Summary

This study was designed to investigate the role of HLA-class I and class II antigens in the etiology of type I diabetes mellitus (TIDM), the cellular changes of peripheral blood lymphocytes(PBL) including phenotyping of surface antigens(CD markers), the functional activities of PBL determined by measurement of proliferative percentage in response to Con-A and in response to other viral antigens which proposed to be involved in the etiology of TIDM like, coxsackie virus type B(CVB5), poliovirus and adenovirus and assessment of other humoral mediators like cytokines, total immunoglobulins(Igs), Complement components, glutamic acid decarboxylase autoantibodies (GADA) and specific anti-viral IgG antibodies for (CVB_5, poliovirus and adenovirus) in order to define if there was previous exposure of TIDM patients to these viral infections.

Sixty TIDM patients who were newly onset of the disease (diagnosed less than five months) were selected from the National Diabetes Center at Al-Mustansiriya University. All the patients were treated with daily replacement doses of insulin.Their age ranged from 3-17 years. They were divided into two groups according to their ages as following: 36 patients equal or less than 10 years old and 24 patients more than 10 years old. Eighty apparently healthy control subjects, matched with age (4-17) years, sex and ethnic backgrounds (Iraqi Arabs) underwent the HLA-typing examination. Finally 50 healthy individuals were selected randomly to undergo the same examination and tests of the study as TIDM patients. They were also divided into two groups according to their ages, 21 individuals equal or less than 10 years and 29 individuals more than 10 years. Another 50 healthy siblings of TIDM patients were available for investigation of HLA-typing, their ages range from 3 to 16 years.

The findings of this study are summarized in the following:

- A remarkable increase of glycosylated hemoglobin $HbA1_C$% with a remarkable decrease of serum C-peptide levels in newly diagnosed TIDM patients in comparison to healthy controls. The age of children had no effect on the metabolic decomposition.
- At HLA-class I region, TIDM patients showed a significant increased frequency of antigen A9 (40.0 vs. 18.75%) and B8 (28.33 vs. 8.75%) as compared to control subjects, while at HLA-class II region, DR3 and DR4 were significantly increased in patients (53.33 vs. 26.25% and 50.0 vs. 12.5% respectively) as compared to controls and they might played an important role in the etiology of the disease. In addition to that, TIDM was significantly associated with DQ2 (33.33 vs. 15%) and DQ3 (40.0 vs. 20%) antigens as compared to controls, suggesting that these haplotypes had a role in disease susceptibility, while the frequency of DR2 and DQ1 antigens were significantly lowered in patients compared to controls (6.66 vs. 25% and 6.66 vs. 22.5% respectively). These molecules might had protective effect. In siblings a significant increase frequency of DR4 antigen (34.0 vs. 12.5%) was observed in comparison to controls, suggesting that it might be much useful for predicting TIDM in affected families.
- TIDM patients showed a remarkable lowering in CD_3^+, CD_4^+ , CD_8^+, $CD_{45}RA^+$, and CD_{56}^+ cells but the decrease in CD_4^+ cells percentage was not significant in patients in comparison to healthy controls. In contrast, a significant elevation of activation markers includes ($CD_{45}RO^+$, CD_{38}^+ and $HLA\text{-}DR^+$ cells were observed in TIDM patients in addition to a significant increase of CD_{19}^+ cell percentage and CD_4^+:CD_8^+ ratio in the patients.
- No significant differences were shown in the PBL proliferative percentage in response to Con-A and tested viruses (CVB_5 and

adenovirus) between TIDM and healthy controls, but PBL proliferative percentage of TIDM patients showed a significant decline in response to poliovaccine. Strong T-cell proliferation in response to the tested viral antigens were observed to be related to HLA-DR4 and HLA-DQ3 antigens.

- By using ELISA technique, serum levels of a Th_1 cell cytokine, interferon gamma (IFN-γ), and Th_2 cell cytokines, interlukin-10(IL-10) and inflammatory cytokine (IL-6) were significantly higher in TIDM patients compared to healthy controls.
- Significant increase in total serum IgM, IgG and IgA levels were observed in patients with TIDM compared to healthy controls while the complement component C_3 and C_4 mean serum levels showed a significant decrease in TIDM patients.
- Anti-GAD autoantibodies were present in 50% of TIDM children especially in older ages and in females more than males. High proportion of GADA was found in the patients carrying HLA-DR3/DR4 heterozygous.
- High proportion of anti-CVB_5 IgG and anti-polio IgG were found in diabetic children compared to controls, while anti-adeno IgG were detected in diabetic patients only.

Introduction

1.1 Introduction

For at least 20 years, diabetes rates in the world have been increasing substantially. According to WHO, in 2000 there were about 668000 diabetics in Iraq alone. It is estimated that by the year 2030 this number will become 2.09 million, while over 18 million Americans have diabetes (WHO, 2005).

Type 1 diabetes mellitus (TIDM) is a chronic disease where insulin-producing beta cells in pancreatic langerhans islet are gradually destroyed. The process which finally leads to complete beta cell loss and onset of clinical disease starts years before any clinical symptoms. It is considered to result from a multifactorial process involving host genes, autoimmune responses and cytokines as well as environmental factors. As an indication of an ongoing autoimmune process at a preclinical stage, beta cell autoantibodies to various islet antigens, insulin glutamic acid decarboxylase 65 (GAD_{65}), tyrosin phosphatase (IA2/IAR) and heat shock protein 60/65, appear in the circulation precede the onset of the disease both in human and in nonobese diabetic mice and they have predictive value in the clinical disease (Brik *et al*., 1996; Winter *et al*., 2002).

The genetic factors that influence disease risk have been subjected to more intensive study. The first diabetes susceptibility genes to be identified were the human leucocyte antigen (HLA) genes located on chromosome 6 referred as IDDM1 locus. This locus is the major determinant of disease risk accounting for 42% of the familial inheritance of TIDM (Kelly *et al*., 2003). The relatively low concordance rate among identical twins (25-53%) suggests that the susceptibility genes have low pentrance that is not all individuals who are genetically "at risk" of TIDM will develop the disease (Kumar *et al*., 1993). Discordance between identical twins may indicate an important non-genetic

(environmental) input to disease susceptibility (Lowe, 1998). Migration studies represent another line of evidence that suggests a role for environmental factors in the development of TIDM. For example, it has been shown that children living in South Asia have a low incidence of TIDM, but migrants from there to the UK have similar rates to the indigenous population (Feltbower *et al*., 2002).

Further support for the environmental factors influences on the disease drives from the marked geographical variations in TIDM incidence. The highest incidence of TIDM is seen in countries in the northern hemisphere, with the lowest incidence found in Asia, followed by Oceania (Australia and New Zeland), South and North America and the highest rate in Europe (Karvonen *et al*., 2000). Taken together these epidemiological and genetic studies are consistent with a model in which a common environmental factor, or set of related factors, operate on a genetically susceptible pool of individuals to give rise to the disease.

Viruses have been traditionally considered prime candidates to trigger and / or accelerate TIDM. Rubella virus, mumps, cytomegalovirus, rota virus and enteroviruses (EVs) (Dahlquist, 1997; Honeyman *et al*., 2000; Varela-Calvino and Peakman, 2003), have all been suggested as environmental factors contributing to TIDM. Several case report studies have shown that EV infections detected as an increase in the antivirus antibody level (Both IgM and IgG) precede the appearance of signs of autoimmunity reflected either by the synthesis of several autoantibodies or the development of clinical disease (Lönnrot *et al*., 1998). There were also indications that maternal EV infections during pregnancy will increase the risk of TIDM in the offspring (Viskari *et al*., 2002). Other studies have shown a higher frequency of EV RNA in the sera of patients with diabetes compared to healthy subjects demonstrating either a recent or a persistent infection in the subject developing TIDM (Craig *et al*., 2003).

1.2 Aims and objectives

In Iraq the diabetes prevalence is increasing compared with the rest of the world. Hence in order to gain more understanding about the role of genes, viral infection and immune system response with initiation of TIDM, this study was conducted to evaluate immunogenetics, viral infection, cellular and humoral immune responses of diabetic patients through:

1. HLA class I and class II polymorphism.
2. Peripheral blood lymphocytes surface antigens (CD markers) (CD_3^+, CD_4^+, CD_8^+, $CD_{45}RA^+$, $CD_{45}RO^+$, HLA-DR^+,CD38+, CD_{56}^+, CD_{19}^+ cells subsets).
3. *In vitro* T-cell proliferation in response to mitogen (Con-A) and in response to different viral antigens using purified CVB_5, poliovaccine (oral sabin) and adenovirus.
4. The possible role of IFN-γ, IL-10 and IL-6 in diabetic patients.
5. Total immunoglobulins and complement components C_3 and C_4.
6. GAD-65 autoantibody.
7. Circulating anti-viral IgG specific to CVB_5, poliovirus and adenovirus.

Literature Review

2.1 Islet Cell Structure

The islets of Langerhans are collections of cells scattered throughout the pancreas; make up about 2% of the volume of the gland which anatomically and functionally separate from pancreatic exocrine tissue that primarily secrets pancreatic enzymes into ducts and drains into the duodenum (Seeley *et al.*, 1992). Normal subjects have about 1-2 million islets with a total weight 1-2 g and constitute less than 2% of the mass of pancreas (Kukreja and Maclarien, 2002).The cells in the islet can be divided into four distinct types: A cells make up 20% of the total, secrete glucagon; β cells which are the most common and account for 60-75% of the islets that secrete insulin; D cells secrete somatostatin and F cells secrete pancreatic polypeptides (Ganong, 1997).

Insulin is a polypeptide hormone consisting of two chains A and B linked by disulfide bridges; the A and B chains have 21 and 30 amino acids respectively (Granner, 1996). Insulin is synthesized as preproinsulin in the ribosomes of rough endoplasmic reticulum, which is cleaved later to proinsulin. The peptide segment connecting the A and B chains, the connecting peptide (C-peptide) consist of 30-35 amino acids (Hoekstra *et al.*, 1982). The proinsulin undergoes a series of site-specific cleavage by two proteases in the granular sacs of Golgi apparatus before secretion (Granner, 1996). Normally 90-97% of the product is insulin with equimolar amount of C-peptide (Kukreja and Maclarien, 2002).

Insulin secretion from β-cells is principally regulated by plasma glucose levels. Increased uptake of glucose by pancreatic β-cells leads to increase in metabolism. This increase is leading to an elevation in the ATP/ADP ratio, which in turn leads to an inhibition of ATP-sensitive K^+ channels. Net result is a

depolarization of the cell leading to Ca^+ influx and insulin secretion (King, 2005).

2.2 Diabetes Mellitus (DM)

Diabetes Mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both (Sherwin, 2000). Deficient insulin action results from inadequate insulin secretion and / or diminished tissue responses to insulin at one or more point in the complex pathway of hormone action (The expert Committee, 2002).

Symptoms of marked hyperglycemia include polyuria, polydispia, weight loss, sometimes with polyphagia. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia (Granner, 1996). Lack of insulin affects the metabolism of carbohydrates, proteins and fat causing a significant disturbance of water and electrolytes (Frier *et al*., 1999). Chronic hyperglycemia causes damage to the eye; kidney; nerves; heart and blood vessels while death may occur during acute metabolic decompensation (Chapel *et al*., 1999). Its etiology and pathogenesis are heterogeneous resulting in different strategies for prevention, diagnostic screening and treatment (The Expert Committee, 2002).

2.2.1 Classification of DM

Two major types of DM are recognized; type I (insulin dependent diabetes mellitus IDDM) and type II (non insulin dependent diabetes mellitus NIDDM). Other known types of diabetes comprise malnutrition diabetes, which is common in the developing countries, and secondary diabetes brought about by pancreatic disease, drug toxicity, endocrine disorders, and genetic diseases (Frier *et al*., 1999).

The world health Organization (WHO) expert committee on diabetes in 1980 proposed a system of classification based on that of National Diabetes Data Group (NDDG) including epidemiologic, clinical, immunologic, genetic

and other factors along with research finding for the last 18 years and was proposing changes to the NDDG / WHO classification scheme as listed in table 2-1 (The Expert Committee, 2003).

The practical approach is to distinguish between type 1 which is an immune-mediated disease and type II which is not immune-mediated (Chapel *et al*., 1999). Some individuals develop a milder form of type I diabetes, characterized by presence of autoantibodies but with clinical classification of type II. This type is classified as the uncommon form, sometimes called type 1.5 diabetes (Boic, 2004). Ketoacidosis is a major feature of untreated type I diabetes and circulating collapse can result from sever acidosis (pH 7.0 or less) (Sherwin, 2000).

Type II diabetes referred to adult-onset diabetes is a term used for individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency (Frier *et al*., 1999). It is principally a disease of the middle aged and elderly and is often associated with strong genetic predisposition (The Expert Committee, 2002).

