains genes that encodes the classical HLA-class I antigens: HLA-A, -B and -Cw. Other class I genes have also been recognized by the 1998 Nomenclature committee, these are HLA-E, -F, -G, -H, -J, -K and -L. Among those, only HLA-E, -F and -G genes are expressed (Dorak, 2002).

HLA-A, -B and -Cw antigens are virtually expressed on the cell surface of most nucleated cells, although the level of expression varies depending on the tissue. The class I molecule is a heterodimer consisting of a heavy chain (alpha; α) and a light chain (β_2-microglubulin; β_2M). The α chain is a transmembrane glycoprotein with a molecular weight of about 45 kilodaltons, and encoded by a structural polymorphic gene located on the short arm of chromosome 6. Based on amino acid analysis, the α-chain is divided into three regions: an extracellular region, which is subdivided into three domains (α1, α2 and α3), a transmembrane region and a cytoplasmic tail (Figure 2-2). The β_2M chain is also a glycoprotein, but with a less molecular weight (12 kilodaltons), and encoded by a non-polymorphic gene located on chromosome 15. The α-chain is non-covalently associated with β_2M, and without the latter chain, class I antigen will not be expressed on the cell surface (Klein *et al*., 1983; Owen and Steward , 1998).

HLA-A, -B and -Cw loci are highly polymorphic, having multiple alleles at each expressed locus, and up-to-date, 24, 48 and 9 alleles have been serologically identified at HLA-A, -B and -Cw loci, respectively (Table 2-1). The polymorphism can be attributed mainly to amino acid substitution in the α1-domain, while α2-domain shows a less degree of amino acid variability. The α3-domain is considered as a conservative part of the chain, because the amino acid sequence in this domain has not shown a variation among HLA-class I antigens so far sequenced (Marsh, 2004).

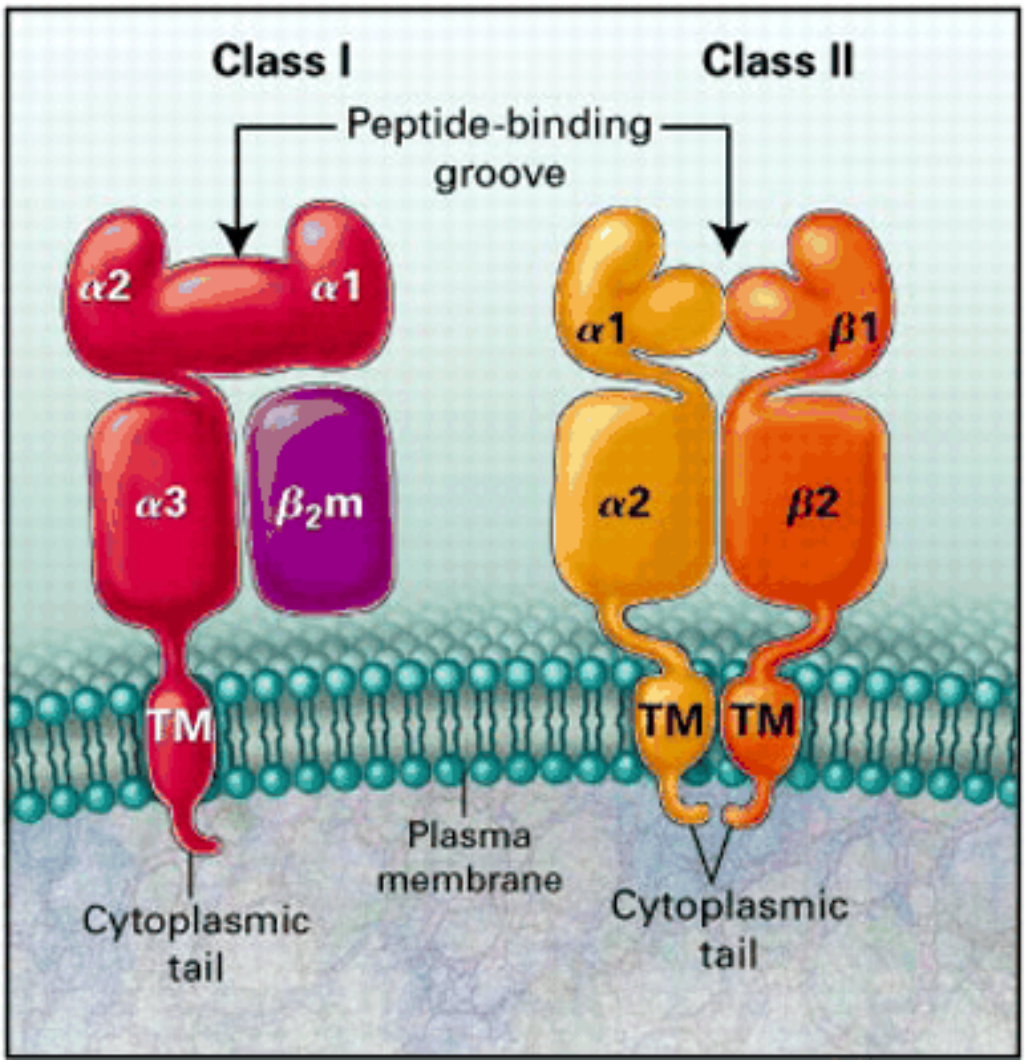

Figure 2-2: Structure of HLA - class I and -class II molecules (Klein & Sato, 2000).

2-1-2: HLA-class II Region

HLA-class II region is located in the centromeric side of the short arm of chromosome 6, and extends over 1000 to 1200 Kbp of DNA (Figure 2-1). Five subregions have been recognized in class II region, and the designation of their loci consists of three letters: the first (D) indicates the class, the second (R, Q, P, O or M) is the family and the third (A or B) refers to the chain (α or β, respectively) (Klein and Sato , 2000).

HLA-DR genes (DRA and DRB) encodes HLA-DR antigens, HLA-DQ genes (DQA1 and DQB2) encodes HLA-DQ antigens, HLA-DP genes (DPA1 and DPB2) encodes HLA-DP antigens, while the HLA-DOB and HLA-DM (DMA and DMB) loci function in recruitment of

antigen by class II molecules. There are also several pseudogenes, which are DRB2, DRB6, DRB8, DRB9, DQA2, DQB2, DQB3, DPA2 and DPB2. Other non-HLA genes whose functions are primarily antigen presentation, are also mapped to HLA-class II region. They are LAMP2 and LAMP7, and TAP1 and TAP2 (Rhodes and Trowsdale, 1998; Margulies, 1999).

The expressed HLA-class II molecule is a heterodimer, which consist of two polypeptide chains: heavy (alpha; α) and light (beta; β). Both chains are encoded by structural genes (A and B, respectively), which are located on the short arm of chromosome 6. Also, they are transmembrane glycoproteins with a molecular weight of 33-35 and 26-29 kilodaltons, respectively. Each chain is divided into extracellular, transmembrane and cytoplasmic regions. The extracellular region is further subdivided into two domains: α1 and α2, and β1 and β2, respectively (Klein *et al*., 1983; Owen and steward, 1998). Both genes (A and B) and their products (α and β) are highly polymorphic in the expressed loci (DR, DQ and DP), but DRA gene is an exception. The gene have shown two allelic forms only (DRA*0101 and DRA*0102) at the molecular level. The polymorphism is mainly ascribed to amino acid substitution in the α1 and β1 domains of α and β chains, respectively (Margulies, 1999). Up-to-date, there have been 20 and 7 alleles, which are serologicaly recognized at HLA-DR and -DQ loci (Marsh, 2004), and at the molecular level, the list can be much further expanded (Table 2-1).

HLA-DR, DQ and DP antigens have a limited tissue distribution. They are expressed on the surface of a subgroup of immune cells that includes B lymphocytes, activated T lymphocytes, macrophages, dendritic cells and thymic epithelial cells (Owen and steward , 1998).

2-1-3: HLA-class III Region

The class III region contains a disparate collection of densely packed genes. Many of them have not been characterizes fully at the functional level, but a role in the innate immune response and inflammation has been suggested (Alper *et al.*, 1986). HLA-class III genes with obvious roles in immunobilogy are members of the complement activation pathways (C2, C4A, C4B and BF), which are also highly polymorphic, especially C4A and C4B loci (Ad'hiah *et al*., 1996). Other genes are also of interest; these are HSP70, TNF and CYP21 genes. The HSP70 genes encode cytosolic molecular chaprons that may function as forerunners of MHC molecules in presenting intracellular contents of cancer cells to the immune system, therefore, HSP70 may behave like a tumor rejection antigen. TNF(A) and TNF(B) genes encode cachectin and lymphotoxin-α molecules, respectively, while CYP21 is the gene for 21-hydroxylase, which is an important enzyme in corticosteroid metabolism (Dorak, 1998).

2-1-4: Inheritance of HLA genes

HLA genes are inherited as a group in a form of a haplotype, and therefore, each individual inherits two haplotypes, one from each parent. Each haplotype contains three class I (A, B and Cw) and three class II (DR, DQ and DP) genes. A heterozygous individual will inherit a maximum of six class I alleles, and a similar number of class II alleles. These alleles are codominantely inherited, and follow the law of segregation set down by Mendel (Klein *et al*., 1983).

Due to their close linkage, alleles on a single chromosome segregate together (*en bloc*), and as mentioned earlier in this section, they represent a haplotype. However, in certain haplotypes, some alleles of different HLA loci occur in a nonrandom association, for instance A1-B8

haplotype. The frequency of HLA-A1 allele in Caucasian populations is 0.17, and that of HLA-B8 is 0.11. Then if these two alleles were associated at random, the expected frequency of their being found together in the population would be the product of their independent frequencies: 0.17 x 0.11 = 0.0187 (1.87%). Inspecting the Caucasian populations for this combination (A1-B8) showed an observed frequency of 0.09 (9%), which is much higher than the expected frequency. This difference between the expected and observed haplotype frequencies is attributed to the phenomenon "Linkage Disequilibrium", which may suggest that selection is favouring certain HLA allele combinations. However, a cross-over between two parental chromosomes at meiosis has also been reported in the HLA region resulting in new recombinant haplotypes, which may scope a further variation in this region (Rhodes and Trowsdale, 1998).

2-1-5: Function of HLA Antigens

In general, HLA molecules are involved in a number of immunological and non-immunological interactions. With respect to the immunological function, HLA molecules bind peptide fragments of antigens degraded inside the antigen presenting cells, and present them to the receptors of T lymphocytes (Trowsdale, 1995). The source and nature of presented peptide impose whether it is associated with class I or class II molecules. Peptides synthesized inside the antigen presenting cells (i.e. viral antigens) are associated with class I antigens. Therefore, when a cell became infected with a virus, the viral proteins are synthesized endogenously using the host cell's machinery, and in the cytosol, the viral proteins are degraded into peptides in proteasomes. Selected peptides are then transported into the cytoplasmic reticulum, where they are loaded onto newly synthesized class I molecules, and the HLA-peptide

complexes are then exported to the cell surface. These complexes are recognized by CD8+ cells, which will be then educated to initiate the process of killing cells having the complexes on their surface (Rhodes & Trowsdale, 1998).