Other specific types of diabetes such as MODY (Maturity Onset Diabetes in the Young), characterized by onset of hyperglycemia at an early age (generally up to 25 years) and constitutes less than 5% of all cases of type II diabetes (Frier *et al*., 1999). They are due to genetic defect in β-cell function. The most common form (MODY3) is associated with mutation on chromosome 12 in a hepatic transcription factor (HNF-1 α) (Byrne *et al*., 1996). The MODY2 is associated with mutation in glucokinase gene on chromosome 7p (Lowe, 1998), while the third one (MODY1) is associated with mutation in the HNF-4 α on chromosome 20 q (The Expert Committee, 2003).

Gestational diabetes applies to women who develop diabetes for the first time during pregnancy; particularly in the 3rd trimester (Frier *et al*., 1999).

Table 2-1: Etiologic classification of diabetes mellitus.

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)
   - A- Immune mediated
   - B- Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance).

III. Other specific types
   - A. Genetic defects of β-cell function
     1. Chromosome 12, HNF-1α (MODY3)
     2. Chromosome 7, glucokinase (MODY2)
     3. Chromosome 20, HNF-4α (MODY1)
     4. Mitochondrial DNA
     5. Others
   - B. Genetic defects in insulin action
     1. Type A insulin resistance
     2. Leprechaunism
     3. Rabson-Mendenhall syndrome
     4. Lipoatrophic diabetes
     5. Others
   - C. Disease of the exocrine pancreas
     1. Pancreatitis
     2. Trauma/pancreatectomy
     3. Neoplasia
     4. Cystic fibrosis
     5. Hemochromatosis
     6. Fibrocalculous pancreatopathy
     7. Others
   - D. Endocrinopathies
     1. Acromegaly
     2. Cushing’s syndrome
     3. Glucagonoma
     4. Pheochromocytoma
     5. Hyperthyroidism
     6. Somatostatinoma
     7. Aldosteronoma
     8. Others
   - E. Drug- or chemical-induced
     1. Vacor
     2. Pentamidine
     3. Nicotinic acid
     4. Glucocorticoids
     5. Thyroid hormone
     6. Diazoxide
     7. β-adrenergic agonists
     8. Thiazides
     9. Dilantin
     10. α-Interferon
     11. Others
   - F. Infections
     1. Congenital rubella
     2. Cytomegalovirus
     3. Others
   - G. Uncommon forms of immune-mediated diabetes
     1. “Stiff-man” syndrome
     2. Anti-insulin receptor antibodies
     3. Others
   - H. Other genetic syndromes sometimes associated with diabetes
     1. Down’s syndrome
     2. Klinefelter’s syndrome
     3. Turner’s syndrome
     4. Wolfram’s syndrome
     5. Friedreich’s ataxia
     6. Huntington’s chorea
     7. Laurence-Moon-Biedl syndrome
     8. Porphyria
     9. Prader-Willi syndrome
     10. Others

IV. Gestational diabetes mellitus (GDM)

2.3 Type I Diabetes Mellitus (TIDM)

Although there are clinical differences between type I and type II diabetes mellitus which was reported first more than a century ago, it was not until the mid-1960's that convincing evidence about involvement of immune system in the pathogenesis of TIDM was reported (Gepts, 1965).

2.3.1 Epidemiology of TIDM

i. Disease Incidence

No country has escape TIDM although the disease incidence varies considerably from one country to another (Karvonen *et al*., 2000). The highest incidence in the world in children <15 years of age ≥20/100,000 per year have been reported from Finland; Sardinia, Sweden, Norway, Portugal, UK and Canada, while the lowest <1/100,000 per year from China, Africa and South America (Karvonen *et al*., 2000; Gale, 2001; 2002). An increase in the incidence of TIDM has been observed in a number of countries, the highest of all in Europe. Sardinia emerging as the Mediterranean "hot spot" with an incidence rate of 36/100,000 for the period from 1989-1993 (Songini *et al*., 1993). Finland also had an incidence rate of 35/100,000 from the period of 1989-2000 (Gale, 2001), but another data point to a figure as high as 1% in Finland and Sweden (Notkins and Lernmark, 2001). Its incidence seems to be increasing in countries around the world and is predicted to be about 40% higher in 2010 than in 1997 (Onkamo *et al*., 1999). High incidence rate are now reported from a number of non-Europid population. Kuwait has the seventh highest rate in the world (Karvonen *et al*., 2000), suggesting that genetic susceptibility may not vary as widely among ethnic groups as was previously believed.

ii. Age and Sex Distribution

The age distribution of newly diagnosed cases of childhood diabetes has been studied extensively (Karvonen *et al*., 1993). This generally shows a peak for clinical onset between 10-14 years of age with a sharp drop in the late teens

(Karvonen *et al.*, 2000). It may be that children susceptible to the disease are exposed to the predisposing factor (s) during their first 14 years of life and that either exposure subsequently decreases or a large proportion of the susceptible individuals has already developed the disease by the time they reach 20 years of age (Gale, 2002). Step rises in the age group under 5 years have been recorded recently (EURODIAB ACE study group, 2000; Gale, 2001). The pubertal peak in onset of TIDM occurs early in girls than boys (Pundziute-Lycka *et al.*, 2002), since the hormonal changes of puberty differ between the sexes. Genes regulated by sex hormones could play an important role in the different patterns of disease presentation (Pottratz *et al.*, 1994). The gene for IL-6 is a possible candidate as its promoter is regulated by 17-β estradiol (E_2) (Gillespie *et al.*, 2005). A slight male predominance (male : female >1) which has been reported in many countries is most pronounced after puberty (Braham and Geevarghese, 1990; Sridhar, 1996; Karvonen *et al.*, 2000). Rais *et al.*, (1996) demonstrated that TIDM seems to be more common in the females as compared with the males (2.5:1) with an earlier age of onset in females.

iii. Seasonality of Diagnosis

Many reports have pointed to increased incidence of TIDM during the cold months in both hemispheres, although this has been observed to be less consistent in children up to 5 years of age (Kimpimaki, 2002). Many reports revealed a significant seasonal variation in clinical presentation even in the youngest age group across all European population studies, with a winter peak that seems to be particularly characteristic of Scandinavian regions (Levy-Marchal *et al.*, 1995), while in Sardinia it shows a decline in the summer (July) and a peak in the autumn (October) (Muntoni *et al.*, 1995).The seasonal incidence of both CVB infection and TIDM peaks in the late summer and autumn (Clement *et al.*, 1995). Thus the seasonal variation in the incidence of TIDM could be due to viral infection which seems to be a predisposing factor to

TIDM, whereas a lack of seasonal variation has been noted in Madras (India) (Ramachandran *et al*., 1996).

2.3.2 Natural Course of TIDM

In 1965, Gepts suggested that the process of beta-cell destruction is slow and that it may take years to destroy enough beta cells to result a clinical symptoms. This is confirmed with observation that beta cells-dysfunction can be demonstrated up to 6-7 years before appearance of symptoms (Merna *et al*., 1999). Subsequently, it was observed that TIDM occurred more frequently in patients with autoimmune disease whom often had organ-specific antibodies (Ten and Maclaren, 2004). The identification of islet cell autoantibodies (ICA) in combination with the observation with an association between the disease and specific alleles of the human leukocyte antigen (HLA) system (Gillespie *et al*., 2002; Graham *et al*., 2002) and infiltration of the islets by lymphocytes and macrophages (Kukreja and Maclaren, 1999), give strong support to the view that TIDM is an autoimmune disease.

The hypothesis of Gepts regarding a chronic rather than an acute process was strengthened by the finding that circulating autoantibodies could be present long before clinical onset of the disease in relatives of patients with TIDM (Winter *et al*., 2002). These finding implies that TIDM is an autoimmune disease caused by multiple interaction between genetic, environmental and immunological factors (Lernmark, 1999). Once initiated the process is insidious and a considerable time may elapse before clinical manifestation (The Expert Committee, 2003).

In the model of beta-cell destruction, the process shows two phases of preclinical diabetes preceding a third phase of clinical diabetes (Ten and Maclaren, 2004) (Figure 2-1). In phase-1, there is no evidence of beta-cell destruction and only genetic predisposition is present (Kukreja and Maclaren, 1999). Due to unknown factors, possibly environmental or other reasons, a selective attack on the beta cells starts (Schatz *et al*., 2000). The duration of this

second phase is unknown; range from months to years. In some individuals this process may come to a half, or regeneration of the beta cell may occur, but in others the destruction process that leads to clinical symptoms would continue (Schatz and Maclaren, 1995).

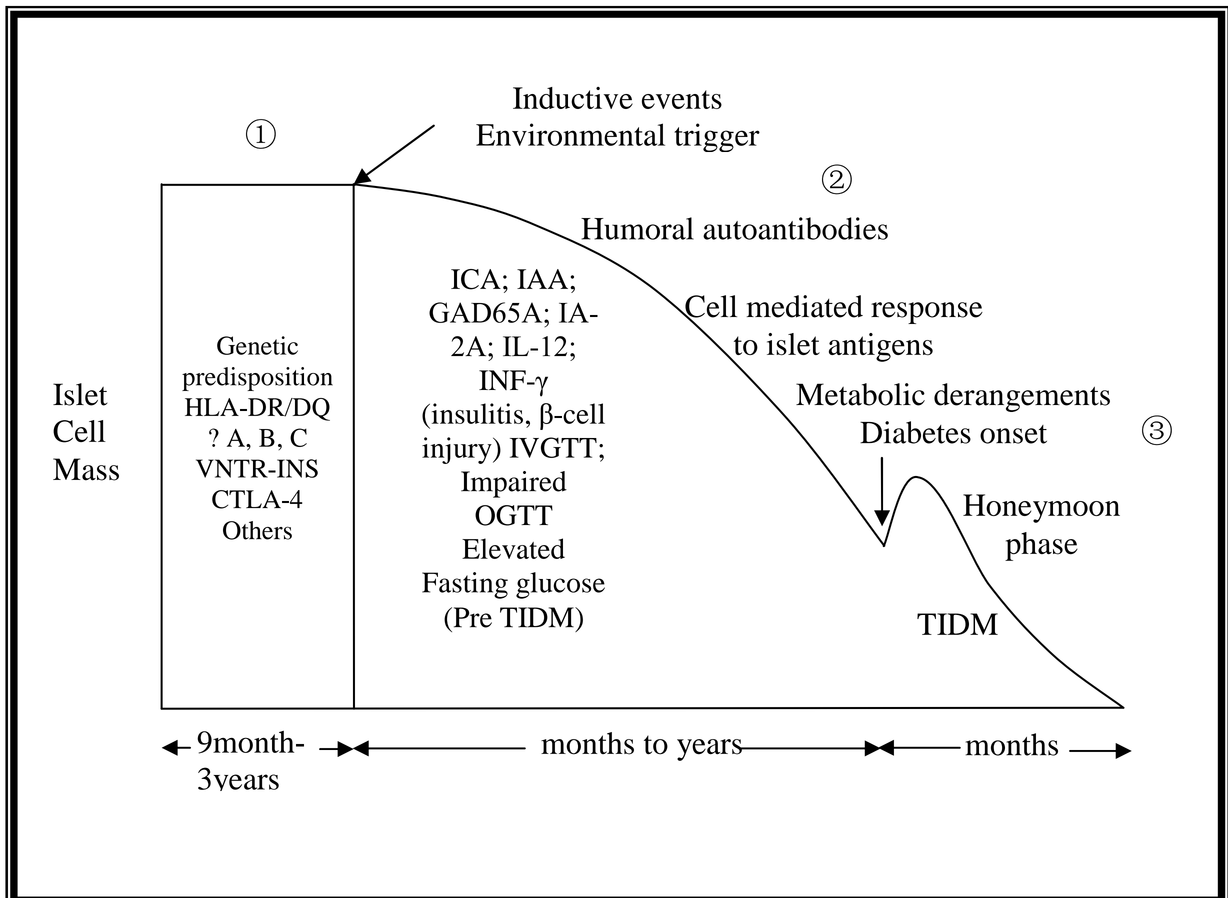

Figure 2-1: The natural history of type 1 diabetes (Cited from Schatz *et al.*, 2000 and Ten and Maclaren, 2004)

The pathogenic process can be identified by the detection of autoantibodies to islet cell antigens often long periods of time before the disease become clinically manifested (Schatz *et al.*, 2000). The presence of ICAs, autoantibodies to glutamic acid decarboxylase (GAD_{65}), insulin autoantibodies (IAAs) and autoantibodies directed to a transmembrane tyrosine phosphatase (IA-2A) may occur individually or in combination (Kimpimaki *et al.*, 2000; Krischer *et al.*, 2003). By the time, around 70-80% of the beta cells have been

lost. Glucose intolerance (OGTT); "Silent" diabetes and symptoms follow this (phase-3) (Kukreja and Maclaren, 1999).

After the initiation of exogenous insulin-therapy comes a phase called honeymoon period, characterized by physiologically significant endogenous insulin secretion initiated; this period is of a limited duration (Ten and Maclaren, 2004).

The remission phase in most children is partial but may be complete in some adult patients who remain normoglycemic without exogenous insulin (Käär *et al*., 1984).

2.3.3 Aetopathogenesis

The aetiology and pathogenesis of TIDM are not completely understood (Lernmark, 1999). The dramatic increase in the incidence of the disease in many parts of the world (Karvonen *et al*., 2000) is unlikely to be explained by increase in the genetic trait in the back ground population (Lowe, 1998); but rather points to the introduction of novel non-genetic factors or an increase in existing exogenous diabetogenic factors (Lernmark, 1999).

i. Genetic Susceptibility of TIDM

a. Major Histocompatibility Complex (MHC) Genes

TIDM is known to be polygenic disease that appears from the interaction of mutations in multiple genes (American Diabetes Association, 2002). TIDM is strongly and genetically linked and associated with HLA on chromosome 6 (designated IDDM1) (Dorman *et al*., 2000). The IDDM1 is located within the MHC. The MHC spans a 3.5 mega base region on the short arm of chromosome 6 that consists of over 200 genes (Goldsby *et al*., 2000), arranged into three subregions encoding three classes of molecules (Figure 2-2).

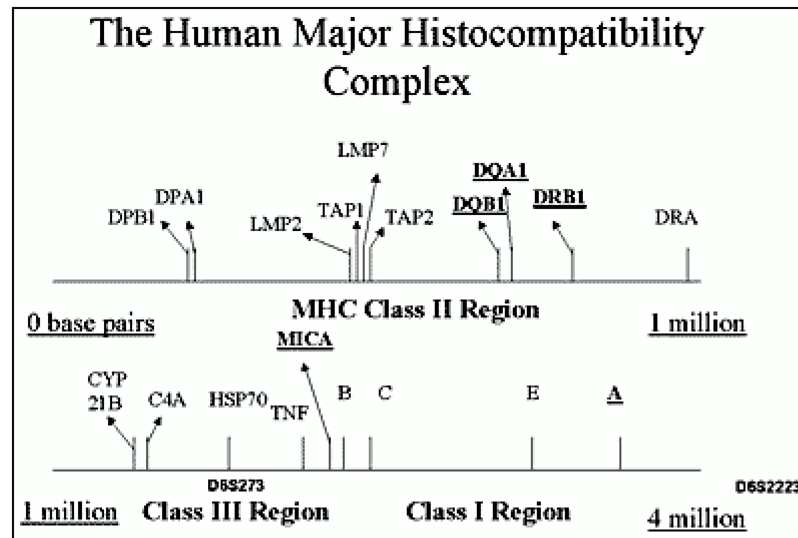

Figure 2-2: A simplified map of the HLA region on the short arm of Chromosome 6(6p21) showing the major genes of class I,II and III (adapted from Eisenbarth, 2004).

1.Class I genes: encode glycoproteins, including a heavy (α) peptide chain associated with a smaller chain called β_2-microglobulin (Figure 2-3). These are expressed on the surface of nearly all nucleated cells and platelets (Goldsby *et al.*, 2000). The HLA class I molecule bind to peptide fragments derived from endogenous antigens and present them for recognition by the T-cell receptor (TCR) of $CD8^+$ T-lymphocytes (Roitt, et al. 1998). Class I genes include the three classical genes; HLA-A (28 alleles); HLA-B (59 alleles) and HLA-C (10 alleles) loci; and the non-classical MHC-1b genes HLA-E; F and G (Williams, 2001), (Table 2-2). They do not display the extensive polymorphism of the classical genes (A, B and C) and appear to have more limited functions in the immune system.

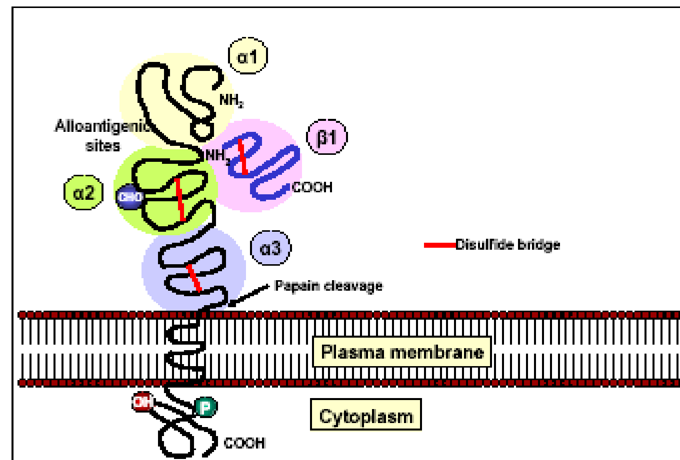

Figure 2-3: Class I MHC molecule (adapted from Pickup and William, 1997)

2.Class II (HLA-D) genes: encode glycoproteins expressed on a few cell types includes B lymphocytes, activated T cells, macrophages, dendritic cells, inflamed vascular endothelium, and some epithelial cells. The MHC class II molecules present processed antigenic peptides to $CD4^{+}$ T-lymphocytes (Chapel *et al*., 1999). The class II genes containing A and B genes encodes for the α and β chains respectively, (Figure 2-4). There are three gene clusters within the class II, designated the HLA-DR (24 alleles), HLA-DQ (9 alleles), and HLA-DP (6 alleles) (Table 2-2) (Dorman and Bunker, 2000). The class I and class II genes are both members of the immunoglobulin gene family (Goldsby *et al*., 2000).

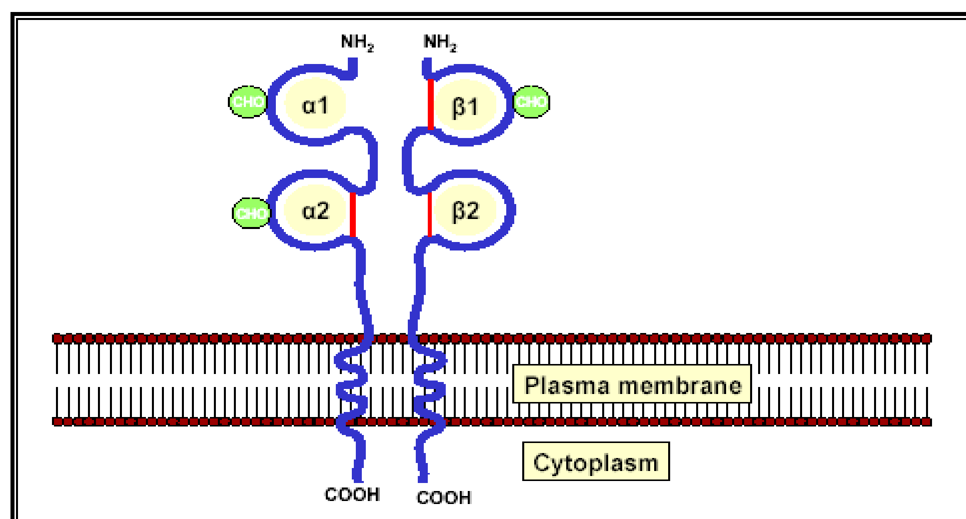

Figure 2-4: Class II MHC molecule (adapted from Pickup and William, 1997)

3.Class III genes: their proteins are not relevant to antigen presentation, and generally encode various secreted proteins that have immune functions, including TNF-α and β; components of the complement factors (C_4A ; C_4B ; C_2 and BF) and heat shock protein (HSP70) (Kelly *et al*., 2003) (Figure 2-2).

The role of HLA alleles in TIDM was first indicated by an association with HLA-B8, B15 and B18 (Singal and Blajchman, 1973).and then with HLA-DR3 and DR4 encoded in the DRB1 locus (Gillespie *et al*., 2002).

Table 2-2: Listing of all recognized serological and cellular HLA specificities. Adapted from HLA informatics group, Anthony Nolan Research Institute (2004)

A	B	C	D	DR	DQ	DP
A1	B5	Cw1	Dw1	DR1	DQ1	DPw1
A2	B7	Cw2	Dw2	DR103	DQ2	DPw2
A203	B703	Cw3	Dw3	DR2	DQ3	DPw3
A210	B8	Cw4	Dw4	DR3	DQ4	DPw4
A3	B12	Cw5	Dw5	DR4	DQ5(1)	DPw5
A9	B13	Cw6	Dw6	DR5	DQ6(1)	DPw6
A10	B14	Cw7	Dw7	DR6	DQ(3)	
A11	B15	Cw8	Dw8	DR7	DQ8(3)	
A19	B16	Cw9(w3)	Dw9	DR8	DQ9(3)	
A23(9)	B17	Cw10(w3)	Dw10	DR9		
A24(9)	B18		Dw11	DR10		
A2403	B21		Dw12	DR11(5)		
A25(10)	B22		Dw13	DR12(5)		
A26(10)	B27		Dw14	DR13(6)		
A28	B2708		Dw15	DR14(6)		
A29(19)	B35		Dw16	DR1403		
A30(19)	B37		Dw17(w7)	DR140415		
A31(19)	B38(16)		Dw18(w6)	DR15(2)		
A32(19)	B39(19)		Dw19(w6)	DR16(2)		
A33(19)	B3901		Dw20	DR17(3)		
A34(19)	B3902		Dw21	DR18(3)		
A36	B40		Dw22			
A43	B4005		Dw23	DR51		
A66(10)	B41		Dw24	DR52		
A68(19)	B42		Dw25	DR53		
A69(28)	B44(12)		Dw26			
A74(19)	B45(12)					

A80	B46					
	B47					
	B48					
	B49(12)					
	B50(21)					
	B51(5)					
	B5102					
	B5103					
	B52(5)					
	B53					
	B54(22)					
	B55(22)					
	B56(22)					
	B57(17)					
	B58(17)					
	B59					
	B60(40)					
	B61(40)					
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	B67					
	B70					
	B71(70)					
	B72(70)					
	B73					
	B75(15)					
	B76(15)					
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	B78					
	B81					
	B					
	Bw4					
	Bw6					

Subsequently the DQB1 and DQA1 genes were shown to be more closely associated with TIDM (Dorman and Bunker, 2000). However the phenotype of TIDM does not exhibit a typical mendelian inheritance that can be attributed to a single locus, and it is therefore an example of a complex genetic disease (Kawasaki *et al.*, 1998), with a multigenic pattern of inheritance and a strong influence of environmental factors which act as modulating (accelerating or delaying) its clinical manifestation (Lernmark, 1999).

In Caucasians, disease susceptibility is associated with two combinations of DQA1 and DQB1 alleles namely: DQA1* 0201. DQB1*0201/ DQA1*0301. DQB1*0302 haplotypes, which encodes the HLA.DQ2 and HLA.DQ8 respectively (Dorman and Bunker, 2000). Other high risk DQ haplotypes include: DQA1*0301. DQB1*0201 among African Americans (Dorman and Bunker, 2000); DQA1*0301.DQB1*0303 in the Japanese and DQA1*0301. DQB1*0401 in Chinese (Awata, 1995; Kawabata *et al.*, 2002). A study conducted by Zalloua *et al.*, (2002) on Lebanese patients reported that 70% and 40% of TIDM patients were positive for DQB1*0201 and DQB1*0302 respectively.

HLA-DQ genes that are in linkage disequilibrium with HLA-DR show the closest association with the disease (Sheehy *et al.*, 1989). There is strong linkage disequilibrium between the various loci in the HLA region, so that DR4 for example usually present together with DQ3, and DR3 together with DQ2 (Klein and Sato, 2000). Up to 90% of patients with diabetes carry one or both of these haplotypes and the highest risk of the disease is conferred by the DR3.DQ2/DR4.DQ8 hetrozygous genotype (Kamulainen *et al.*, 1999; Tanaka *et al.*, 2002).A Study conducted by Mezal, (1988) showed that A1, B8, and DR3 were the high risk antigens, while Al-Samarria, (2001) found that A24,B8,B15,DR3,DR4,DQ2, and DQ3 were highly associated with TIDM among Iraqi patients.

Strong natural protection against TIDM is also conferred by the DR2.DQ6 haplotypes (Moghaddam *et al.*, 1998), which occurs in approximately 20% of

the healthy white population but it is rarely found among patients with diabetes. These molecule can prevent progression of diabetes even after the onset of islet autoimmunity, suggesting that it may have an immunomodulatory role (Kimpimaki *et al*., 2001; Zalloua *et al*.,2002). However, its influence is not absolute because patients with diabetes who were positive for DQ6 have been reported (Pugliese *et al*., 1999). DR2; DQ1;DQ4 and B35 were found among the protective alleles in Iraqi patients (Mezal, 1988; AL-Samarrai, 2001). It is of interest that HLA-B35 and Cw4 antigens were found to be protective against type I and type II diabetes mellitus in Iraqi patients(AL-Samarrai, 2001). The association of DQ alleles with diabetes is related to the absence of amino acid asparatate at position 57 on β chain and the presence of arginine at position 52 on α chain (Khalil *et al*., 1990).DPB1 genes were shown to influence susceptibility to TIDM (Noble *et al*., 2000).