Peptides derived from outside antigen presenting cell (exogenous antigens; bacteria) are taken in by endocytosis, processed using proteolytic enzymes and presented with class II molecules, and such processes are a pivotal event in the initiation of immune responses. Class II molecules and associated peptides are then presented for T-helper lymphocytes (CD4+), which in turn initiate an immune activation presented by cytokine productions that direct cellular and humoral immune responses against the challenged antigen (Owen & Steward, 1998).

An additional function of HLA molecules that is not directly related to T-lymphocyte recognition has been recognized in recent years. They serve as elements for signal transduction to natural killer (NK) cells. The expression of HLA class I molecules by a target cell can protect the target from killing by the NK cells, and target cells defective in the expression of these molecules are susceptible to NK-cell lysis (Rook & Balkwill, 1998).

With respect to the non-immunological functions of HLA antigens, it has been augmented that these molecules interact with other receptors on the cell surface, for instances transferrin receptor, epidermal growth factor and various hormone receptors (Schafer *et al*., 1995).

2-1-6: Methods of HLA Typing

HLA typing is carried out at the phenotypic level (i.e. what types of protein are expressed on the cell surface) and the genotypic level (i.e. what DNA sequence is present in the genes). The former approach is

termed serological HLA phenotyping, and employs a technique called microlymphocytotoxicity test, which was developed by Terasaki and McClelland (1964). In this technique, anti-HLA antibodies recognize and react with HLA molecules on the surface of lymphocytes. The reacted cells will activate the classical pathway of the added rabbit complement, and consequence in their death, which indicates that these cells express the HLA molecule defined by that antiserum (Ad'hiah, 1990).

In 1984, HLA alleles were analyzed at the genotypic level, and the use of restriction fragment length polymorphism (RFLP) was introduced during the ninth histocompatibility workshop (Albert , 1984). In this workshop, HLA genes were studied at the DNA level, and such presentation demonstrated the power and utility of DNA technology in HLA typing, and paved the way for its use in subsequent workshops. In 1991, the eleventh histocompatibility workshop recognized the introduction of polymerase chain reaction (PCR) technology with sequence specific oligonucleotide probe (SSOP) hybridization as an HLA-DNA typing method (Forbes and Trowsdale ,1999). These methods were further examined and developed in the subsequent workshops, and their applications revolutionized the HLA polymorphism especially in the class II region (Robinson *et al,* 2003).

2-1-7: HLA and Diseases

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

Chapter Three: Subjects, Materials and Methods

3-1: Subjects

Sixty–five Iraqi patients with inflammatory bowel disease (IBD) were investigated. During the period May-December, 2004. The disease was clinically diagnosed by the consultant medical staff at Al- kadhamyiah Teaching hospital and Gastrointestinal Tract (GIT) and Liver Disease Center in Baghdad. The diagnosis was based on a clinical evaluation using colonoscopy and a histopathological examination of a biopsy. According to the point view of consultants, the patients were clinically subdivided into ulcerative colitis (ULC) and Crohn's disease (CRD) (Table 2.1).

Table 3.1: Numbers and percentage frequencies of IBD patients (UCL and CRD) and controls divided by sex, and their age ranges.

Parameter		IBD patients (No.= 65)						Controls (No. = 67)	
		Total (No. = 65)		UCL (No.= 50)		CRD (No. = 15)			
		No.	%	No.	%	No.	%	No.	%
Sex	Males	29	44.6	25	50.0	4	26.7	30	44.8
	Females	36	55.4	25	50.0	11	73.3	37	55.2
Age Range (years)		13-65		13-35		19-65		18-65	

Sixty–seven individuals (apparently healthy) were further investigated, and were considered as a control sample. They were matched

with patients for age, sex and ethnic background (Arab Muslims) (Table 2.1).

3-2: Biological Materials and Kits

1. HLA class I and class II antisera and control sera: Biotest, Germany.
2. Mouse antihuman CD markers (CD3, CD4, CD8 and CD19): Serotec, U.K.
3. Lyophilised rabbit complement: Duxted Rabbit, U.K.
4. Blood group kit (anti-A and anti-B antibody): Biotest, Germany.

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., 1.5 pound/in^2, 20 minutes) and stored in the refrigerator (4ºC.) until use.
- Hank's balanced salt solution (HBSS): The following materials were dissolved in 1000 ml of distilled water. After adjusting the pH to 7.2, the solution was autoclaved and stored at 4ºC until use (Hudson & Hay, 1980).

    1. Sodium chloride (NaCl): 8.00 grams.
    2. Potassium chloride (KCl): 0.40 gram.
    3. Calcium chloride ($CaCl_2$): 0.20 gram.
    4. Magnesium sulphate ($MgSO_4.2H_2O$): 0.20 gram.
    5. Potassium di-hydrogen phosphate (KH_2PO_4): 0.10 gram.
    6. Sodium bicarbonate ($NaHCO_3$): 1.27 gram.
    7. Glucose ($C_6H_{12}O_6$): 2.00 grams

- 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).
- Wright stain: The stain solution was ready-prepared (The Institutes of Sera and Vaccines, Baghdad).
- Nitroblue tetrazolium (NBT) stain: An amount of 0.2 gram of the stain powder was dissolved in 100 ml of sterile physiological saline. Then, the solution was stored in sterile dark bottle at 4º C until use (Metcalf *et al*., 1986).
- 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 Media: 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 in heparinzed test tube, and divided into two aliquots (1.5 and 8.5 ml). The first aliquot was used for total and differential counts of leucocytes, phagocytosis and blood group phenotypes. The second one was employed for the detection of HLA polymorphism and lymphocyte subpopulations. The blood was manipulated for laboratory investigation in less than two hours.

3-4-2: Total and Differential 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:

$$\text{Total Count (cells/cu.mm.blood)} = \left(\frac{\text{Number of Cells Counted}}{4} \right) \text{x } 20 \text{ x } 10$$

3-4-2-2*: Differential Count of leucocytes*

A blood smear was made on a clean slide and left for air drying. Then the slide was stained with Lishman'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 count of each type of leucocytes was calculated according to the following equation:

$$\text{Total Count (cells/cu.mm.blood)} = \left(\frac{\text{Percentage of Cells x Total Count}}{100} \right)$$

3-4-3: HLA phenotyping

3-4-3-1: *Principles*

The test was performed in the Histocompatiblty Laboratory of the 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 Infections 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.

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 3.4.1). The following steps were followed:

- The blood (8.5ml) 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 salin 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 rmp) for 30 minutes in a cooled centrifuge.
- After centrifugation, the lymphocytes were visible as cloudy band between the plasma and lymphoprep layeres.
- 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 resuspended in 5ml 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 most 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 (DR and DQ) 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 and -DQ 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 3.4.3.2) was transferred to the barrel of nylon-wool, and both ends of the syringe barrel were sealed with parafilm and 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 antigen.

- The nylon-wool adherent B-lymphocytes were isolated by adding a worm washing medium (10 ml) to the syringe-barrel, and the nylon-wool was squeezed by the syringe piton 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- class II antigens.

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

An aliquot of 10 µl of cell suspension (section 3.4.3.3) 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 lid and stored in a freezer (-20ºC) until required. The wells contained antisera specific for HLA-class I (A and B) and -class II (DR and DQ) antigens, which were available in the Histocompability laboratory at the Al - Karama hospital (Table 3-2).

Table 3-2: Listing of used HLA antisera

HLA –A	HLA-B	HLA-DR	HLA-DQ
A1	B7	DR1	DQ1
A2	B8	DR2	DQ2
A3	B12	DR3	DQ3
A9	B13	DR4	
A10	B14	DR5	
A11	B15	DR6	
A28	B16	DR7	
A29	B17	DR8	
A30	B18	DR10	
A31	B21		
A33	B22		
	B27		
	B35		
	B40		
	B41		
	B42		
	B48		
	B51		

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-classII 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 eosine 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 were as the following:

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: Phenotyping of Blood Groups

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 on the cell surface of erythrocytes.

3-4.5: Lymphocyte Subtyping 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 & 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 (3.4.3.2) 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 Almonium 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: Phagocytosis

The method of Metcalf and colleagues (1986) was adapted to carry out the procedure of assessing phagocytosis in the peripheral blood. The method is based on assessing the percentage of polymorphonuclear cells that phagocytose heat-killed yeast, as well as, their ability to reduce the substance nitroblue tetrazolium (NBT).

3-4-6-1: *Preparation of Heat-Killed Yeast*

Ten grams of the yeast (*Saccharomyces cervisiae*) were suspended in warm (37ºC) physiological saline (150 ml). The cell suspension was heated in boiling water both for 60 minutes. After heating, the cells suspension was cooled to 37ºC, and filtered using sterile double layers of gauze. The filtered cell suspension was assessed for yeast cell viability by dye exclusion test (trypan blue) to assure that all cells were dead. Then, the cell suspension was divided into aliquots (5 ml) and stored at -20ºC until use.

3-4-6-2: *Phagocytic Index*

To determine the phagocytic index (PI), an aliquot of the heat killed yeast suspension was obtained from the freezer and left in a water bath (37ºC) for

thawing. Then, the cell suspension was washed twice with physiological saline and the yeast cells were adjusted to a concentration of 1 x 10^7 cells/ml. Heparinized blood (0.25 ml) was mixed with 0.15 ml of yeast suspension together with 0.20 ml of Hank's balanced salt solution, and the mixture was incubated for 30 minutes at 37ºC, with gentle shaking every 5 minutes. After incubation, the mixture was mixed thoroughly, and a blood film was made and stained with Wright stain for 3 minutes. Then, the slide was washed with tap water and air-dryed. The slide was inspected for yeast-phagocytic cells using light microscope, and for each samples at least 100 polymorphonuclear cells (phagocytic and non-phagocytic cells) were randomly counted, and the percentage of yeast-phagocytic cells was scored according to the following formula:

$$\text{Phagocytic Index (\%)} = \left(\frac{\text{Number of Phagocytic cells}}{\text{Total Count}} \right) \text{x } 100$$

3-4-6-3: *Nitroblue Tetrazolium Index*

The procedure of Metcalf *et al*. (1986) was followed to assess the nitroblue tetrazolium index. The following materials were mixed in a test tube and incubated at 37ºC for 25 minutes:

1- Heparinized blood : 0.25 ml
2- HBSS : 0.20 ml
3- NBT solution : 0.10 ml
4- Yeast suspension : 0.05 ml

After that, a blood smear was prepared on a slide and left for air-drying. The slide was stained with Wright stain (3 minutes), washed with tap water and air-dryed. Then, the slide was examined under light microscope to determine the polymorphonuclear cells that reduced the NBT substance, and

their percentage was calculated (NBT index; %) using the equation of phagocytic index.