The mechanism of HLA-conferred disease susceptibility and protection has remained open, although various hypotheses have been proposed:

1. Antigen binding in the periphery: It has been proposed that susceptibility to TIDM and protection form may be detected from individual affinities in the interaction between diabetogenic peptide and various class II molecules (Nepom, 1990).
2. Molecular stability and thymic deletion of autoreaction T-cells: this model suggests that the HLA effect may depend on the failure of the immune system to maintain tolerance to pancreatic beta cells (Sheehy, 1992).
3. Influence on T-cell phenotype: The immuno-modulatory hypothesis perform that the HLA molecule may interact differently with the TCRs of autoreactive T-cells, affecting the phenotype of T-cells (Proinflammatory versus regulatory) or their activation status (Proliferative versus anergised) (Kelly *et al*., 2003).

Methods for Studying Genetic Association

Population testing has been utilized to study the association between HLA markers and the disease based on screening in families and screening in the general population (Dorman, 2000).

In general population studies, it is investigated whether one or more of the HLA antigens occur in different frequencies in patients compared to the corresponding frequencies in healthy unrelated controls matched for sex, age and region (Ad'hiah, 1990). Difference in frequency between patients and controls is expressed in terms of a relative risk (RR) (Svejgaard and Ryder, 1994).

The RR value indicates how many times as frequently the disease occurs in individuals carrying the antigen relative to those having lack of it. Accordingly, a risk above 1 means increased frequency of the antigen in the patients (positive association); whereas the decrease frequency gives a risk value below 1 (negative association) (Cruse and Lewis, 2000). The statistical significance in both cases is normally evaluated by the chi-square test and for smaller numbers Fisher's exact probability is used.

Further estimates were etiological (EF) and preventive (PF) fractions (Kockum, 1995). The EF value indicates the role of disease associated factor in evoking the disease state and it may show some information about the degree of linkage disequilibrium between HLA genes and a hypothetical disease predisposing gene. The EF can only be used for positive association (RR is more than 1). However, when RR is less than 1 (negative association), an estimation of the PF value can be used. Both the EF and PF values can vary between 0 (no association) and 1 (maximum association) (Svejgaard and Ryder, 1994).

Another approach to study genetic association is through screening for the high risk alleles in affected individuals and their family members (Dorman, 2000). The family study includes investigation of affected family with a

possibility of determining whether observed distribution of the haplotypes sharing differs from the expected one (Svejgaard and Ryder, 1994).

Although most intervention trials are based on first degree relatives, about 90% of those who develop the disease have a negative family history (Dorman, 2000).

b. Other Susceptibility Genes

Approximately other twenty genes loci-large stretches of DNA are suspected of contributing to the development of TIDM, but few specific genes have been pointed (American Diabetes Association, 2002), among these the polymorphism within the insulin gene region on chromosome 11 (Awata *et al*., 1998; Metcalf *et al*., 2001). Insulin gene contains the second major susceptibility locus for TIDM (IDDM2) (Dorman, 2000). Upstream of the insulin gene are variable numbers of tandem repeats (VNTR) region. Three classes of VNTR alleles have been identified, segregated according to the number of repeats of 14-15 bp sequence, (Kelly *et al*., 2003); class I alleles (26-63 repeats); class II alleles (64-139 repeats) and class III alleles (140-210 repeats). Class I is generally associated with susceptibility to TIDM with the highest risk while class III alleles are associated with dominant protection (Boic, 2004). The mechanism by which the insulin VNTR polymorphisms influence the risk of TIDM is unclear (Dorman, 2000). The protective effect of class III alleles may be explained by higher concentrations of proinsulin mRNA in the thymus, perhaps to enhance immune tolerance to preproinsulin, a key autoantigen in TIDM pathogenesis (Lernmark, 1999). However, this locus has been shown to regulate the expression of two downstream genes that may be relevant to disease pathogenesis, namely; the insulin gene and the insulin-like growth factor 2 (IGF2) gene (Kelly *et al*., 2003). Insulin and its precursor are potential target autoantigens for beta cells destruction, (Boic, 2004).

The cytotoxic T-lymphocyte antigen gene (CTLA-4) on chromosome 2 is also involved in the susceptibility of TIDM (Awata *et al*., 1998). The linkage of

TIDM to the CTLA-4 is not understood; however it has speculated that a gene polymorphism involving a AT repeat at the C terminus on the `3 end of the gene may affect the mRNA stability of the CTLA-4 mRNA (Lernmark, 1999). The longer the repeat, the less stable CTLA-4 mRNA. As CTLA-4 is critical to T-cell apoptosis, it has been speculated that long AT repeat might lead to T-cell survival because the CTLA-4 protein is not formed (Lernmark, 1999).

ii. Environmental Factors

Only minority of genetically susceptible individuals develops clinical disease (Rewers *et al.*, 1996). Based on data from the American Diabetes Autoimmune Study in the Young (DAISY), it was estimated that at least 30% of the general population have some degree of increased genetic risk of TIDM, but only about 0.5% develop the disease. The lack of 100% concordance in monozygotic twins suggests that the environmental factors also make significant contributions to the pathogenesis of the disease (Lowe, 1998). Environmental triggers of TIDM are postulated to include viral infections; dietary factors; environmental toxins; psychological stress and season of the year (American Diabetes Association, 2002). However, no single trigger has been conclusively identified.

a. Viral Infections

A causal relationship has been shown between TIDM and high frequency of infectious disease (Blom *et al.*, 1991); although an inverse association between the exposure to infections during the first year of life and subsequent TIDM has been reported (Gibbon *et al.*, 1997). The timing and diabetogenity of the infective agent may be crucial for the initiation of the pathogenetic process (Davidkin *et al.*, 1998).

The evidence that viral infection might cause TIDM is derived from studies where virus particles known to cause cytopathic or autoimmune damage to beta cells have been isolated from the pancreas (Yoon *et al.*, 1979). Several viruses have been implicated including infection with mumps;

coxsackie B virus, retroviruses, rubella (in utero, cytomegalovirus, and epistien-bar virus (Haslett *et al*., 1999).

Congenital rubella has been observed to be associated with an approximately 12-20% absolute risk of TIDM in individuals express HLA-A8 (Menser, 1974; Rubinstein *et al*., 1982) and in diabetic patients express the HLA-class II DR3 or DR4 haplotypes with ICAs (Schopfer *et al*., 1982). The virus acts by forming rubella-specific immune complexes which can and do act on the pancreas (Coyle *et al*., 1982).

Case reports and *in vitro* studies with human islet cell cultures suggest that mumps might also be involved (Prince *et al*., 1978; Jenson *et al*., 1980). The childhood diabetes study (Di Me) in Finland showed increase anti-mump IgG class antibody titer in children with newly diagnosed TIDM (Hyoty *et al*., 1993). Large epidemiological studies demonstrate parallel curves between outbreaks of mumps and new cases of TIDM (with a lag of 2-3 years) (Stratton *et al*., 1993).

MMR (measles; mumps; rubella) vaccine especially its mump and rubella components has been implicated in the causation of TIDM (Coulter, 1997). Another study conducted by Davidkine *et al*., (1998) confirm that measles or rubella-like illness in MMR vaccinated children are caused by other viruses like parvovirus, enterovirus, and adenovirus. In contrast, children with previous exposure to rubella had higher levels of ICAs, and no evidence was found that MMR vaccination during adolescence might be trigger autoimmunity (Lindberg *et al*., 1999).

The human cytomegalovirus (hCMV) may also play a role in the development of TIDM (Haslett *et al*., 1999). A Finnish study found comparable levels of hCMV IgG and IgM antibodies in children with newly diagnosed TIDM (Hiltunen *et al*., 1995). An Iraqi study conducted by Wahbi, (1998) has demonstrated anti-rubella IgM and anti-hCMV IgM in 7.5% and 5% respectively of newly diagnosed TIDM patients. Hiemstra *et al*., (2001) showed that hCMV-derived epitope can be naturally processed by dendritic cells and recognized by GAD_{65} reactive T-cells, thus involved in the loss of T-cell

tolerance to autoantigen GAD_{65}. In contrast, Hiltunen *et al*., (1995) found no differences of hCMV IgG and IgM levels between early onset TIDM patients and control group. Itoh and his team (1995) also failed to detect hCMV and Epstein-Bar virus genome in pancreatic biopsy of recent onset TIDM.

Rotaviruses are the major cause of human infantile gastroenteritis worldwide (Haslett *et al*., 1999). Sever rotavirus gastroenteritis have been associated with pancreatitis in two children, whereas pancreatic ICAs were detected in acute-phase but not convalescent-phase serum (Nigro, 1991). An Australian study noticed that Rotavirus of VP7 serotype 1 and 3 contain peptide sequences highly similar to T-cell epitope in the islet autoantigen GAD and IA-2, and the levels of anti-rotal IgA and IgG increase significantly with the increase levels of IA-2 and GAD antibodies in the serum of genetically susceptible children (Honeyman *et al*., 2000). Coulsen *et al*., (2002) have shown that rhesus rotavirus was replicated in the pancreatic islet of NOD mice, suggesting that infection of pancreas *in vivo* by rotavirus might be possible.

Enteroviruses (EVs) have also been indicated to be associated with the onset of TIDM in both epidemiological, serological as well as by the studies of the viral antigen (Dahlquist, 1997). Ylipaasto *et al*., (2004) demonstrated a definite islet-cell tropism of EVs in the human pancreas.

The EVs comprise a large genus belonging to the picornaviridae (Brooks *et al*., 2001).They are small, non enveloped, single strand RNA, ranging in size from 7.2-8.4 kb (Nester *et al*., 2004). EVs of human origin include: Coxsackie viruses of group A (CVA), type 1-24 (there is no type 23); Coxsackie viruses of group B (CVB), type 1-6; Polioviruses, type 1-3; Echoviruses, type 1-33 (no type 10, 22; 23 or 28) and Enteroviruses, type (68-71), (Minor *et al*., 1986; Brooks *et al*., 2001). EVs are transient inhabitants of the human alimentary tract and may be isolated from the throat or lower intestine (Nester *et al*., 2004). EVs infection in humans may result in a wide range of acute symptoms involving the cardiac and skeletal muscles; central nervous system; pancreas; skin and mucous

membranes (Meinlick, 1996; Muir *et al*., 1998), and have been associated with such chronic disease as TIDM (Yoon, 1990).

Epidemiological studies point to an increased incidence of TIDM after EV epidemics (Gamble and Taylar, 1969). Results from Scandinavian prospective studies suggest that EV exposure in childhood and even in utero may increase the risk of TIDM and initiate beta cell autoimmunity (Dahlquist *et al*., 1995; Hyôty *et al*., 1995; Viskari *et al*., 2002). EV infections were detected during a six month observation period preceding the first emergence of diabetes associated autoantibodies almost twice as often in autoantibody positive children than the matched antibody negative controls (Lönnert *et al*., 2000a).

Many investigators reported a frequent occurrence of EV mRNA in serum samples taken from children at the time of diagnosis (Clement *et al*., 1995; Yin *et al*., 2002; Craig *et al*., 2003; Bourlet *et al*., 2003; Paananen *et al*., 2003); whereas no excess of acute EV infection was found in children with newly diagnosed TIDM (Hyôty *et al*., 1995; Lönnert *et al*., 2000b).

A longitudinal study conducted by Buschard and Madsbad, (1984) found that CVB4 antibody titer fell from the diagnosis and the 5 months to the 2 years study in TIDM patients, and the average titer was also lower than in healthy control individuals. A Turkish study conducted by Emekdas *et al*., (1992) found no significant differences of neutralizing antibody levels of CVB serotypes 1,2,3,4,5 and 6 in sera obtained from TIDM patients and from control group. In contrast, Juhela *et al*., (2000) and Yin *et al*., (2002) demonstrated high levels of specific IgM antibodies to CVB in most newly diagnosed TIDM children.

In Finland another study found that non of the children vaccinated against poliomyelitis had antibodies to the diabetes associated epitope to tyrosine phosphatase IA2, but the same diabetic children had high levels of specific IgM antibodies to poliovirus derived VP1 peptide at onset of TIDM (Härkonen *et al*., 2003).

Davidkin *et al*. (1998) demonstrated that 4% of children (less than 4 years old) vaccinated with MMR had IgM and IgG specific antibodies to adenovirus in their sera.

Adenoviruses are medium-sized 80-110 in diameter and non-enveloped, containing a linear genome of double-stranded DNA (36-38 kbp) (Brooks *et al*., 2001). Adenoviruses are divided into six groups (A-F) containing 41 serotypes. They commonly infect human causing acute illness, mainly of the respiratory (the common cause of colds with fever) and intestinal tract (Nesler *et al*., 2004).

Pierce *et al*., (2003) reported that autoimmune TIDM could be inhibited in NOD mice transgenically expressing adenovirus early region 3 antiapoptotic genes through inhibiting Fas or tumor necrosis factor (TNF)-α-induced apoptosis and TNF-α-induced NF-kB activation.