3-5: Statistical Analysis

The statistical manipulations included three main analyses, which were assessment of HLA gene frequencies, HLA and disease association and assessment of significance between means. These analyses are presented by Ad'hiah (1990).

3-5-1: Gene Frequency of HLA alleles

The gene frequency of HLA alleles was assessed by employing the formula of Square Root Method:

$$\text{Gene Frequency [P]} = 1 - \sqrt{1 - \text{Antigen Frequency}}$$

From gene frequencies, the expected numbers of alleles were estimated using the formula:

$$\text{Expected Number} = [2 \times N \times P (1 - P) + (N \times P^2)]$$

3-5-2: HLA and Disease Associations

The association between a marker and a disease is expressed in terms of relative (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 is positive, the EF is calculated, while if it is negative, the PF is calculated. The significance of such association (positive or negative) is assessed by either Chi-Square test or Fisher's exact probability. The latter assessment is more preferred, because it

allows for the correction of probability, moreover, it is not affected by small numbers (less than 5). The mathematical calculations of such parameters are as the following:

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

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

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

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.

3-5-3: Differences between Means

For total and differential counts of leucocytes, lymphocytes subpopulations and phagocytosis (PI and NBT indices), the means were given. Differences between means of patients and controls were assessed by ANOVA test and the Least Significant Difference (LSD), These analyses were carried out using the computer programme; Statistical Package for Social Sciences (SPSS).

Significant differences in the distribution of blood group phenotypes were assessed by Chi-square test, which was calculated according to the following formula:

$$\text{Chi - square} = \frac{(\text{Observed - Expected})^2}{\text{Expected}}$$

Chapter Five: Discussion

The present study demonstrated that immunogenetic predisposition may be considered as an important requirement for the development of IBD, and HLA antigens are in favour of such generalization, in which several markers of human MHC showed different distributions in patients and controls. These markers are belonging to two regions of human chromosome 6. They are HLA-class I and -class II loci, in which a highly polymorphic status is recognized (Dorak, 2002).

At HLA-class I region, remarkable deviations were observed for the antigens A9, A11 and B41. Both A9 and B41 were in favour of increased frequencies in the patients. The antigen A9 was observed in around 50% of the patients, while B41 antigen was much higher (about two-thirds of the patients). Such two deviations scored RR values of 5.03 and 30.78, respectively, and maintained EF values of 0.42 and 0.63, respectively. Inspecting other HLA-IBD-association studies carried out in other world populations revealed associations with other HLA-class I antigens; B5 in Japanese, and B27 and B44 in Caucasians (Brown *et al*., 1995). Such discrepancy can 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*., 2004). 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 A9 (0.42) and B41 (0.63) support the forthcoming statement, and 37 and 58% contributions of other factors in association with A9 and B44, respectively, are required in the development of IBD, but what are these factors?. The answer may be augmented if we consider the immunological roles of HLA-class I antigens. These antigens are virtually expressed on most nucleated cells, and are involved in antigen presentation carried out by macrophages, especially those antigens of a viral origin (Klein *et al*., 1983). A default in such mechanism may render an individual vulnerable to any immunologically-mediated morbidity (Owen and Stewant, 1998, 1998). In this respect, different viruses (Herpes viruses, cyto megalovirus and influenza viruse) have been encountered in different populations (Xia *et al.*, 1998), and although none of them is a conclusive causative agent, their importance can not be ignored if the immunological role of HLA-class I antigens in antigen presentation is considered. In this respect, the hypothesis of molecular mimicry may have the clue, because each potential virus may share epitopes with HLA antigens, and such sharing may be different in different populations due to the environmental impact, which is certainly varied from region to region in the globe (Parkes and Jewell, 2001). Therefore, the positive association of A9 and B41 with IBD in Iraq could not be confirmed by previous studies.

A further inspection of HLA-class I antigens in subgroups of IBD (ULC and CRD) revealed a similar picture, and both A9 and B41 antigens were positively associated with the two clinical entities of IBD. In this sense, these two antigens may confer an immunogenetic predisposition to the general IBD irrespective of its clinical types, although the strength of the two associations was different. The B41 was

more important than A9 in ULC (RR = 40.5 *vs*. 3.6; EF = 0.70 *vs*. 0.31), while the opposite outcome was observed in CRD, in which the A9 is the most important class I antigen (RR = 6.88 ; EF =0.51). Furthermore, and with the attempt to characterize each clinical type in terms of HLA antigens, one important finding was observed. The antigen B16 was present in only 2% of ULC patients, while its frequency in CRD patients reached 33.3%. So, can we consider B16 as a differentiating marker between the two clinical types of IBD, a further confirmation is certainly required before reaching a substantial conclusion.

At HLA-class II region, further antigens were in favour of positive associations with IBD, these were DR4 (RR = 3.3; EF = 0.23), DR5 (RR = 6.6; EF = 0.14) and DR8 (RR = 4.5; EF = 0.24), but these associations were limited to DR8 after correction of probability (Pc=0.018). Although, these associations were not strong as that of HLA-class I region, the polymorphism of HLA-class II loci has gain much more interest in HLA-disease association studies, because both α and β chains, which are coded by structural genes on chromosome 6, are highly polymorphic especially at HLA- DQ and -DP subregions (Margulies, 1999). Accordingly, it has been suggested that susceptibility to IBD is partially genetically determined, and the HLA-class II genes are candidates for a role in genetic susceptibility to IBD, because their products play a central role in the immune response (Stokkers *et al*., 1999). The present results strongly support such theme, however, multiple studies have reported associations between HL-DR and -DQ phenotypes and IBD, either ULC or CRD, but much of the data are still contraversal (reviewed by Zheng *et al*., 2003). These studies have demonstrated that DR1 (Toyoda *et al*., 1993; Danze *et al*., 1996), DR4 (Matake et al., 1992), DR5 (Fujita et al., 1984) and DR7 (Reinshagen *et al*., 1996) are positively associated with CRD, while DR2 (Matake et al.,

1992), DR3 Forcione *et al*., 1996) and DR8 (Purrmann *et al*., 1990) are negatively associated. For ULC, positive associations have been found with DR2 (De La Concha *et al*., 1997), DR6 (Leidenius *et al*., 1995) and DR12 (Satsangi *et al*., 1994), and negative associations with DR2 (De La Concha *et al*., 1997), DR6 (Toyada *et al*., 1993) and DR7 (McConnell, 1983) have been observed. For HLA-DQ locus, the studies have reported further associations, and ULC has been associated positively with DQ2 and negatively with DQ3, while an increased frequency of DQ3 and DQ4 were observed in patients with CRD, and in the same disease, decreased frequencies of DQ1 and DQ6 have been reported (Reviewed by Zheng et al., 2003). None of these findings was confirmed in the present study, and in contrast, DR8 was positively associated with IBD, either total or clinical sub types. However, some of these associations were observed in the present IBD patients (IBD: DR4, DR5, DR6, DR7 and DQ2; ULC: DR4, DR5, DR6, DR7, DQ1 and DQ2; CRD: DR5, DR6 and DQ1), but the significance was lost when the probability was corrected for the number of antigens test at each locus, and such statistical application is important to exclude a chance occurrence of an association due to many comparisons that were made (Ad'hiah, 1990). Therefore, the discrepancy can be ascribed to either racial differences (different associations in different populations), low sample size (the level of significance is affected) or environmental impacts (different causative pathogens). With respect to the latter factor, the phenotypic expression of HLA-class II antigens is also involved in antigen presentation by macrophages, but with antigens of a bacterial origin, and such pathogens may have adapted different epitpes in different populations (Owen and Steward 1998).Therefore, a similar argument, as in HLA-class I antigens, can be upgraded, and the hypothesis of molecular mimicry can also be put forward to explain the cellular destruction at the sites of IBD in the

intestine. In agreement with this scope some bacterial pathogens has been described as putative causative agents in IBD, for instances *Mycobacterium paratuberculosis* and *Helicobacter pylori*, therefore epitope sharings may be considered as a an important explaining mechanism, especially if we consider that HLA-DR positivity of mucosal cells is related to a disease activity in IBD patients (Brown *et al*., 1995).

Comparing the two clinical forms of IBD with each other in terms of HLA antigen and gene frequencies revealed that the antigen B16 was significantly different in ULC and CRD. Although such observation has not been recorded, it may help to answer crucial questions regarding the genetics of IBD, and how the two clinical forms are related to each other. The answer can be augmented if we consider family studies carried out in ULC and CRD families. Such studies have demonstrated that the two clinical forms do not always segregate independently within families (Parkes and Jewell, 2001). Accordingly, it has been suggested that there are three genetic forms of IBD, one leading to ULC alone, one to CRD alone, and a third leading to both ULC and CRD. Fine mapping of the HLA region in IBD families may support this, and explain some the discrepancies in HLA-IBD association studies (Toyoda *et al.*,1993). Furthermore, an evidence for linkage of both ULC and CRD around the HLA region on the short arm of chromosome 6 has been presented (Stokkers *et al.*, 1999). Moreover, these studies have described further predisposing loci on different chromosomes (Parkes and Jewell, 2001), and accordingly the disease heterogeneity has been highlighted.

The classical blood group antigens are a further subject of immunogenetics, although with limited allelic polymorphism. The present study demonstrated that blood group phenotypes were similarly distributed in patients (total IBD, ULC and CRD) and controls. However, such approach may underestimate the role of these antigens in the

pathogenesis of IBD, because the disease is limited to the mucosa of the intestine, 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. These are secretor, which secrets blood group antigens in their secretions, and non-secretor, which does not (Nora and Fraser 1989). The question is, can these antigens protect the mucosa or predispose it to inflammatory responses, the question waits an answer, and further investigations are required.

The aetiopathogenesis of IBD can also explained in the ground of immunology, which is one of the most actively studied aspects in IBD, and disturbances of immune reactions often has been observed in the patients, and suggestions have proposed that an abnormal immune response to a normal stimulus in a genetically susceptible host may initiate the scope of IBD (Konstantinos *et al*.,1999). Several aspects of immunity were tackled in the present study, and the start was with total and differential counts of leucocytes, as these cells in the peripheral blood stream may give a general picture of the immunological status of patients (Lydyard and Grossi, 1998). The total count showed approximate counts in patients and controls (range: 6569.71-6928.01 cells/cu. mm. blood), and such range is considered as normal. However, the differential count showed an opposite picture, and some leucocytes showed different counts in the patients and controls. Significantly increased counts of neutrophils in total IBD and ULC patients were observed, and such findings may impact the importance of these cells in the pathogenesis of IBD. Konstantinous and colleagues (2003) have recently demonstrated that neutrophils are activated in IBD patients, and the intestinal inflammation observed in the patients is characterised by an influx of neutrophils into the intestinal mucosa. Furthermore, anti-neutrophil cytoplasm antibodies (p-ANCA) have been considered as a characterizing marker for ULC but

not CRD (Rowe , 2004). The main function of neutrophils is phagocytosis (Jones *et al*., 1999), and if these cells are activated, there may be an increased phagocytic function. An examination the present data of phagocytosis revealed that phagocytosis of heat-killed yeast was significantly increased in the patients, total and subgroups, but the reducing effect of these cells on the substance NBT was significant in the total patients and ULC patients. Therefore, these data may confirm that neutrophils were activated in the IBD patients in terms of ability to phagocytosis and digesting the engulfed objects. Their activation may have a trigger, but what is the nature of that object, it is a matter of speculations. The antigenic challenge to the intestinal immune system is enormous, including pathogenic bacteria, normal resident intestinal flora, bacterial products, toxins, viruses, ingested chemicals, and even food and drinks. Thus, the immune system of the gut, which is originated in the blood stream, has evolved at least two directions. On one hand, it has to provide the host with protective mechanisms against invasion of pathogens across the surface of the mucosa, and on the other hand it has to take up large nutrient substances and tolerate the normal intestinal flora, all of which may potentially be immunogenic (Xia *et al*., 1998).