Hypothetically, a virus may trigger or accelerate an autoimmune process in the beta cells through various mechanisms. These include:

1. **Molecular Mimicry:**

A number of viruses have been shown to possess antigenic determinants that are identical or similar to normal host (self-protein) which leads to activation of T-cell population against the viral antigen and the development of autoimmune disease (Goldsby *et al*., 2000). The major peptide determinant of islet cells autoantigen (GAD_{65}), recognized by patients with diabetes has a significant sequence similarity to a 15 amino acid sequence within the P2-C protein of CVB (Varela-Calvino *et al*., 2000). In addition to P2-C, the viral coat proteins VP2, VP3 and VP1 represent major targets (Varela-Calvino, 2002). Antibodies to the P2-C protein cross react with GAD_{65} and vice versa, leading to up regulation of antigen presentation and could lead to direct perforin-mediated killing of beta cells (Leech, 1998).

2. **Virus-Induced Cytolysis (Killing of Beta Cells)**

Some viruses may have direct cytotoxic effects on beta cells by infecting and damaging these cells in experimental animals (Mena *et al*., 2000).

Roivainen *et al*., (2000) found that CVB causes functional impairment and beta-cells death to human islet culture characterized by nuclear pyknosis, in addition to many other EV serotypes like poliovirus; parechovirus 1 and CVA13 (Roivainen *et al*., 2002). Horwitz *et al*., (2004) demonstrated on their research on mice that CVB4 infection of beta cells does not directly cause beta-cell death, instead the beta cells are phagocytes by macrophages following infection, suggesting that macrophages are the initiating pathogenic cells during virus-mediated autoimmune diabetes.

3. Formation of a novel Antigen:

Retroviral expression of superantigen (Sags) may be able to activate clonal expansion of autoreactive T-cell clones.The lymphocyte stimulated antigen of the retroviral sags expressed by beta-cells interact with the development T-helper cells of both Th1 and Th2 subsets in mice (Kukreja and Maclaren, 2002). Furthermore, incubation of peripheral blood mononuclear cells from TIDM patients with CVB-infected lysate induced a selective expansion of T-cells with TCR VB7 chains (Varela-Calvino and Peakman, 2003), which may cause a slowly progressing inflammation and tissue damage (Horwitz *et al*., 2002).

4. **Induction of MHC:**

Through release of autoimmune mediators like proinflammatory cytokines IL-1β; TNF-α; IL-2 and IFN-α (Horwitz *et al*., 1998; Chehadeh *et al*., 2000), these immune mediators can induce cells that normally do not express class II HLA antigen to do so. Thus destruction occurs as cytokine-mediated death (Seewaldt *et al*., 2000). Carollo *et al*., (1992) reported that infection with measles and mumps viruses induced the release of cytokine by the human insulinoma cell line and led to the expression of class I and II HLA antigen.

b. Dietary Factors

Breast feeding; nicotinamide; zinc and vitamins C, D and E have been reported as possibly protecting against TIDM, whereas N-nitrosom compounds; cow milk; increased linear growth and obesity may increase the risk (Virtanen and Knip, 2003).

An inverse relationship between duration of breast feeding and the development of TIDM was reported for the first time in 1984 by Borch-Johnsen *et al*., (1984). A greater decrease in risk of TIDM was seen among children who had been breast-feeding more than or equal to 12 months (Mayer *et al*., 1988). Gerstein, (1994) found that a short duration of breast feeding may be associated with a weak risk of TIDM in a meta analysis of 13 retrospective studies. Breast feeding has a protective effect against infections via maternally transferred immunoglobulins (Howie *et al*., 1990) and may thus provide protection from other potential triggers of beta-cell autoimmunity.

A series of studies have suspected the early introduction of cow's milk protein as possible risk factor. Saukkonen *et al*., (1998) has reported increased levels of bovine serum albumin (BSA) IgA antibodies and β-lactoglobulin IgA antibodies in newly diagnosed TIDM children. Interestingly BSA share an amino acid sequence (ABBOS) with an islet protein P69 and the antibodies against this BSA have been shown to cross-react with P69 (Karjalainen *et al*., 1992). Oral exposure to cow's milk induced bovine insulin-binding antibodies that cross-react with human insulin (Vaarala *et al*., 2000), so that dietary bovine insulin appears to be an environmental trigger of primary immune response to a beta-cell specific antigen in healthy children (Virtanen and Knip, 2003). Kimpimaki, (2002) reported that a long duration of exclusive breast feeding and late introduction of cow's milk may protect genetically susceptible children form progressive beta-cell destruction during the first years of life.

It has been known from the animal studies that nitrosamine compounds are toxic to beta cells probably by reducing their nicotinamide adenine dinucleotide (NAD) content, which is preventable by pretreatment with

nicotinamide (Helgason, 1982). The results of a population study found a relationship between the risk of TIDM and food intake or water rich in nitrites that are partly converted into nitrosamines in the gastrointestinal tract (Virtanen *et al*., 1994).

c. Un Specific Infections and Stress

The results of both case-control study and extensive survey showed a relationship between the total numbers of infections experienced during the year preceding the clinical onset of diabetes and its risk (Cardwell *et al*., 2003). Atobelli *et al*., (2003) found that multiple exposure of infection might increase the risk of diabetes as a potential accelerating factor for clinical manifestation of TIDM. Infection could promote beta-cells destruction by increasing cytokine activity (Nerup *et al*., 1988).

Therlund *et al*., (1995) has shown that psychological stressful life events during the year prior to the onset of their disease were significantly more common in individuals with TIDM than in matched controls. This association could be due to an increased insulin requirement due to excess cortisol and catecholamines release by psychological stress (Dahlquist, 1997).

2.3.4 Mechanisms of Beta Cell Destruction

i. Cellular Autoimmunity

TIDM is characterized by infiltration of mononuclear cells into and around the pancreatic islets with specific beta-cells destruction (Notkins and Lernmark, 2001). Local lymphocyte infiltration of the islet, termed **insulitis** implies an important role for abnormal lymphocytic regulation in the pathogenesis of this autoimmune disease (Goldsby *et al*., 2000). It has been suggested that the autoimmune processes may be caused by initial beta-cell damage, leading to islet antigen release and the production of cytotoxic cytokines by helper (Th) lymphocytes (Bach, 1988). The target of the

autoimmune process may be islet cell proteins such as autoantigens (Ellerman and Like, 1999).

After possible environmental exposure, an antigen is presented to a T-cell receptor by APCs in association with HLA class II molecule (Horwitz *et al*., 2002), that is considered to be the first step in the initiation of the disease processes (Boic, 2004). Activated T-cells expressing the HLA-DR antigen or other markers of activation can be detected in increased levels during the prediabetic period (Faustman *et al*., 1989). The formation of a complex between the MHC molecule which attached autoantigen and TCR results in the activation of CD_4^+-Th lymphocyte, Figure(2-5).

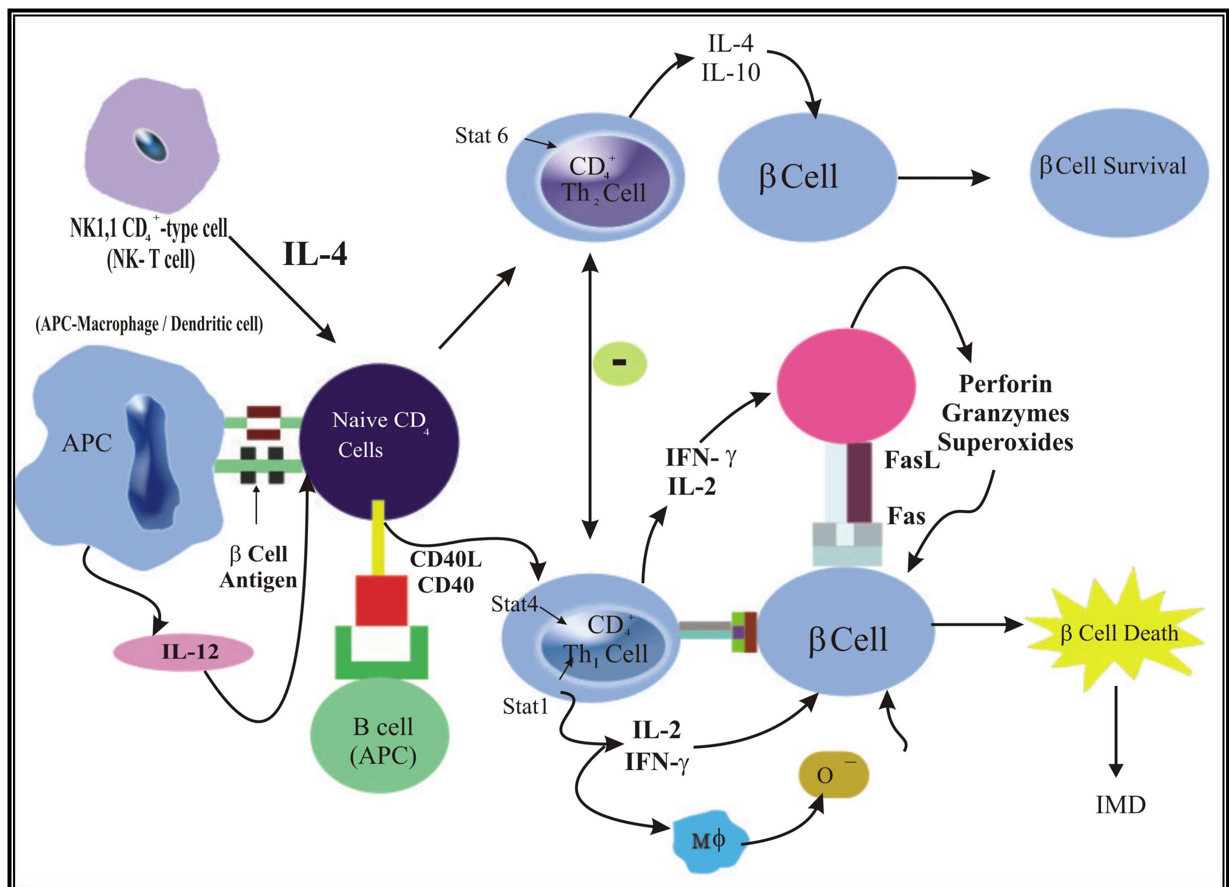

Figure 2-5: A schematic description of the roles of various subsets of T cells and their cytokines in Beta cell destruction leading to diabetes (adapted from Kukreja and Macleren, 1999)

The function of T-cells is mainly mediated via cytokines. Macrophages secrete IL-12, stimulating CD_4^+ T-cells (Boic, 2004). Activated CD_4^+ Th1 cells are known to release predominantly proinflammatory cytokines such as IL-2and IFN-γ. IFN-γ stimulates other resting macrophages to release in turn other cytokines such as IL-1β; TNF-α; nitric oxide and other oxygen radicals are toxic to pancreatic beta cells (Kraine and Tisch, 1999). The CD_4^+ Th2 cells release IL-4; IL-5; IL-10 and IL-13 and provide help for antibody production (Liblau *et al.*, 1995). During this process, cytokines induced the migration of beta cell autoantigen specific CD_8^+ cytotoxic (Tc) cells (Boic, 2004). On recognizing specific autoantigen on beta cells in association with MHC class I molecule, these CD_8^+ Tc cells cause beta-cells damage by releasing perforin and granzyme (Kassem *et al.*, 2000) and by Fas-mediated apoptosis of the beta-cells (Mauricio and Mandrup-Poulsen, 1998).

Both direct and indirect killing are thought to occur by apoptosis following activation of caspases (Mandrup-Poulsen, 2001), or via reactive oxidative stress, but necrosis also might play some roles (Notkins, 2002). Th1 cells which are associated with cellular immunity may promote the disease, whereas the role of Th2 cells which are mainly involved in humoral immunity is contraversal (Liblau *et al.*, 1995). It has proposed that Th1-type and Th2-type cytokines may interact to induce or suppress the destructive process of pancreatic beta-cells (Kolb, 1997).

The APCs include: Dendritic cells (DCs); B-lymphocytes and macrophages. All of this subpopulation has been shown to play roles in the progression of insulitis (Horwitz *et al.*, 2004).

Takahashi *et al.*, (1998) reported that DCs are both phenotypically as well as functionally impaired in humans at risk for TIDM. Alternatively B-lymphocytes secrete autoantibodies that bind to pancreatic beta-cell antigens and may subsequently trigger autoreactive T-cells through an antibody-dependent cell mediated cyotoxicity response (Serreze *et al.*, 1998). A study conducted by Wong *et al.*, (2004) in NOD mice shows that the antigen presenting function of

B-lymphocytes that was critical in the development of TIDM presenting sequestered antigens to CD_4^+ T-cells.

a. Lymphocytes

It was observed in 1984 that activated T-cells are present in the peripheral blood of patients with newly diagnosed TIDM (Alviggi *et al*., 1984), with significant abnormalities of lymphocyte subpopulations including: reduced percentage of CD_4^+ cells (helper / inducer) (Michalkova *et al*., 2000), or normal percentage (Faustman *et al*., 1989) or predominant activation of CD_8^+ cells (cytotoxic / suppressor) (Drell and Notkins, 1987), expressing activation markers such as HLA-DR (Hehmke *et al*., 1995). Moreover, abnormal elevated levels of HLA-DR, CD_8^+ T-cells demonstrated in the peripheral blood of monozygotic twins patients in prospective studies, whereas twins that remained normoglycemic had lower levels of activated CD_8^+ T-cells (Peakman *et al*., 1996).