Other lineage of polymorphonuclear cells is eosinophils, and in the present study these cells were significantly decreased in the patients whether total or subgroups. These cells are also involved in the non-specific cellular immune response, but their function is normally related to allergic reactions, which are immunologically mediated (Hyde, 2000). However, their reduced count may suggest some immunological reactions in the sense of immune suppression, but no further investigation to confirm or contrast such finding, and further investigations are invited to clarify the relationship between eosinophils and pathogenesis of IBD. The same theme may also be highlighted if we consider the count of

monocytes, and similarly these cells were significantly decreased in the patients. However, these cells are involved in antigen presentation, which enhances the function of lymphocytes, and a defect in their function may effect the immune response (Owen and Steward, 1998). The lymphocytes, which are the arms humoral and cellular immune responses, showed similar counts in patients and controls, and no significant difference was recorded. However, the lymphocytes can be better understood in terms of their subgroups, which are defined by surface receptors collectively called CD markers. Accordingly, the lymphocytes can be CD3+ (pan T-lymphocytes), CD4+ (T-helper lymphocytes), CD8+ (T-cytotoxic lymphocytes) and CD19+ (B-lymphocytes) cells (Hyde , 2000). CD3+ lymphocytes were increased in the patients, but a significant level was observed in total IBD and ULC patients. It has been suggested that one of the important immunoregulatory abnormalities in IBD is related to CD3+ lymphocytes, and activation of T-cells by different stimuli increases their expression of surface markers (Xia *et al* ., 1998). Isolated T-lymphocytes from the peripheral blood or lamina propria of intestinal mucosa in IBD patients have revealed their early activation markers, which are IL-2R, CD98 and HLA-DR antigens (Konstantious *et al* ., 1999). The present study focused on T-lymphocytes with a special reference to their CD markers; CD4 and CD8. Both cells showed significant increased percentages in the patients, but the CD8+ cells showed a clearer increase than CD4+ cells, and such deviation contributed in shifting the CD4/CD8 ratio to be reduced in IBD patients (total and subgroups) as compared to controls. It is difficult to explain such finding, but most of recent studies have explained these observations in terms of cytokine production (Zheng *et al*, 2003), and unfortunately the present study could not have tackled their profiles. An understanding of cytokine profile will certainly help in revealing the observed cellular

abnormalities observed in IBD patients, and such approach may also explain the significant increased percentage of CD19+ cells in the peripheral blood of patients.

Chapter Four: Results

4-1: HLA Antigens

The frequencies of HLA antigens (A, B, DR and DQ) were compared between IBD patients (totals and subgroups) and controls. Two clinical subgroups of IBD were considered; ULC and CRD. The disease heterogeneity of IBD was also inspected through comparisons of HLA antigen frequencies between ULC and CRD.

4-1-1: Inflammatory Bowel Disease

The distributions of HLA-A, -B, -DR and -DQ antigens in total IBD patients and controls are presented in tables 4-1, 4-2, 4-3 and 4-4, respectively, while antigens showing significant variations between patients and controls are given in table 4-5.

At HLA-A locus, four antigens (A9, A11, A30 and A33) showed significant deviations when comparisons between patients and controls were made. The antigen A9 was significantly ($P = 4.7 \times 10^{-5}$) increased in the patients (52.3 *vs*. 17.9%), and such difference associated with RR value of 5.03 and PF value of 0.42. Moreover, this positive association remained significant ($Pc = 5.1 \times 10^{-4}$) after correction for the number of antigens tested (11). In contrast, the antigens A11, A30 and A33 showed negative associations with IBD, and significant (P = 0.001, 0.021 and 0.045, respectively) decreased frequencies of these antigens (3.1 *vs*. 22.4%, 6.2 *vs*. 20.9% and 4.6 *vs*. 16.4%, respectively) were observed in the patients. But, correcting the probabilities rendered one negative significant association, which was between A11 and IBD (Pc = 0.011).

At HLA-B locus, B41 antigen was significantly increased in the patients (66.1 *vs*. 5.9%, $P = 7.8 \times 10^{-14}$, RR = 30.78, PF = 0.63), while

antigens B35 and B51 were significantly (P = 0.039 and 0.009, respectively) decreased in the patients (6.2 vs. 17.9% and 16.9 vs. 37.3%, respectively). Correcting the probabilities of these associations gave one significant positive association, which was between B41 and IBD (Pc = 1.4×10^{-12}).

At HLA-class II region (DR and DQ loci), five antigens showed different frequencies in patients and controls, these were DR4, DR5, DR7, DR8 and DQ2. Increased frequencies of DR4 (33.8 *vs*. 13.4%), DR5 (16.9 *vs*. 3.1%), DR8 (30.8 *vs*. 8.9%) and DQ2 (24.6 *vs*. 10%) were observed in the patients. These positive associations scored RR values of 3.3, 6.6, 4.5 and 2.8, respectively, and PF values of 0.23, 0.14, 0.24 and 0.16, respectively. However, one positive association remained significant after correction (Pc = 0.018), and this was with DR8. In contrast, the DR7 antigen was significantly decreased in the patients (7.7 *vs*. 23.9%), but such negative association also failed to retain a significant level after correction (Pc = 0.144). It was also interesting to note that DR6 was not detected in the control sample, while its frequency in the patients was 10.7%. Such positive association was significant before correction (P = 0.006), and felt short of significance after correction (Pc = 0.054).

Table 4-1: Observed and expected numbers, percentage and gene frequencies of HLA-A antigens in IBD patients and controls.

HLA-A antigens	IBD Patients (number = 65)					Controls (number = 67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
A1	31	20.0	13.0	20.0	0.106	9	13.4	8.9	13.3	0.071
A2	27	41.5	27.0	41.5	0.235	21	31.3	21.0	31.3	0.171
A3	14	21.5	13.9	21.4	0.114	6	8.9	5.4	8.8	0.046
A9	34	52.3	33.9	52.2	0.309	12	17.9	12.0	17.9	0.094
A10	6	9.2	6.0	9.2	0.047	9	13.4	9.0	13.4	0.070
A11	2	3.1	2.0	3.1	0.016	15	22.4	15.0	22.4	0.119
A28	5	7.7	4.9	7.5	0.039	5	7.5	5.0	7.5	0.038
A29	1	1.5	0.9	1.4	0.008	2	3.0	1.9	2.9	0.015
A30	4	6.2	4.0	6.2	0.031	14	20.9	13.9	20.8	0.111
A31	5	7.7	4.9	7.5	0.039	2	3.0	2.9	2.9	0.015
A33	3	4.6	2.9	4.5	0.023	11	16.4	11	16.4	0.086
Others	–	–	4.1	6.3	0.033	–	–	20.3	30.3	0.165

Table 4-2: Observed and expected numbers, percentage and gene frequencies of HLA-B antigens s in IBD patients and controls.

HLA-B antigens	IBD Patients (number = 65)					Controls (number = 67)				
	Observed		expected		Gene frequency	Observed		Expected		Gene frequency
	No	%	No.	%		No	%	No.	%	
B7	ND	ND	ND	ND	ND	6	8.9	5.4	8.9	0.046
B8	11	16.9	11	17	0.083	3	4.5	3.0	4.5	0.023
B12	7	10.7	7	10.7	0.055	8	11.9	7.9	11.9	0.061
B13	1	1.5	0.9	1.4	0.008	3	4.5	3.0	4.5	0.227
B14	2	3.1	2.0	3.1	0.016	2	3.0	1.9	2.9	0.015
B15	ND	ND	ND	ND	ND	3	4.5	3.0	4.5	0.023
B16	6	9.2	6.0	9.2	0.047	3	4.5	3.0	4.5	0.023
B17	7	10.7	7	10.7	0.055	ND	ND	ND	ND	ND
B18	1	1.5	0.9	1.4	0.008	2	3.0	1.9	2.9	0.015
B21	4	6.2	4.0	6.2	0.031	ND	ND	ND	ND	ND
B22	2	3.1	2.0	3.1	0.015	1	1.4	1.0	1.4	0.007
B27	1	1.5	0.9	1.4	0.007	3	4.5	3.0	4.5	0.023
B35	4	6.2	4.0	6.2	0.031	12	17.9	12.0	17.9	0.093
B40	2	3.1	2.0	3.1	0.015	1	1.4	1.0	1.4	0.007
B41	43	66.1	42.9	66	0.417	4	5.9	3.8	5.7	0.030
B42	1	1.5	0.9	1.4	0.0008	ND	ND	ND	ND	ND
B48	1	1.5	0.9	1.4	0.0008	ND	ND	ND	ND	ND
B51	11	16.9	10.97	16.88	0.0008	25	37.3	25.0	37.3	0.208
Others	–	–	13.0	20.0	0.106	–	–	25.6	38.2	0.214

ND: not detected.

Table 4-3: Observed and expected numbers, percentage and gene frequencies of HLA-DR antigens in IBD patients and controls.

HLA-DR antigens	IBD Patients (number = 65)					Controls (number = 67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
DR1	12	18.5	11.9	18.4	0.097	8	11.9	7.9	11.9	0.061
DR2	23	35.4	22.7	35.1	0.194	18	26.8	18	26.7	0.144
DR3	15	23.5	15	23	0.123	14	20.9	13.9	20.8	0.111
DR4	22	33.8	22	33.8	0.187	9	13.4	9.0	13.4	0.069
DR5	11	16.9	11	17	0.083	2	3	1.9	2.9	0.015
DR6	7	10.7	7	10.7	0.055	ND	ND	ND	ND	ND
DR7	5	7.7	4.9	7.5	0.039	16	23	16.1	23.1	0.128
DR8	20	30.8	20	30.8	0.168	6	8.9	5.9	8.9	0.046
DR10	4	6.2	4.0	6.2	0.031	1	1.4	1	1.4	0.007
Others	–	–	2.9	4.5	0.023	–	–	44.3	66.1	0.419

ND: not detected.