In contrast Michalkova *et al*., (2000) reported unbalanced activation of CD_4^+ and CD_8^+ T-cells with a sharp decrease of CD_8^+ lymphocytes percentage in newly diagnosed TIDM children, but the percentage was elevated significantly after 1 month and 7 months of diagnosis in parallel with decreased percentage of CD_4^+ lymphocytes (Buschard *et al*., 1990). Lymphocytes possessing HLA-class II molecules and receptors for IL-2 have been detected in peripheral blood at the time of diagnosis (Pozzilli *et al*., 1983; Hayward and Hexberg, 1984). An Iraqi study conducted by Al-Samarrai (2001) reported low percentage of peripheral blood CD_3, CD_4, CD_8 and MCH class II molecules with a decreased CD_4 / CD_8 ratio in early onset and long term TIDM patients.

T-lymphocytes has been seen to be activated mostly in the prediabetic state and to fade with increasing destruction of beta cells (Faustman *et al*., 1989), suggesting that a combination of cellular and humoral immune changes with their tendency to persist may be highly predictive of progression to clinical TIDM (Durinovic-Bello *et al*., 1996). Kretowski *et al*., (2000) found significant increasing of gamma and delta TCR^+ , CD_8^+ T-cells in the peripheral blood of

patients with TIDM, treated with insulin for 3-6 months, believing that it may play a role of regulatory T-cells and could be induced by delivery of exogenous insulin.

Some Characteristic Markers of Lymphocytes in DM Patients

–CD_{38} is a single chain type II transmembrane glycoprotein expressed on the surface of monocytes, platelets, NK-cells, T and B lymphocytes, myeloid cells, vascular endothelium and many tissues, used as a phenotypic markers of differentiation and activation of hematopoietic cells (Goldsby *et al*., 2000). CD_{38} (ADP-ribosyl cyclase / cyclic ADP ribose hydrolase) a surface and cytosolic enzyme that catalyzes the conversion of NAD^+ and cyclic ADP-ribose (Mehta *et al*., 1996). In pancreatic beta-cells, this protein appears to play a role in glucose-induced insulin release (Antonelli *et al*., 2001), through a mechanism that involves its cyclase activity leading to increase cytoplasmic Ca^{+2} concentration (Pupilli *et al*., 1999), and insulin release. It was detected that anti-CD_{38} autoantibodies were found in 9.7% of type II diabetic patients and in 13.1% of TIDM patients vs. 1.3% in control group (Pupilli *et al*., 1999). Marchetti *et al*., (2002) found that prolonged exposure of human pancreatic islets to sera containing CD_{38} antibodies impairs their function and viability.

Natural Killer (NK) Cells: make up to 10-15% of the recirculating lymphocyte population (Chapel *et al*., 1999), express neither TCR nor BCR-Ag receptor. Most surface antigens detectable on NK-cells by mAbs are shared with T-cells (CD_{56}) or macrophages (CD_{16}) (Goldsby *et al*., 2000). Because NK-cells produce number of immunologically important cytokines like IFN-γ, IL-4, IL-13 they can also play important roles in immune regulation (Roitt *et al*., 1998).

Michalkova *et al*., (2000) reported a decreased number of NK-cells in TIDM children at onset, while Kukreja *et al*., (2002) showed that both before and after clinical onset of TIDM, the level of NK-cells in peripheral blood reduced. In other hand some investigators demonstrated that the level of NK-cells in peripheral blood of TIDM patients remain normal (Faustman *et al*.,

1989; Hehmke *et al*., 1995). The data of T-cell reactivity to various diabetes associated autoantigens in diabetic patients and non-diabetic controls have remained inconsistent. Tun *et al*., (1994) reported an increased proportion of activated T-lymphocytes in the peripheral circulation which is associated with the presence of IAAs . Increased T-cells responses to diabetes associated autoantigens were detected in newly diagnosed patients with TIDM and in antibody positive first degree relatives as compared with healthy controls (Durinovic-Bello *et al*., 1996). In contrast Schloot *et al*., (1997a) found no differences in T-cell responses to insulin between newly diagnosed TIDM patients and first degree relatives or normal individuals. Furthermore, a highly frequency of T-cell responses to diabetes associated autoantigens had been described in healthy persons. Thus it been regarded so far as an unreliable indicator of the risk of TIDM.

T-cell responses to CVB antigen have been reported to be lower in newly diagnosed TIDM in comparison with longterm diabetic children and healthy controls (Juhela *et al*., 2000; Varela-Calvino *et al*., 2002).

b. Cytokines

Cytokines are important for coordinating the immune responses and a disturbance in the balance between autoreactivity and tolerance that can result in autoimmunity (Azar *et al*., 1999).

Cytokines are chemical messengers, protein molecules produced by a variety of activated cells that act as regulators and mediators of immune responses (Chapel *et al*., 1999). The term cytokines was first used by Stanley Cohen in 1974. It's action includes the ability to induce growth; differentiation; cytolytic activity; apoptosis and chemotaxis (Paul, 1999).

Recently, cytokines have been classified into several different groups (Rabinovitch, 1998; Kukreja and Maclaren, 1999; Goldsby *et al*., 2000). There are as follows:

1.Type 1 cytokines (T-helper 1) such as:Interlukin-2(IL-2); Interferone gamma(IFN-γ); TNF-β.

2.Type 2 cytokines (T-helper 2) such as: IL-4; IL-5; IL-6; IL-10 and IL-13.

3.Type 3 cytokines (T-helper 3) such as: Transforming growth factor (TGF-β).

The dominance of Th1 and proinflammatory cytokines have been implicated in TIDM by promoting cell destruction in autoimmune diabetes in animal models (Amrani *et al*., 2000), and in human islets (Heitmeier *et al*., 2001).

IFN-γ which was originally secreted by Th1;cytotoxic Tc and NK cells plays a central role in many immunoregulatory processes, including the regulation of mononuclear phagocytes; B-cell switching to certain IgG classes, and the support or inhibition of the development of Th2-cell subsets (Goldsby *et al*., 2000). IFN-γ plus IL-1 enhance the expression of nitric oxide synthase (iNOS), which impairs the oxidative metabolism of islet cells that leading to beta cells necrosis (Thomas *et al*., 2002). Karlsen *et al*., (2000) demonstrated that IFN-γ induces the expression of cystine protease IL-1 β converting enzyme (ICE) in human, rat and mouse islets which is a key proapoptotic caspases. Moreover, Suk *et al*., (2001) indicated that a combination of IFN-γ and TNF-α induced caspases dependent apoptosis through STAT1/IFN regulatory factor 1 pathway *in vivo* as well as *in vitro*. Hussain and Colleagues, (1996) reported elevated levels of cytokines namely: IL-2, IFN-γ; TNF-α and IL-1α in recently diagnosed patients with TIDM, but no difference in the levels of IL-4 and IL-10 was recorded. They reported in another study (Hussain *et al*., 1998), increased levels of TNF-α, IL-2 and IL-1 in non-diabetic family members and their affected relatives. IL-1 receptor appears to be present on beta cells (Eizirik *et al*., 1991). Hostens *et al*., (1999) reported elevated proinsulin levels in prediabetic individuals in company with high levels of IL-1β plus IFN-γ.

Based on many studies, it was concluded that Th2 cytokines IL-4 and IL-10 protect from TIDM in NOD mice either by reverse T-cell unresponsiveness (Rappoport *et al*., 1993) or via decreased Th1 cytokines IFN-γ to Th2 cytokine IL-4 ratio within T-cell infiltrated pancreatic islet (Serreze *et al*., 2001). Marselli *et al*., (2001) reported that treatment of isolated human islet with Th2 cytokines IL-4 and IL-10 partially protected the islet cells form cytostatic and cytotoxic action of proinflammatory and Th1 cytokines by reducing the production of nitrous oxide (NO).

Other reports pointed against the anti-inflammatory action of Th2 cytokines. It was demonstrated that local production of IL-10 but not IL-4 accelerated autoimmune destruction of β-cells (Pakala *et al*., 1997). In addition NOD mice were protected from development of diabetes by a neutralizing anti-IL-10 monoclonal antibodies (mAbs) but not anti-IL-4 mAbs, which were described to be ineffective in altering the course of Th2 autoimmune destruction of pancreatic beta islet cells (Pakala *et al*., 1997). Interestingly, IL-10 is characterized as a potent β-cell activator and enhances MHC class II expression on B-cells which in turn accelerates the disease progress (Gianani and Sarvetnick, 1996). In contrast another study conducted by Balasa *et al*., (1998); found that IL-10 was essential for an early phase of diabetes in NOD mice via CD_8^+ T-cell pathway with out the participation of B-cells. In any event, Th2 cytokines can no longer be viewed as "Protective" of TIDM (Almawi *et al*., 1999).

The inflammatory cytokines IL-6 originally secreted by macrophage, monocytes and Th2 cells (Goldsby *et al*., 2000) was a powerful inducer of the hepatic acute-phase protein (Yudkin *et al*., 2000). Serum levels of IL-6 have been found to be higher (Myrup *et al*., 1996) or normal (Kulseng *et al*., 1999) in TIDM patients compared with those of healthy controls. Another study conducted by Targher *et al*., (2001) found that serum levels of IL-6 were elevated markedly in young TIDM patients without clinical evidence of microvascular and macrovascular complication versus healthy controls.A further

study demonstrated that blood levels of IL-6 were higher in hyperketonemic diabetic patients than in normoketonemic patients and healthy controls. These results confirmed in the same study by reporting high levels of IL-6 in monocytic cell culture in a ketonic media (Jain *et al*., 2003). *In vitro* studies indicating an additive effect of several cytokines includes: IL-6, TNF, lymphotoxin and IFN-γ acting alone or in synergetic operating (Sandler *et al*., 1990).

Macrophages are among the first immune cells to be found in the islet (Bach, 1988), it can function as APCs and exert a cytotoxic effect by releasing cytokine (IL-12) that attract other immune cells and accelerate autoimmune diabetes (Trembleau *et al*., 2003).

Cytokines and Viral Infections

The activation of antiviral IFN-α and it's relation with CVB infection have been analyzed in TIDM patients (Chehadeh *et al*., 2000b). Chehadeh *et al*., (2000a) demonstrated elevated levels of IFN-α in the plasma of TIDM patients, which in turn induced T-cells to secrete IFN-γ (Kadowaki *et al*., 2000). In this way type 1-IFN bridges the innate to the adaptive immune system.

It was demonstrated that dsRNA was formed during viral replication, activates the antiviral responses in infected islet cells through direct interaction with IFN-γ to stimulate beta-cells nitric oxide synthase (iNOS) expression, leading to beta-cells dysfunction, (Heitmeier *et al*., 1999; 2001), or induction of islet cell apoptosis through the dsRNA-dependent protein kinase (PKR) (Scarim *et al*., 2001), or might be via both chemokine (IL-15) expression and nuclear factor (NF-kB) dependent apoptosis (Liu *et al*., 2002). Seewaldt *et al*., (2000) infected islet cells isolated from mice by lymphocyte choriomeningitis virus (LCMV), and found that the perforin-mediated killing of beta-cells by autoreactive Tc-lymphocytes is not sufficient to lead to diabetes but requires a direct effect of IFN-γ.

ii. Abnormalities of Humoral Immunity

a. Abnormalities of Autoantibodies

Although antibodies have turned out to be excellent diagnostic and predictive markers for TIDM, it is generally known that they play only a minor role in the pathogenesis of the disease (Notkins, 2002). Four autoantibodies have emerged as the most useful autoimmune markers of TIDM, ICAs, IAAs, GADAs, and Insulinoma Associated protein-2 Antibodies(IA-2As) while the latter include ICA_{512} and IA-2C autoantibodies (Winter *et al*., 2002).

1. Islet Cell Autoantibodies (ICAs)

ICAs are mainly polyclonal autoantibodies of immunoglobulin G (IgG) type, that react with all cells of the islet such as α, β, γ, δ and pp cells (Gorus, 1996). ICAs were classified as a first identification in association with TIDM in 1974, when they were detected in adult patients with autoimmune polyendocrine disease (Botazzo *et al*., 1974). Subsequently they were found in children and adults with newly diagnosed TIDM (Botazzo *et al*., 1980). ICAs may be detected in the peripheral circulation for several years before the diagnosis of TIDM (Gorsuch *et al*., 1981). At onset of TIDM around 73-88% of patients tested positive for ICAs (Bingley *et al*., 1997) but their frequency declines following diagnosis and no more than 5-10% of TIDM patients remain ICA-positive after 10 years (Winter *et al*., 2002). In respect to Iraqi TIDM patients, 62.5% of the patients showed ICAs in their sera (Wahbi, 1998).
The prevalence of ICAs in first degree relatives of patients with TIDM varies from 2.6-6.9% (Krischer *et al*., 2003), while in non diabetic persons varying from 0.24-4.1% (Kulmala *et al*., 2001). Positivity for ICAs have been observed to be associated with HLA-DQB1*02/*0302 (Kimpimaki *et al*., 2002; Kupila *et al*., 2002), HLA-DRB1*03.DQA1*0501.DQB1*02 hetrozygous (Gillespie *et al*., 2002) and DQ8 (Graham *et al*., 2002). Savola *et al*., (2001) observed in siblings of children with TIDM that there were high titers of ICAs associated with HLA DR_3 / DR_4 heterozygosity.