Table 4-4: Observed and expected numbers, percentage and gene frequencies of HLA-DQ antigens in IBD patients and controls.

HLA-DQ antigens	IBD Patients (number = 65)					Controls (number =67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
DQ1	29.0	44.6	29.0	44.6	0.256	12.0	18.0	12.0	18.0	0.140
DQ2	16.0	24.6	16.0	24.6	0.132	7.0	10.0	7.0	10.0	0.053
DQ3	16.0	24.6	16.0	24.6	0.132	16.0	23.0	16.0	23.1	0.128
Others	–	–	47.4	72.9	0.480	–	–	60.0	89.6	0.679

Table 4-5: Antigens of HLA – class I class II regions showing significant variations between IBD patients and controls.

HLA Antigens	patients		Controls		RR	EF	PF	P	Pc
	No	%	No	%					
A9	34	52.3	12	17.9	5.03	0.42	__	4.7×10^{-5}	5.1×10^{-4}
A11	2	3.1	15	22.4	0.11	__	0.21	0.001	0.011
A30	4	6.2	14	20.9	0.25	__	0.16	0.021	0.231
A33	3	4.6	11	16.4	0.24	__	0.13	0.045	0.495
B41	43	66.1	4	5.9	30.78	0.63	__	7.8×10^{-14}	1.4×10^{-12}
B51	11	16.9	25	37.3	0.34	__	0.23	0.011	0.198
DR4	22	33.8	9	13.4	3.3	0.23	__	0.007	0.063
DR5	11	16.9	2	3.1	6.6	0.14	__	0.008	0.072
DR6	7	10.7	N.D.	N.D.	__	__	__	0.006	0.054
DR7	5	7.7	16	23.9	0.26	__	0.18	0.016	0.144
DR8	20	30.8	6	8.9	4.5	0.24	__	0.002	0.018
DQ2	16	24.6	7	10.0	2.8	0.16	__	0.040	0.12

4-1-2: Ulcerative Colitis

The distributions of HLA-A, -B, -DR and -DQ antigens in ULC patients and controls are respectively given in tables 4-6, 4-7, 4-8 and 3-9, while antigens showing significant variations between patients and controls are summarised in table 4-10.

At HLA-A locus, two antigens (A3 and A9) showed increased frequencies in the patients, and other two antigens (A11 and A30) showed decreased frequencies. The antigen A3 was present in 26% of the patients, while its frequency in the control group was 8.9%. Such positive association was significant before correction (P = 0.021) but not after (Pc = 0.231), although RR and PF values of 3.5 and 0.19 were respectively recorded. The antigen A9 was also positively associated with ULC, and

an antigen frequency of 44% was present in the patients, while in the control subjects, the frequency was 17.9%. Such deviation associated with RR value of 3.6 and EF value of 0.31, moreover, the probability of such observation was significant before (P = 0.004) and after (Pc = 0.044) correction. In contrast, the antigens A11 and A30 showed negative associations with ULC, and each of the two antigens scored a frequency of 4% in the patients, while in the controls, the frequencies were 22.4 and 20.9%, respectively. Although, these two associations were significant (P = 0.007 and 0.0121, respectively), the corrected probabilities failed to attain a significant level (Pc > 0.05).

At HLA-B locus, the antigen B41 showed a significant (P = 2.2 x 10^{-14}) increased frequency (72 vs. 5.9%), and such positive association was highly significant after correction (Pc = $3.9x10^{-13}$), with RR value of 40.5 and EF value of 0.7.

At HLA-class II region (DR and DQ loci), several deviations (increased or decreased) in antigen frequencies were observed in the patients when comparisons were made with control subjects. Increased frequencies of antigens DR4 (36 *vs*. 13.4%), DR5 (16 *vs*. 3%), DR8 (28 *vs*. 8.9%), DQ1 (40 *vs*. 18%) and DQ2 (26 *vs*. 10%) were observed in the patients. The RR values of such positive associations were 3.0, 6.19, 3.95, 3.05 and 3.01, respectively, and the EF values were 0.24, 0.13, 0.21, 0.26 and 0.17, respectively. These associations were significant before correction, but after correction, only the association with DQ1 remained significant (Pc = 0.036). A negative association was also observed between DR7 and ULC. The antigen was present in 6% of the patients, while its frequency in controls reached 23%. Such deviation was significant before correction (P = 0.011) but not after (Pc = 0.108).

Table 4-6: Observed and expected numbers, percentage and gene frequencies of HLA-A antigens in ULC patients and controls.

HLA-A antigens	ULC Patients (number = 50)					Controls (number =67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
A1	10	20.0	10	20.0	0.106	9	13.4	8.9	13.3	0.071
A2	22	44.0	22	44.0	0.251	21	31.3	21.0	31.3	0.171
A3	13	26.0	13	26.0	0.140	6	8.9	5.4	8.8	0.046
A9	22	44.0	22	44.0	0.251	12	17.9	12.0	17.9	0.094
A10	5	12.0	6	12.0	0.062	9	13.4	9.0	13.4	0.070
A11	2	4.0	2	4.0	0.020	15	22.4	15.0	22.4	0.119
A28	4	8.0	4.0	8.0	0.041	5	7.5	5.0	7.5	0.038
A29	1	2.0	1	2.0	0.010	2	3.0	1.9	2.9	0.015
A30	2	4.0	2	4.0	0.020	14	20.9	13.9	20.8	0.111
A31	2	4.0	2	4.0	0.020	2	3.0	2.9	2.9	0.015
A33	3	6.0	3	6.0	0.030	11	16.4	11	16.4	0.086
Others	–	–	4.7	9.5	0.049	–	–	20.3	30.3	0.165

Table 4-7: Observed and expected numbers, percentage and gene frequencies of HLA-B antigens in ULC patients and controls.

HLA-B antigens	ULC Patients (number = 50)					Controls (number =67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
B7	ND	ND	ND	ND	ND	6.0	8.9	5.4	8.9	0.046
B8	9.0	18.0	8.9	17.9	0.094	3.0	4.5	3.0	4.5	0.023
B12	5.0	10.0	5.0	10.0	0.051	8.0	11.9	7.9	11.9	0.061
B13	ND	ND	ND	ND	ND	3.0	4.5	3.0	4.5	0.227
B14	2.0	4.0	2.0	4.0	0.020	2.0	3.0	1.9	2.9	0.015
B15	ND	ND	ND	ND	ND	3.0	4.5	3.0	4.5	0.023
B16	1.0	2.0	1.0	2.0	0.010	3.0	4.5	3.0	4.5	0.023
B17	6.0	12.0	6.0	12.0	0.062	ND	ND	ND	ND	ND
B18	1.0	2.0	1.0	2.0	0.010	2.0	3.0	1.9	2.9	0.015
B21	3.0	6.0	3.0	6.0	0.030	ND	ND	ND	ND	ND
B22	5.0	10.0	5.0	10.0	0.051	1.0	1.4	1.0	1.4	0.007
B27	1.0	2.0	1.0	2.0	0.010	3.0	4.5	3.0	4.5	0.023
B35	5.0	10.0	3.0	6.0	0.030	12.0	17.9	12.0	17.9	0.093
B40	1.0	2.0	1.0	2.0	0.010	1.0	1.4	1.0	1.4	0.007
B41	36.0	72.0	36.0	72.0	0.471	4.0	5.9	3.8	5.7	0.030
B42	1.0	2.0	1.0	2.0	0.010	ND	ND	ND	ND	ND
B48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B51	10.0	20.0	10.0	20.0	0.106	25.0	37.3	25.0	37.3	0.208
Others	–	–	6.8	13.7	0.071	–	–	26.6	38.2	0.214

ND: not detected.

Table 4-8: Observed and expected numbers, percentage and gene frequencies of HLA-DR antigens in ULC patients and controls.

HLA-DR antigens	ULC Patients (number = 50)					Controls (number =67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
DR1	10	20	10.0	20.0	0.106	8	11.9	7.9	11.9	0.061
DR2	17	34	17.0	34.0	0.188	18	26.8	18	26.7	0.155
DR3	13	26	13.0	26.0	0.140	14	20.9	13.9	20.8	0.111
DR4	18	36	18.0	36.0	0.200	9	13.4	9.0	13.4	0.069
DR5	8	16	8.0	16.0	0.083	2	3	1.9	2.9	0.015
DR6	4	8	4.0	8.0	0.040	ND	ND	ND	ND	ND
DR7	3	6	3.0	6.0	0.030	16	23	16.1	23.1	0.128
DR8	14	28	14.1	28.0	0.151	6	8.9	5.9	8.9	0.046
DR10	2	4	2.0	4.0	0.020	1	1.4	1	1.4	0.007
Others	–	–	4.11	8.2	0.042	–	–	44.3	66.1	0.419

ND: not detected.

Table 4-9: Observed and expected numbers, percentage and gene frequencies of HLA-DQ antigens in ULS patients and controls.

HLA-DQ antigens	ULC Patients (number = 50)					Controls (number =67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
DQ1	20	40	20.0	40.0	0.225	12	18	12	18	0.140
DQ2	13	26	13.0	26.0	0.140	7	10	7	10	0.053
DQ3	12	24	12.1	12.1	0.128	16	23	16.0	23.1	0.128
Others	–	–	24.2	48.5	0.282	–	–	60.0	89.6	0.679

Table 4-10: Antigens of HLA – class I class II regions showing significant variation between ULC patients and controls.

ULC Antigens	patients		Controls		RR	EF	PF	P	Pc
	No	%	No	%					
A3	13	26.0	6	8.9	3.5	0.19	__	0.021	0.231
A9	22	44.0	12	17.9	3.6	0.31	__	0.004	0.044
A11	2	4.0	15	22.4	0.14	__	0.19	0.007	0.077
A30	2	4.0	14	20.9	0.15	__	0.18	0.012	0.132
B41	36	72.0	4	5.9	40.5	0.70	__	2.2×10^{-14}	3.9×10^{-13}
DR4	18	36	9	13.4	3	0.24	__	0.007	0.063
DR5	8	16	2	3	6.19	0.13	__	0.018	0.162
DR6	4	8	N.D.	N.D.	__	__	__	0.031	0.279
DR7	3	6	16	23	0.2	__	0.19	0.011	0.099
DR8	14	28	6	8.9	1.8	0.12	__	0.012	0.108
DQ1	20	40	12	18	3.05	0.26	__	0.012	0.036
DQ2	13	26	7	10	3.01	0.17	__	0.045	0.135

N.D.: not detected

4-1-3: Crohn's Disease

The distributions of HLA-A, -B, -DR and -DQ antigens in CRD patients and control subjects are shown in tables 4-11, 4-12, 4-13 and 3-14, respectively, while antigens showing significant variations between patients and controls are summarised in table 4-15.

At HLA- A locus, the antigen A9 reached a frequency of 60% in the patients, while in the controls such frequency was 17.9%. Such deviation was significant before (P = 0.002) and after (Pc = 0.022) correction, and associated with RR value of 3.6 and EF value of 0.18.

At HLA-B locus, three antigens (B16, B41 and B51) exhibited variations between patients and controls. Increased frequencies of B16 (33.3 *vs*. 4.5%) and B41 (46.6 *vs*. 5.9%) were observed in the patients.

Such deviations elevated the RR values to 10.6 and 13.7, respectively, and EF values to 0.29 and 0.42, respectively. The probabilities of such positive associations were significant before correction (P = 0.004 and 0.0004, respectively), but after correction, the association between B41 and CRD remained significant (Pc = 0.007). The antigen B51, in contrast, showed a significant (P = 0.029) decreased frequency in the patients (6.6 *vs*. 37.3%), with a PF value of 0.30, but the difference lost the significance after correction (Pc = 0.522).

At HLA-class II region, increased frequencies of DR5 (20 *vs*. 3%), DR8 (40 *vs*. 8.9%), and DQ1 (60 *vs*. 18%) were observed in the patients. Such variations associated with RR values of 8.1, 6.7, and 6.8, respectively, and EF values of 0.17, 0.34, and 0.51, respectively. These positive associations were significant before correction (P = 0.04, 0.007, and 0.03, respectively), but not after (P > 0.05). It is also interesting to note that DR6 was detected in 20% of the patients, but none of the control subjects showed the antigen. Such difference associated with a probability value of 0.005, which was also significant after correction (Pc = 0.045).

Table 4-11: Observed and expected numbers, percentage and gene frequencies of HLA-A antigens in CRD patients and controls.

HLA-A antigens	CRD Patients (number = 15)					Controls (number =67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
A1	3	20.0	3.0	20.0	0.106	9	13.4	8.9	13.3	0.071
A2	5	33.3	4.9	32.9	0.181	21	31.3	21.0	31.3	0.171
A3	1	6.6	1.0	6.6	0.034	6	8.9	5.4	8.8	0.046
A9	9	60.0	9.0	60	0.368	12	17.9	12.0	17.9	0.094
A10	ND	ND	ND	ND	ND	9	13.4	9.0	13.4	0.070
A11	ND	ND	ND	ND	ND	15	22.4	15.0	22.4	0.119
A28	1	6.6	1.0	6.6	0.034	5	7.5	5.0	7.5	0.038
A29	ND	ND	ND	ND	ND	2	3.0	1.9	2.9	0.015
A30	2	13.3	2.0	13.3	0.067	14	20.9	13.9	20.8	0.111
A31	3	20	3.0	20.0	0.106	2	3.0	2.9	2.9	0.015
A33	ND	ND	ND	ND	ND	11	16.4	11	16.4	0.086
Others	–	–	2.9	20.0	0.104	–	–	20.3	30.3	0.164

ND: not detected.

Table 4-12 : Observed and expected numbers , percentage and gene frequencies of HLA-B antigens in CRD patients and controls.

HLA-B antigens	CRD Patients (number = 15)					Controls (number = 67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
B7	ND	ND	ND	ND	ND	6	8.9	5.4	8.9	0.046
B8	2.0	13.3	2.0	13.3	0.069	3	4.5	3.0	4.5	0.023
B12	2.0	13.3	2.0	13.3	0.034	8	11.9	7.9	11.9	0.061
B13	1	6.6	1.1	6.6	0.034	3	4.5	3.0	4.5	0.227
B14	ND	ND	ND	ND	ND	2	3.0	1.9	2.9	0.015
B15	ND	ND	ND	ND	ND	3	4.5	3.0	4.5	0.023
B16	5	33.3	4.9	33.3	0.181	3	4.5	3.0	4.5	0.023
B17	1	6.6	1.0	6.6	0.034	ND	ND	ND	ND	ND
B18	ND	ND	ND	ND	ND	2	3.0	1.9	2.9	0.015
B21	1	6.6	1.0	6.6	0.034	ND	ND	ND	ND	ND
B22	ND	ND	ND	ND	ND	1	1.4	1.0	1.4	0.007
B27	ND	ND	ND	ND	ND	3	4.5	3.0	4.5	0.023
B35	1.0	6.6	1.1	6.6	0.034	12	17.9	12.0	17.9	0.093
B40	1.0	6.6	1.1	6.6	0.034	1	1.4	1.0	1.4	0.007
B41	7.0	46.6	7.0	46	0.265	4	5.9	3.8	5.7	0.030
B42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B48	1.0	6.6	1.1	6.6	0.034	ND	ND	ND	ND	ND
B51	1.0	6.6	1.1	6.6	0.034	25	37.3	25.0	37.3	0.208
Others	–	–	5.0	33.0	0.18	–	–	25.6	38.2	0.214

Table 4-13 : Observed and expected numbers , percentage and gene frequencies of HLA-DR antigens in CRD patients and controls.

HLA-DR antigens	CRD Patients (number = 15)					Controls (number = 67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
DR1	2	13.3	2.0	13.3	0.069	8	11.9	7.9	11.9	0.061
DR2	5	33.3	4.9	33.0	0.181	18	26.8	18	26.7	0.144
DR3	2	13.3	2.0	13.3	0.069	14	20.9	13.9	20.8	0.111
DR4	4	26.6	3.9	26	0.140	9	13.4	9.0	13.4	0.069
DR5	3	20	3.0	20.0	0.106	2	3	1.9	2.9	0.015
DR6	3	20	3.0	20.0	0.106	ND	ND	ND	ND	ND
DR7	2	13.3	2.0	13.3	0.069	16	23	16.1	23.1	0.128
DR8	6	40	6.0	40.0	0.225	6	8.9	5.9	8.9	0.046
DR10	2	13.3	2.0	13.3	0.069	1	1.4	1	1.4	0.007
Others	–	–	5.8	39.4	0.217	–	–	44.3	66.1	0.419

ND: not detected.

Table 4-14: Observed and expected numbers , percentage and Gene frequencies of HLA-DQ antigens in CRD patients and controls.

HLA-DQ antigens	CRD Patients (number = 15)					Controls (number =67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
DQ1	9	60	9	60	0.368	12	18	12	18	0.140
DQ2	3	20	3.0	20.0	0.106	7	10	7	10	0.053
DQ3	4	26.6	3.9	26	0.140	16	23	16.0	23.1	0.128
Others	–	–	9.3	62.2	0.386	–	–	60.0	89.6	0.679

Table 4-15: Antigens of HLA – class I class II regions showing significant variation between CRD patients and controls.

HLA Antigens	patients		Controls		RR	EF	PF	P	Pc
	No	%	No	%					
A9	9	60	12	17.9	0.73	0.18	__	0.002	0.022
B16	5	33.3	3	4.5	10.6	0.29	__	0.004	0.072
B41	7	46.6	4	5.9	13.7	0.42	__	0.0004	0.0072
B51	1	6.6	25	37.3	0.12	__	0.30	0.029	0.522
DR5	3	20	2	3	8.1	0.17	__	0.040	0.36
DR6	3	20	N.D.	N.D.				0.005	0.045
DR8	6	40	6	8.9	6.7	0.34	__	0.007	0.063
DQ1	9	60	12	18	6.8	0.51	__	0.030	0.09

4-1-4: Immunogenetic Heterogeneity of Inflammatory Bowel Disease

The immunogenetic heterogeneity of IBD was assessed by comparing the antigen frequencies, as well as, gene frequencies of HLA-A, -B, -DR and -DQ loci between ULC and CRD patients. Several antigens (A2, A3, A9, A30, B16, B41, B51, DR4, DR6, DR7, DR8 DR10, DQ1 and DQ2) showed different distributions in the two groups of the patients, but none of these differences maintained a significant level (Table 3-16). The antigen B16 was an exception in this regard. Out of 50 ULC patients, only one subject (2%) expressed B16, while 5 subjects (33.3%) out of 15 CRD patients expressed this antigen. Therefore, the gene frequency of B16 was also different (ULC = 0.010; CRD = 0.181). Such difference associated with a probability value of 0.002, which was significant even after correction for the number of antigen tested at HLA-B16 locus (Pc = 0.036) table (4-16).

Table 4-16: Antigens of HLA class I and class II that show significant differences between ULC and CRD patients.

HLA-A antigens	ULC Patients (number = 50)					Controls (number =67)				
	Observed		expected		Gene frequency	Observed		expected		Gene frequency
	No.	%	No.	%		No.	%	No.	%	
A1	10	20.0	10	20.0	0.106	9	13.4	8.9	13.3	0.071
A2	22	44.0	22	44.0	0.251	21	31.3	21.0	31.3	0.171
A3	13	26.0	13	26.0	0.140	6	8.9	5.4	8.8	0.046
A9	22	44.0	22	44.0	0.251	12	17.9	12.0	17.9	0.094
A10	5	12.0	6	12.0	0.062	9	13.4	9.0	13.4	0.070
A11	2	4.0	2	4.0	0.020	15	22.4	15.0	22.4	0.119
A28	4	8.0	4.0	8.0	0.041	5	7.5	5.0	7.5	0.038
A29	1	2.0	1	2.0	0.010	2	3.0	1.9	2.9	0.015
A30	2	4.0	2	4.0	0.020	14	20.9	13.9	20.8	0.111
A31	2	4.0	2	4.0	0.020	2	3.0	2.9	2.9	0.015
A33	3	6.0	3	6.0	0.030	11	16.4	11	16.4	0.086
Others	–	–	4.7	9.5	0.049	–	–	20.3	30.3	0.165

4-2: Blood Groups

The distributions of blood group phenotypes in total IBD patients and controls are presented in table 4-17, while those for ULC and CRD patients are given in tables 4-18 and 4-19, respectively. As shown in table 3-9, the most frequents blood group phenotype in IBD patients was O (47.7%), followed by blood group phenotypes A, B and AB (20.0, 18.5 and 13.8%, respectively). The controls shared a similar picture, although blood group B was more frequent than blood group A (22.9 *vs.* 17.1%). Chi-square analysis of these distributions in Total IBD patients and controls revealed no significant difference (X2 = 0.638, D.F. = 3, P .

0.05). Dividing the patients into two groups (ULC and CRD) ended in a similar theme, and no significant differences were observed between the two groups of patients and controls (Tables 4-18 and 4-19). However, the results of CRD may impact some interest, because summing A, B and AB blood groups in one group gave a frequency of 60%, while such frequency in controls was 51.4%. Such difference is mainly attributed to a decreased frequency of AB blood group phenotype (6.6 *vs.* 11.4%) and increased frequencies of A (26.7 *vs.* 17.1%) and B (26.7 *vs.* 22.9%) blood group phenotypes in CRD patients as compared to controls.

Table 4-17: Observed and expected numbers, and percentage frequencies of blood group phenotypes in total IBD patients and controls.