2. Insulin Autoantibodies (IAAs)

The first islet autoantigen and beta cell specific autoantigen reported was insulin (Palmer *et al*., 1983).

At onset of TIDM, IAAs occur in 54% of children less than 12 years (Sabbah *et al*., 1999). Komulainen *et al*., (1999) found that IAAs titer seems to be the highest in children <2 years of age compared to older children 2-15 years of age. IAAs are usually the first autoantibodies to appear in children followed the birth for development of TIDM (Ziegler *et al*., 1999). They can appear in the first 6 months of life (Ziegler *et al*., 1999). More than 90% of children developing TIDM prior to age 5 have IAAs which appears earlier than ICAs, GADAs or IA-2A (Kimpimaki, 2002), while less than 50% of children developing diabetes after age 12 have such autoantibodies (Eisenbarth, 2004). In a study on Iraqi patients, 40% of them showed IAAs positive (Wahbi, 1998).

The prevalence of IAAs was 1.4-7.2% among sibling of children with TIDM (Kimpimaki *et al*., 2000) and from 0.9-3% among children from the background population (Kulmala *et al*., 2001). Prospective studies targeting siblings showed that an increased prevalence of IAAs was closely associated with the DR4 / and DQB1 (Kimpimaki *et al*., 2001) and DR3 / DR4 phenotypes (Savola *et al*., 2001), thereas the highest frequencies of IAAs were seen in children carrying HLA-DQB1*02 / *0302 genotype diagnosed before 5 years of age (Komulainen *et al*., 1999).

3. Glutamic Acid Decarboxylase autoantibodies (GADAs)

GAD is neither beta-cell nor islet specific. GAD is expressed predominantly in the nervous system. Other tissues that express GAD include testes, ovary, adrenal, pituitary, thyroid and kidney (Winter *et al*., 2002). GAD which exists in two isoforms GAD_{65} and GAD_{67} is expressed in the human pancreatic cells an enzyme which catalyse the conversion of glutamic acid to gamma amino butyric acid (GABA) (Baekkeskov *et al*., 1990). The frequency of

GADAs has been reported to vary from 0.5-3% among children from background population (Kulmala *et al*., 2001), from 6.4-13% among siblings of children with TIDM (Kulmala *et al*., 1998; Kimpimaki *et al*., 2000) and from 62-84% among patients with newly diagnosed disease (Sabbah *et al*., 1999). GADA has been reported to be associated with the HLA-DQA1*0501 / DQB1*0201 (DQ2) alleles (Sabbah, 2000) and the DR3.DQB1*02 haplotypes (Kulmala *et al*., 2000). Because GADA are more persistent than ICAs after the diagnosis of TIDM (Winter *et al*., 2002), and may have preferred combing GADAs and ICAs for screening strategy in a population of non-diabetic relatives of a proband with TIDM (Krischer *et al*., 2003). GADAs may play a significant role in the processing and presentation of T-cell epitope from the human GAD-65 autoantigen to T-cells through increasing the efficiency of antigen capture by APCs (Reijonen *et al*., 2000). GADAs are also more frequent in girls than in boys (Rais *et al*., 1996), and in individuals more than 10 years of age (Sabbah, 2000).

4. Insulinoma Associated Protien-2 autoantibodies (IA-2As)

IA-2 is a member of the protein tyrosine phosphatase (PTP) family and is expressed in pancreatic islets, in nervous tissue, and the pituitary (Winter *et al*., 2002). IA-2As develop later than GADAs and thus may serve as markers of beta-cell destruction associated with rapid progression to TIDM (Genovese *et al*., 1996) . The prevalence of IA-2As has been observed to vary from 0.2-2% among children from the back ground population (Kulmala *et al*., 2001), and from 1.5-5.3% in siblings of children with TIDM (Kimpimaki *et al*., 2000; 2002), while in patients with newly diagnosed disease they were 54-86% (Sabbah *et al*., 1999). Positivity for IA-2As has been reported to be associated with the DR4 allele and DR3 / 4 hetrozygosity (Kulmala *et al*., 2000), and with DR4-DQB1*0302 (DQ8) haplotype (Sabbah *et al*., 1999; Graham *et al*., 2002).

5. Antibodies to other antigens

In addition to insulin, GAD, IA-2, a number of autoantigens have been identified as potential targets for an autoimmune attack on beta cells progressing to TIDM. These include: carboxypeptidase H, beta cell glucose transporter (GLUT-2) and insulin receptor (Winter *et al*., 2002).

2.4 Immunological Marker(s) assay

2.4.1 HLA Tissue Typing

i. Serological HLA Typing (Microlymphocytotoxicity) Test

HLA typing is carried out at phenotyping level using antibodies to HLA type I and HLA type II (DQ and DR) molecules (Dorman, 2000). Lymphocytes are tested with a set of HLA specific antisera either polyclonal or monoclonal in a complement-dependent lympholysis technique (Stocker and Bernoco, 1979). These antibodies are usually obtained from multiparous women, multitransfused patients and patients who have rejected transplanted organs (Thompson, 1978). This method required living T and B lymphocytes obtained from the peripheral blood samples (Dorman, 2000).

ii. Cellular Detection of Class II molecules:

Via mixed lymphocyte culture, used for typing HLA-D antigens (Bach and Van Rood, 1976) and Primed Lymphocyte Testing (PLT) which was introduced by Sheehy *et al*., (1975) was used to type DP antigens (Johnson *et al*., 1996).

iii. Molecular Typing Method

Various DNA hybridization techniques have replaced serology and lymphocyte culture based methods used earlier. Molecular typing based on genomic DNA can be easily isolated from a variety of sources such as lymphocytes, dried blood spots, buccal brushes (Dorman, 2000).

Restriction **F**ragment **L**ength **P**olymorphism (**RFLP**) was analyzed with long radioactive cDNA probes which were originally used for genome based typing, but then replaced by **P**olymerase **C**hain **R**eaction (**PCR**) based methods (Ilonen *et al*., 2002).

2.4.2 Phenotypic characteristics of Cell Surface Markers

It has been found that surface markers are associated with cell development stages and have important biological functions for normal cell physiology such as cell-cell interaction and cell-matrix interaction (Goldsby *et al*., 2000). These markers include: CD_3, CD_4, CD_8, CD_{19}, CD_{56}, CD_{38}, CD_{45}RA (naive cells) and CD_{45}RO (memory cells).

To enumerate and differentiate individual lymphocyte subsets, Immunofluorescence (IF) and Immunoperoxidase (IP) methods can be used (Thompson, 1978). In IF technique, fluorescent mAbs to specific cell surface antigen can be used. The most commonly used fluorescent dyes are fluorescien and rhodamine, but other highly fluorescent substances such as phycoerythrin (PE) and phycobiliprotein have also been in use (Thompson, 1978). The dyes can be conjugated to the Fc region of an antibody and emits light under the fluorescein microscopy. Fluorescent-antibody staining of cell membrane molecules can be done directly or indirectly (Goldsby *et al*., 2000).

The IP method, a modification of the technique of IF has chemically linked the specific anti-tissue antibody to enzyme such as horse radish peroxidase thus no need for fluorescien microscopy and cells can be viewed by using ordinary microscope (Thompson, 1978).

Flow cytometry, a version of which is called **FACS** (**F**luorescence **A**ctivated **C**ells **S**orter), which has designed to automate the analysis with separation of cells according to their fluorescence and light scattering properties using a laser beam and light detector to count single intact cells in suspension (Chapel *et al*., 1999).

2.4.3 Assessment of Functional Activities of Lymphocytes

These methods are based on the evaluation of the *in vitro* responsiveness of B and T-lymphocytes to a specific antigen. These tests can be done by using either whole blood or separated lymphocytes. The most currently used technique is "Induced lymphocyte proliferation or blastogensis" (Hickling, 1998).

This technique is based on the capability of the lymphocytes for responding to an antigen (specific response). A few small resting lymphocytes respond by changing into blast cells showing DNA synthesis over a few days, because of the lymphocyte capability to reacting to different lectins or mitogens that will be used as a proliferation control (Haeney, 1985). The proliferative response is measured by colorimetric assay based on tetrazolium salt MTT that measures only living cells (Mosmann, 1983) or radiolabelled tritiated thymidine (^{3}H-Thy) incorporated into DNA (Hickling, 1998) or by the expression of cell-surface markers such as CD_{69}, CD_{25}, CD_{71} and HLA-DR found on activated cells after a few hours (Chapel *et al*., 1999).

Another approach is the measurement of the cytotoxic activity of CD_8^+ T-lymphocytes to ward target cells that was labeled with chromium 51. After incubation together for few hours, the supernatant was measured using gamma counter (Hiclking, 1998). All these tests needed tissue culture facilities.

2.4.4 Cytokines Release

Many reports indicated that cytokines are released by lymphocyte subsets (IFN-γ, IL-10), and mononuclear cells IL-6 occur during the pathogenesis of TIDM (Rabinovitch, 1998). Cytokine levels in body fluids can be measured directly or alternatively as populations of T-cells can be stimulated and cultured *in vitro* and the quantities of cytokines that are produced can be determined (Goldsby *et al.*, 2000). In either situation, both bioassays and immunoassays can be used (Hickling, 1998). Cytokine can be measured also by the enzyme-linked immunospot (ELISPOT) assay which is an adaptation of the ELISA, which measures the local concentration of cytokines that are released from an activated T-cell (Hickling, 1998).

2.4.5 Estimation of Total Immunoglobulins(Igs) and complement.

i. Estimation of Total Igs

Functional activities of Igs are well demonstrated in many aspects as in host defense. Hence it is used as diagnostic tool for assessing the levels of antimicrobial antibodies (Ganong, 1997).

Different techniques are used for detection of Igs, as single radial immunodiffusion (SRID) was originally described by Mancin. A precipitating antiserum specific for the heavy chain of the class of Ig being measured, is mixed with melted agar. The agar-antiserum mixture is poured into a glass plate. Holes are punched in the agar and filled with test or control sera (Chapel *et al.*, 1999). Other techniques include: Immunoprecipitation, IF, ELISA and Radial Immune Assay (RIA) (Goldsby *et al.*, 2000).

ii. Estimation of Complement Components

Immunochemical assay of C3 and C4 are the most useful. Low levels of C3 and C4 are more relevant clinically than high levels. As all complement components can act as a cute-phase reactants while the rates of synthesis rise in any inflammatory conditions (Haeney, 1985). Many techniques are used for

measuring total levels of individual complement proteins including: RIA, ELISA and SRID which use antibody specific for the protein under investigation (Roitt *et al*., 1998).

iii. Estimation of GADAs

Measurement of islet autoantibodies can assist in the diagnosis of TIDM and the detection of these markers in non-diabetic individuals indicates a significantly increased risk for the subsequent development of TIDM (Winter *et al*., 2002).

Detection of circulating GADAs commonly involves several methods: Indirect IF, ELISA and Radioimmunoassay (Chapel *et al*., 1999).

iv. Determination of Antibodies to Viral Antigens

Also detection of antibodies to microorganisms has been used in the diagnosis of infection. The presence of circulating antibody indicates that the antigen has been met previously (Buschard and Madsbad, 1984). Antibodies (IgG) to CVB, Polio virus and Adeno virus are usually detected by ELISA (Chapel *et al*., 1999).

2.5 Hematological Assays

2.5.1 Estimation of Glycosylated Hemoglobin (HbA1c)

HbA1c has a glucose attached to the terminal valine in each beta chain of polypeptide in the hemoglobin molecule, and its quantity in the blood increases in poorly controlled diabetes mellitus (Ganong, 1997).

Different methods are used for measuring HbA1c by: electrophoresis, immunoassays, and chromatography(Mayer and Freedman, 1983).

2.5.2 Estimation of Serum C-peptide

The importance of C-peptide marker is to identify the IDDM and NIDDM. Decreasing of C-peptide level in blood is a marker for decreasing insulin level in blood. In addition C-peptide is more stable than insulin and its level is more than insulin for 5-6 times, so its measurement is very helpful. It is measured by RIA (Hoekstra *et al*., 1982; Hamad *et al*., 1997).

Materials and Methods

3.1 Subjects

This study was conducted on the following main groups:

3.1.1 Patients Study Group

Sixty Iraqi type I diabetic patients (28 males and 32 females) were subjected to this study. The patients were attending to National Diabetes Center at Al-Mustansiriya University / College of Medicine. Clinical examination was performed by a committee of physician and ophthalmologist. Their ages range from 3-17 years, and they were new onset of the disease (diagnosis was from one week up to five months).