Blood Groups	IBD Patients (number = 65)				Controls (Number = 70)			
	Observed		Expected		Observed		Expected	
	Number	%	Number	%	Number	%	Number	%
A	13	20.0	12.0	18.5	12	17.1	13.0	18.6
B	12	18.5	13.5	20.8	16	22.9	14.5	20.7
AB	9	13.8	8.2	12.6	8	11.4	8.8	12.6
O	31	47.7	31.3	48.1	34	48.6	33.7	48.1
X^2 = 0.638 D.F. = 3 P > 0.05 (Not significant)								

Table 4-18: Observed and expected numbers, and percentage frequencies of blood group phenotypes in ULC patients and controls.

Blood Groups	ULC Patients (number = 50)				Controls (Number = 70)			
	Observed		Expected		Observed		Expected	
	Number	%	Number	%	Number	%	Number	%
A	9	18.0	8.7	17.4	12	17.1	12.3	17.6
B	8	16.0	10.0	20.0	16	22.9	14.0	20.0
AB	8	16.0	6.7	13.4	8	11.4	9.3	13.3
O	25	50.0	24.6	49.2	34	48.6	34.4	49.1
X^2 = 1.148 D.F. = 3 P > 0.05 (Not significant)								

Table4-19: Observed and expected numbers, and percentage frequencies of blood group phenotypes in CRD patients and controls.

Blood Groups	CRD Patients (number = 15)				Controls (Number = 70)			
	Observed		Expected		Observed		Expected	
	Number	%	Number	%	Number	%	Number	%
A	4	26.7	2.8	18.7	12	17.1	13.2	18.8
B	4	26.7	3.5	23.3	16	22.9	16.5	23.6
AB	1	6.6	1.6	10.7	8	11.4	7.4	10.6
O	6	40.0	7.1	47.3	34	48.6	32.9	47.0
X^2 =1.208 D.F. = 3 P > 0.05 (Not significant)								

4-3: Total and differential count of leucocytes

Total and differential counts (means) of leucocytes in IBD patients (total, ULC and CRD) and control subjects are given in table 4-20.

Total count: Similar means of total leucocyte count were observed in the investigated groups, although the total IBD patients and ULC patients showed higher means (6850.8 and 6928 cells/cu.mm.blood, respectively) than controls and CRD patients (6569.7 and 6593.3 cells/cu.mm.blood, respectively). Therefore, the statistical analysis revealed no significant differences ($P > 0.05$).

Neutrophils: The total IBD and ULC patients showed increased means of neutrophils (4557.6 and 4606.1 cells/cu.mm.blood, respectively) as compared to controls (4074.2 cells/cu.mm.blood). Both deviations were significant (P_1=0.01). However, comparing ULC and CRD patients revealed no significant difference ($P_2 > 0.05$) between the means of neutrphils.

Lymphocytes: Lymphocytes count showed similar means in IBD patients (total =2027.3, ULC=2017.2, CRD= 2060.2 cells/cu.mm.blood) and controls (2026.1 cells/cu.mm.blood), and no significant difference was observed.

Monocytes: The monocyte count showed a decreased mean in IBD patients (total=169, ULC=183.1, CRD=121.8 cells/cu.mm.blood) as compared to controls (353.2 cells/ cu.mm.blood). Such deviations were highly significant and associated with P_1 values of 0.002, 0.005 and 0.0001, respectively. Although, the ULC mean of monocyte was higher than CRD mean, the difference failed to reach a significant level ($P_2 > 0.05$).

Eosinophils: As in the count of monocytes, the eosinophils count also showed a decreased mean in IBD patients (total=47.2, ULC=48, CRD=44.4 cells/cu.mm.blood) as compared to controls (186

cells/cu.mm.blood). Such deviations were highly significant and associated with P_1 value of 0.0002, 0.0001 and 0.0001, respectively. Although, the ULC mean of eosinophils was higher than CRD mean, the difference failed to reach a significant level ($P_2 > 0.05$).

Basophiles: Basophiles count showed similar means in IBD patients (total=18.8, ULC=22.2, CRD=7.6 cells/cu.mm.blood) and controls (17.1 cells/cu.mm.blood), and no significant difference was observed

Table 4-20: Total and differential counts (means) of leucocytes in IBD patients (total, ULC and CRD) and controls.

Cells	Groups	Number	Cells/cu.mm.blood				P_1	P_2
			Mean	S.E.	Min.	Max.		
Total	Controls	70	6569.7	172.5	4000	9600		
	Total IBD	65	6850.8	164.3	4200	9600	N.S	
	ULC	50	6928.0	186.3	4800	9600	N.S	
	CRD	15	6593.3	351.9	4200	8600	N.S	N.S
Neutropijls	Controls	70	4074.2	124	1640	7216		
	Total IBD	65	4557.6	128	2688	7104	0.01	
	ULC	50	4606.1	147	2970	7104	0.01	
	CRD	15	4394.5	268	2688	6278	N.S	N.S
Lymphocytes	Controls	70	2062.1	777	924	6279		
	Total IBD	65	2027.3	56.64	244	2994	N.S	
	ULC	50	2017.2	66.23	244	2816	N.S	
	CRD	15	2060.2	110.79	1372	2994	N.S	N.S
Monocytes	Controls	70	353.2	191.3	54	968		
	Total IBD	65	169.0	40.62	0	2720	0.002	
	ULC	50	183.1	52.57	0	2720	0.005	
	CRD	15	121.8	15.20	52	258	0.0001	N.S
Eosinophils	Controls	70	186.0	146.9	0	704		
	Total IBD	65	47.2	4.64	0	136	0.0002	
	ULC	50	48.0	5.44	0	136	0.0001	
	CRD	15	44.4	9.00	0	86	0.0001	N.S
Basophils	Controls	70	17.1	36.6	0	163		
	Total IBD	65	18.8	3.87	0	92	0.402	
	ULC	50	22.2	4.69	0	92	0.317	
	CRD	15	7.6	5.29	0	66	0.756	N.S

P_1: Patients *vs*. Controls **P_2**: ULC *vs*. CRD N.S.: Not Significant

4-4: Subpopulations of Lymphocytes

The percentage of CD3+, CD4+, CD8+ and CD19+, lymphocytes, as well as, the mean of CD4/CD8 ratio are given in table 4-21.

CD3+Lymphocytes: Increased percentage means of CD3+ lymphocytes were observed in total IBD patients (82.65%) and ULC patients (82.47%) as compared to controls (79.4 %). Both deviations were significant (P_1=0.03 and 0.05, respectively). The CRD patients also showed an increased mean of CD3+ cells (83.2 *vs*. 79.4%), but the standard error (1.36) limited the difference to reach a significant level ($P_2 > 0.05$).

CD4+Lymphocytes: Increased percentage means of CD4+ lymphocytes were observed in total IBD patients (42.40%) and ULC patients (42.67%) as compared to controls (39.55%). Both deviations were significant (P_1=0.01 and 0.01, respectively). The CRD patients also showed an increased mean of CD4+ cells (41.6%), but the difference failed to reach a signification level ($P_2 > 0.05$).

CD8+Lymphocytes: The CD8+ cells showed increased percentage means in IBD patients (total= 28.7%, ULC=28.27%, CDR=30%) as compared to the control group (22%). These differences were highly significant ($P_1=3.6\times10^{-8}$, 4.7×10^{-7} and 6.8×10^{-6}, respectively).

CD19+Lymphocytes: Total IBD patients and ULC patients showed increased means of CD19+ lymphocytes (21.9 and 22.13%, respectively) as compared to the controls group (18.7%). Both differences were significant and associated with P_1 value of 0.002 and 0.0007, respectively. The CRD patients also showed an increased percentage

mean of CD19+ cells (21.2 ***vs.*** 18.7%), but the different failed to reach a significant level ($P_2 > 0.05$).

CD4 / CD8 ratio: the CD4/CD8 cells ratio showed increased percentage means in IBD patients (total = 1.499%, ULC=1.524%, CRD= 1.425%) as compared to control group (1.818 %). these differences were highly significant ($P_1=1\times10^{-5}$, 0.0004, 0.001, respectively).

Table 4-21: Percentage means of lymphocyte subpopulations in IBD patients (total, ULC and CRD) and controls.

Cells	Groups	Number	Percentages of Cells				P_1	P_2
			Mean	S.E.	Min.	Max.		
CD3+	Controls	20	79.40	1.10	70	87		
	Total IBD	20	82.65	0.97	75	90	0.03	
	ULC	15	82.47	1.23	75	90	0.05	
	CRD	5	83.20	1.36	78	86	N.S	N.S
CD4+	Controls	20	39.55	0.59	36	45		
	Total IBD	20	42.40	0.87	36	50	0.01	
	ULC	15	42.67	1.14	36	50	0.01	
	CRD	5	41.60	0.68	40	43	N.S	N.S
CD8+	Controls	20	22.00	0.60	18	27		
	Total IBD	20	28.70	0.76	22	34	3.6×0^{-8}	
	ULC	15	28.27	0.75	23	34	4.7×0^{-7}	
	CRD	5	30.00	2.10	22	34	6.8×0^{-6}	N.S
CD19+	Controls	20	18.70	0.69	14	24		
	Total IBD	20	21.90	0.76	17	26	0.002	
	ULC	15	22.13	0.75	17	26	0.0007	
	CRD	5	21.20	2.10	19	26	N.S	N.S
CD4/CD8 Ratio	Controls	20	1.818	0.054	1.41	2.11		
	Total IBD	20	1.499	0.055	1.18	2.04	1×10^{-5}	
	ULC	15	1.524	0.057	1.27	2.04	0.0004	
	CRD	5	1.425	0.013	1.18	1.95	0.001	N.S

P_1: Patients *vs*. Controls **P_2**: ULC vs. CRD N.S.: Not Significant

4-5: Phagocytosis

The percentage means of phagocytic index and NBT index in IBD patients (Total, ULC and CRD) and control subjects are presented in table 4-22.

Phagocytic Index: The phagocytic index showed increased percentage means in IBD patients (total = 19.1, ULC=20.2, CRD= 16.9) as compared to controls (12.3). Such deviations were highly significant and associated with P_1 values of 2.1×10^{-9}, 3.6×10^{-10} and 0.0009, respectively. The phagocytic index in ULC patients was higher than its counter part in CRD, and the difference was significant (P_2= 0.02).

NBT Index: The total IBD patients and ULC patients showed increased percentage means of NBT Index (11.37 and 12.70 %) respectively. Both deviation were highly significant ($P_1=3.4\times 10^{-5}$ and 4.1×10^{-7} , respectively) . The NBT index in CRD shared a similar mean with the control groups (8.7 and 7.8%, respectively) and no significant ($P > 0.05$) difference was observed between the two groups. However, comparing ULC and CRD show a decreased level of NBT index was observed in CRD patients, and the difference was significant.

Table 4-22: Percentage means of phagocytic index and NBT index in IBD patients (total, ULC and CRD) and controls.

Index	Groups	Number	Percentages of Cells				P_1	P_2
			Mean	S.E.	Min.	Max.		
Phagocytic Index	Controls	20	12.30	0.60	8	19		
	Total IBD	30	19.10	0.70	13	24	2.1×10^{-9}	
	ULC	20	20.20	0.75	13	24	3.6×10^{10}	
	CRD	10	16.90	1.23	13	23	0.0009	0.02
NBT Index	Controls	20	7.85	0.32	5	11		
	Total IBD	30	11.37	0.61	7	16	3.4×10^{-5}	
	ULC	20	12.70	0.68	7	16	4.1×10^{-7}	
	CRD	10	8.70	0.70	7	14	N.S.	0.0004

P_1: Patients *vs*. Controls **P_2**: ULC vs. CRD N.S.: Not Significant

Conclusions

The present study reached the following conclusions:

1. The HLA-class I (A and B) and -class II antigens play an important role in the aetiology of IBD with a special reference to the antigens A9, A11, B41 and DR8. The same picture is also applied to the clinical forms (ULC and CRD) of IBD, however, the two clinical forms are differentiated by the antigen B16.
2. Blood group phenotypes showed similar distributions in patients (total and subgroups), as well as, controls.
3. Although, the total count of leucocytes showed no significant differences between patients and controls, the differential counts showed some differences, especially for neutrophils, monocytes and eosinophils.
4. The lymphocyte count of patients approximated the corresponding count in controls, but defining these cells by means of CD markers gave (CD3, CD4, CD8 and CD19) better outcomes.
5. The phagocytic activity of peripheral blood phagocytes deviated positively in the patients.

Recommendations

Based on the findings of the present study, and after reviewing the literature, the following recommendations can be suggested:

1. The role of HLA polymorphism in the aetiology of IBD in Iraqi patients requires further investigations, especially at the molecular levels by means of DNA technologies.
2. The forthcoming approach can be fruitful if the patients are investigated in terms of twin (sib-pair analysis) and family (linkage analysis) studies.
3. The blood group phenotypes require further investigations, but with emphases on the secretor status of their antigens to determine their role in protecting the intestinal mucosa from inflammatory responses in relation to some pathological agents.
4. Some HLA phenotypes (positive and negative associations) of patients require further investigations in terms of cytokine profiles, and correlate these findings with the subpopulations of lymphocytes that are defined by CD markers.
5. Expand researches regarding the neutrophil functions, especially their enzymatic constituents.
6. Sibce our present study strongly support the finding that the two clinical forms of IBD can be differentiated by the antigen B16 as a differentiating marker.

Appendix I: general laboratory Equipments

Equipment or Instrument	Company	Country
Centrifuge	Rem	India
Cooled centrifuge	Sorvall	U.S.A.
Horizontal shaker	Shaker	Italy
Water distillater	Manesty	U.K.
Incubator	Memmert	Germany
Water bath	Memmert	Germany
Autoclave	Hiryama	Autoclave
Sensitive balance	Mettler	U.K.
Light microscope	Olympus	Japan
Phase contrast inverted microscope	Wild	Switzerland
Fluorescent microscope	Olympus	Japan
Oven	Gallen kamp	U.K.
Micropipette	Biokit	Finland
Graduated glass cylinder	Supc ORIior	Germany
Pasteur pipette	Biomerieu	France
Graduated pipette	Sterilin	U.K.
Terasaki plates	Terasaki	Germany
Glass test tube(10ml)	Sterilin	U.K.
Heparin tubes(10ml)	Sterilin	U.K.
Eppendroff tubes	Afam-Dispo	Spain
Disposable syringes(10ml)	Unolok	U.K.
Slides	Inter leaved	China
Cover slips	Inter leaved	China
Heamocytometer	Thoma	Germany
Pipette tips(100ul and 100ul)	Sterilin	U.K.
Parafilm	Americans-company	U.S.A.
Filter paper	Sweicher and schuell	Germany
Hamilton syringes	Hamilton bondaduz	U.K.

Appendix II:Data sheet

<table>
<tr><td colspan="2">Name</td><td colspan="2"></td><td>Sex</td><td></td><td>D.O.B.</td><td>Number</td><td colspan="2"></td></tr>
<tr><td colspan="10">Clinical diagnosis Of Inflammatory Bowel Disease</td></tr>
<tr><td colspan="3">Ulcerative Colitis</td><td colspan="3"></td><td colspan="2">Crohn s Disease</td><td colspan="2"></td></tr>
<tr><td colspan="10">Demographic Data</td></tr>
<tr><td rowspan="2">Age of Onset</td><td rowspan="2"></td><td colspan="7">Family history</td><td colspan="2" rowspan="2">Duration of disease</td></tr>
<tr><td>Father</td><td></td><td>Mother</td><td></td><td>Broth</td><td></td><td>Sister</td><td></td><td>Other</td><td></td></tr>
<tr><td rowspan="9">Laboratory Examination</td><td rowspan="3">Leucocyte</td><td>Total Count</td><td>N</td><td>L</td><td>M</td><td>E</td><td>B</td></tr>
<tr><td>%</td><td>%</td><td>%</td><td>%</td><td>%</td><td>%</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>HLA-Class I Antigen</td><td colspan="3">A</td><td colspan="3">B</td></tr>
<tr><td></td><td colspan="3"></td><td colspan="3"></td></tr>
<tr><td rowspan="2">HLA-Class II Antigen</td><td>DR</td><td colspan="2">DQ</td><td rowspan="2">Blood Group</td><td colspan="2" rowspan="2"></td></tr>
<tr><td></td><td colspan="2"></td></tr>
<tr><td rowspan="2">Phagocytosis</td><td colspan="3">Phagocytic Index</td><td colspan="3">NBT Index</td></tr>
<tr><td colspan="3"></td><td colspan="3"></td></tr>
<tr><td rowspan="3"></td><td>CD 3</td><td>CD4</td><td>CD8</td><td>CD19</td><td colspan="2">CD4/CD8 ratio</td></tr>
<tr><td>%</td><td>%</td><td>%</td><td>%</td><td colspan="2">%</td></tr>
<tr><td></td><td></td><td></td><td></td><td colspan="2"></td></tr>
<tr><td colspan="2">Address</td><td colspan="8"></td></tr>
</table>

Chapter Four

Results

الخلاصة :

صممت الدراسة الحالية لبحث دور مستضدات التطابق النسيجي – الصنف الاول (B , A) والصنف الثاني (DQ , DR) والطرز المظهرية لمجاميع الدم في الاصابة بمرض المعي الالتهابي (IBD) في عينات لمرضى عراقيين.
كما درست أيضاً معايير مناعية أخرى شملت العدد الكلي والتفريقي لخلايا الدم البيض والمجاميع الثانوية للخلايا اللمفية (خلايا CD3+ , CD8+ , CD4+ , CD19+) والبلعمة.

شملت الدراسة (٦٥) مريضاً مصابين بمرض المعي الالتهابي و فُحصوا خلال الفترة أيار – كانون الاول ٢٠٠٤ . شُخص المرض بواسطة الكادر الطبي في مستشفى الكاظمية التعليمي ومركز أمراض الجهاز الهضمي في بغداد. أعتمد التشخيص على الفحص الطبي بأستعمال ناظور القولون والفحص النسجي. أعتماداً على وجهة نظر الكادر الطبي قسم المرضى سريرياً الى مرض التهاب القولون التقرحي (ULC ; ٥٠ مريض) ومرض كرون (CRD ; ١٥ مريض) بالاضافة الى ذلك فقد شملت الدراسة عينة سيطرة مكونة من ٦٧ فرد (بدو أصحاء) متطابقون من حيث العمر ، الجنس والعامل العرقي (مسلمين عرب).

بالنسبة لمستضدات التطابق النسيجي- الصنف الاول ، أظهر العدد الكلي للمرض زيادة معنوية في تكرارA9 (٥٢,٣ ضد ١٧,٩%) و B41 (٦٦,١ ضد ٥,٩%) ونقصان معنوي في تكرار All (٣,١ ضد ٢٢,٤%) عند المقارنة مع عينة السيطرة ، ولوحظت نتائج مشابهة عند أعتماد الانواع السريرية للمرض (CRD , ULC). بينما أظهرت مستضدات التطابق النسيجي الصنف الثاني زيادة معنوية في المستضد DR8 في العدد الكلي للمرضى (٣,٨ ضد ٨,٩%) لم يظهر النوعين السريريين للمرضى مثل هذا الارتباط . كما تصاحب مرض التهاب القولون أرتبط معنويا مع DQ1 ، وعند

مقارنة مرضى التهاب القولون الترحي مع مرض كرون ظهر أختلاف معنوي في المستضد B16 (٢٠ ضد ٣٣,٣%).

أظهرت الطرز المظهرية لمجاميع الدم (O,AB,B,A) تكرارات متشابهة في المرضى (العدد الكلي والأنواع الثانوية) والسيطرة ، لم يلاحظ فرق معنوي ملحوظ كما وجد وفق تحليل مربع كاي . ولكن عند جمع مجاميع الدم AB , B , A أرتفع ترددها إلى ٦٠% في مرضى الكرون بينما كان تكرارها في عينة السيطرة ٥١,٤%.

أظهر العدد الكلي لخلايا الدم البيض عدم وجود أختلاف معنوي بين المرضى (العدد الكلي و الانواع الثانوية) وعينة السيطرة بينما أظهر العدد التفريقي بعض الاختلافات ، حيث حصلت زيادة معنوية في عدد الخلايا العدلة في العدد الكلي للمرضى ومرض التهاب القولون التقرحي بينما أظهرت الخلايا وحيدة النواة و الخلايا الحمضة نقصان معنوي في المرضى (العدد الكلي والانواع الثانوية).

أظهرت الخلايا اللمفية الموجبة للواسمات CD19, CD4 , CD3 زيادة معنوية في نسبتها في العدد الكلي للمرضى بالاضافة الى مرضى التهاب القولون التقرحي بينما كانت خلايا الـ +CD8 مرتفعة معنوياً في العدد الكلي للمرضى و المجاميع الثانوية .الا أن نسبة خلايا CD8/CD4 كانت منخفضة معنوياً في المرضى (العدد الكلي والانواع الثانوية) .

أظهر معامل البلعمة في المرضى (العدد الكلي والانواع الثانوية) زيادة معنوية مقارنة بعينة السيطرة ، بينما كان معامل NBT مرتفع معنويا (العدد الكلي للمرضى ومرضى التهاب القولون التقرحي).

دراسة وراثية مناعية لمرض المعي الالتهابي في مرضى عراقيين

رسالة مقدمة إلى قسم التقانة الاحيائية

كلية العلوم – جامعة النهرين

كجزء من متطلبات نيل درجةالماجستير في التقانة الاحيائية.

من قبل همسة أحمد جاسم

ربيع الثاني

١٤٢٦هـ

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