All the patients were treated with daily replacement doses of insulin at the time of blood sampling. This study was conducted during the period May 2004 to October 2005.

The patients were divided into two groups according to their ages: 36 child equal or less than 10 years and 24 child from 10.1 up to 16 years (Table 3-1).

Table 3-1: Numbers and percentage frequencies of T1DM patients and controls divided by sex and their mean ages.

Parameters		**T_IDM patients**				**Controls**			
		Total No. = 60				**Total No. = 50**			
		≤10 years No. = 36		**>10 years No. = 24**		**≤10 years No. = 21**		**>10 years No. = 29**	
Age range		Mean	SE	Mean	SE	Mean	SE	Mean	SE
		6.74	1.12	13.11	2.68	7.62	1.66	13.9	2.58
Sex	**Males**	No	%	No	%	No.	%	No.	%
		13	36.1	15	62.9	9	42.9	16	55.2
	Females	23	63.9	9	37.5	12	57.1	13	44.8

3.1.2 Healthy Control Group

For the purpose of comparisons, 80 healthy control subjects matched for age (4-17 years old), sex and ethnic back ground (Iraqi Arabs) were selected who have no history or clinical evidence of type I diabetes or any chronic disease and obvious abnormalities were selected as a control group for HLA typing. Out of these 80 controls, 50 healthy subjects (25 males and 25 females) were randomly selected for further investigations which include: phenotypic and functional characterization of peripheral blood lymphocytes (PBLs), humoral mediators, cytokine measurement in serum and other hematological tests(Table 3-1)

3.1.3 Siblings Group

Fifty healthy siblings of type I diabetic patients were available for investigation of HLA-typing including (25 males and 25 females). Their ages range form 3 to 16 years.

Patients as well as control group were subjected to questionnaire shown in appendix I, which include detailed history related to DM, and through clinical examination in conjugation with various relevant laboratory investigations to delinate the aetiological factors associated with this disease.

3.2 Collection of Blood Samples

Ten to fifteen ml of venous blood was collected from each patient as well as siblings and controls. The aspirated blood was immediately transferred into different test tubes. Two ml of blood was added in EDTA tube (1.5 mg / ml) for HbA1c estimation. Eight ml of blood was put in heparinised test tube (10 U/ml) used for lymphocyte separation for the detection of HLA polymorphism, lymphocyte proliferation, and lymphocyte phenotyping to detect CD markers. Heparinised blood was processed as soon as possible. When immediate processing was not possible, samples were left overnight in an incubator at room

temperature (20-22°C), however the interval between bleeding and processing never exceeded 18 hrs (Biotest data sheet, 1989).

The remaining blood was collected into plain test tubes, then the serum was separated by centrifugation at 2500 rpm for 10 min, divided into aliquot and kept at-20°C for the evaluation of other parameters(Johnstone and Thrope, 1987).

3.3 Materials

3.3.1 Instruments

Instrument	Company (Origin)
Cell-Dye-1700 with printer	Abbott diagnostics (USA)
Autoclave	AMSCO (USA)
Variant (Hemoglobin testing system)	Bio-RAD (Germany)
pH meter	Corning (Germany)
Tray viewer device, Magnetic stirrer, magnetic bar	Electronic (UK)
Printer for microculture plates	Epson (UK)
Incubator	Fisher Scientific (USA)
Improved Neubauer counting chamber	Haemocytometer (Germany)
Hamilton multichannel syringe holding 50 µl each and delivering 1 µl at one time. Hamilton microtiter syringe of 50 µl and 250 µl delivering 1 µl and 5 µl at each time respectively	Hamilton (USA)
CO_2-Incubator	Heraaus (UK)
Moisture chamber	Iraq
Horizantal or orbital shaker >280 rpm	Labcoo (Germany)
Water bath, Oven	Memmert (Germany)

Centrifuge-minor 35	MSE (Germany)
Light microscope, Fluorescent microscope	Olympus (Japan)
Automatic micropipette, precision adjustable with different sizes	Salmed (Germany)
Laminar flow cabinate	San EI, Seisakusha , Ltd
Refrigerated centrifuge	Sorvall (USA)
Vortex mixer	Stuart Scientific (UK)
Microculture plate washer, Microculture plate reader with different filters.	Thermo-electron (UK)
Gamma Counter	Wallac-Wizard (Germany)
Phase contrast inverted microscope	Zeiss (Germany)

3.3.2 Chemicals

Chemical	Company (Origin)
Ammonium chloride (NH_4Cl); Acetone; Acetic acid analar (CH_3COOH); Disodium hydrogen phosphate (Na_2HPO_4); Ethylene diaminetetra acetic acid (EDTA); Formaldehyde solution analar 37-40% w/v; glycerol; isopropanol; magnesium chloride ($MgCl_2$); Potassium dihydrogen phosphate (KH_2PO_4); potassium hydrogen carbonate ($KHCO_3$); Potassium chloride (KCl); Sodium hydrogen carbonate ($NaHCO_3$); Sodium chloride (NaCl); Sodium carbonate anhydrous (Na_2CO_3); Tris-base (hydroxymethyl amino methane); Tris-sodium citrate; HCl; H_2SO_4	BDH (England)
Substrate Solution (tetramethyl benzidine TMB / H_2O_2)	Biomaghreb (Tunisia)
Roswell park memorial institute-1640 medium (RPMI-1640) with L-glutamine	Euroclone (UK)
Eosin stain; Trypan blue stain	Fluka chemical (Switzerland)
Ficoll-Isopaque separation fluid-lymphoprop specific gravity 1.077; HEPES powdered; Liquid paraffin; specific gravity 0.87-0.89	Flow-Laboratories (UK)
(MTT)1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide	Sigma (Germany)

3.3.3 Sera, Antisera and Biological Materials

Material	Company (Origin)
HLA-antisera and control sera reconstituted with equivalent volume of diluent; Lyophilzed rabbit complement reconstituted with diluent before use.	Biotest (Germany)
Human reference sera for immunoglobulin IgA, IgG, IgM and complement C_3 and C_4; Endoplates (12 wells) for immunoglobulins and Complement (agarose gel containing monospecific antisera), RID plates.	Biomaghreb (Tunisia)
Common antibody diluent	BioGenex Laboratories (California, USA)
Human AB Serum (Pooled)	Baghdad Blood-Transfusion Center
Fetal Calf Serum (FCS)	Flow laboratorises (UK)
• Lyophilized Adeno KBR-CF antigen type 3, 4, 7, Vero; • Lyophilized Coxsackie type B_5, KBR-CF antigen Vero; • Lyophilized human positive adenovirus serum • Lyophilized human negative adenovirus serum • Lyophilized human positive coxsackie B_5-serum • Lyophilized human negative coxsackie B_5-serum	Institute Virion (France)
Anti-human IgG (γ-chain specific) with horse-reddish peroxidase (HRP) conjugate; Bovine serum albumin (BSA)	Sigma (Germany)
Mouse antihuman CD_3, CD_4, CD_8, CD_{19}, $CD_{45}RA$, $CD_{45}RO$, CD_{56}, CD_{38} HLA-class II monoclonal antibodies (mcAbs). All the mcAbs were purified IgG conjugated to fluorescein isothiocynate isomer-1 (FITC).	Serotec (UK)
Polioral Trivalent; Chiron, S.P.A.	WHO, Aventis

3.3.4 Antibiotics

Antibiotic	Dose	Company (Origin)
Crystalline penicillin	1,000,000 IU	Pharma-intersprl (Belgica)
Streptomycin Sulfate	1 gm	

3.3.5 Kits Used

i. Human IFN-γ ELISA Kit: (Immunotech-Beckman Coulter)

This kit was intended for the quantification of hIFN-γ in plasma, serum or culture supernatants. The kit consists of:

- Microculture plate with 96 wells coated by hIFN-γ.
- Lyophilized standard.
- Biotinylated monoclonal antibody.
- Streptavidin HRP conjugate.
- Diluent.
- Washing solution.
- Substrate; (TMB / H_2O_2)

ii. **Human IL-10 ELISA Kit; (Mabtec)**

hIL-10 kit was intended for quantitative determination of native and recombinant hIL-10 in serum; plasma and cell culture supernatant. It consists of:

- hIL-10 mcAb 9D7 concentration: 1 mg/ml.
- Biotinylated mcAb 12G8 concentration 1 mg/ml.
- Streptavidin-Alkaline phosphatase (strepavidin-ALP).
- Recombinant hIL-10 standard.
- Substrate p-nitrophenyl phosphate (PNPP) tablets.

iii. Human IL-6 ELISA Kit; (Mabtec)

hIL-6 kit was used for the quantitative determination of native and recombinate hIL-6 in serum; plasma and cell culture supernatent. It consist of the following:

- hIL-6 mcAb 13A5, concentration: 1mg /ml
- Biotinylated mcAb 39C3, concentation: 1mg /ml
- Streptavidin- ALP
- Recmbinant hIL-6 standered
- Substrate (PNPP) tablets

iv. C-peptide IRMA Kit: (Immunotech. Beckman Coulter Company)

C-peptide kit is immunoradiometric assay for the *in vitro* determination of C-peptide in human serum, plasma and urine.The kit consists of:

- Anti-C-peptide mcAb coated tubes.
- ^{125}I-labeled monoclonal anti-C-peptide antibody.

  ^{125}I-labeled immunoglobulin in buffer containing BSA and sodium azide <0.1% and a dye.
- C-peptide dilution buffer (Contains BSA and sodium azide <0.1%).
- Calibrators, six vials contain from 0 to 6400 PM of C-peptide in buffer.
- Control sera: two vials contain C-pepetide lyophilized in BSA and sodium azide.
- Washing solution.

v. Anti-GAD IRMA Kit: (Immunotech. Beckman Coulter)

Anti-GAD kit is immunoradiometeric assay for the quantitative determination of anti-GAD autoantibodies in human serum. The kit contains:

- Protein A suspension; lyophilized in buffer containing BSA and sodium azide.
- ^{125}I-labeled GAD; the vial contains 50 KB9 of radiolabeled recombinant lyophilized GAD.
- Standards; seven vials contain from 0 to 300 U/ml of anti-GAD antibodies in human serum and sodium azide <0.1%.
- Control samples: Two vials contain anti-GAD antibodies in human serum and sodium azide.
- Assay buffers: contains buffer with BSA and sodium azide.

vi. Variant Hemoglobin A1c Testing System. (Bio-RAD Diagnostic Group. Hercules)

It was used to improve quantification of HbA_{1c} using ion exchange high-performance liquid chromatography (HPLC). It consists of:

- Sample vials (1.5 ml volume).
- Buffer 1: Sodium phosphate buffer pH: 5.9, contains <0.1% sodium azide as a preservative.
- Buffer 2: Sodium phosphate buffer pH: 5.6, contains <0.1% sodium azid as a preservative.
- Whole blood primer: Ten vials of lyophilized human red blood cells hemolysat with gentamycin: tobramycin and EDTA as preservative.
- Wash solution: Deionized distilled water with <0.05% sodium azide as preservative, pH: 6.6.
- Hemolysis Reagent: Citrate solution; pH: 5.0 contains <0.05%, sodium azide as a preservative.
- Hemoglobin A1c Calibrator / Diluent Set, it consist of:
    - ♦ Six vials of lyophilized human red blood cell hemolysate containing gentamycin, tobramycin and EDTA as preservative.

- Diluent containing deionized distilled water plus EDTA and potassium cyanide as preservatives, pH: 7.2

3.3.6 Solution and Buffers

i. Physiological Saline (Nile company)

The solution was readily prepared, (Nile Company)

ii. Phosphate Buffer Saline (PBS) (Johnstone and Thrope, 1987)

Phosphate buffer saline was prepared by dissolving the following materials in 1L distilled water.

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

pH was adjusted to be 7.2-7.4, then the solution was autoclaved (121°C, 15 pound/In^2 for 20 minutes) stored in the refrigerator (4°C) till used.

iii. Solutions and Stains for HLA Typing

a. Trypan Blue Stain Solution (2%)

Two grams of trypan blue stain powder was dissolved in 50 ml physiological saline, then the volume was completed to 100 ml. Stain was filtered before use .

b. Eosin Stain Solution (5%) (Metcalf *et al.*, 1986)

Five grams of eosin Y powder were dissolved into 50 ml of physiological saline. Then the volume was completed to 100 ml, mixed well and placed in shaking water bath at 70°C for 30 min.. Later it was filtered by filter paper, dispensed into 5 ml volume and stored at 4°C.

c. **Washing Solution for Lymphocytes**

Containing 5 ml of heat inactivated (56°C for 30 min) FCS and 95 ml of RPMI culture medium.

iv. Solutions Used for Lymphocyte Phenotyping

a. Fixative Solution for Direct IF Technique (Johnstone and Thrope, 1987)

Buffered Formal Acetone (BFA), prepared as follows:

PBS (3.2.6.2)	8 ml
Distilled Water	38 ml
Formalin 37-40%	33.2 ml
Acetone	60 ml

The solution was stored in the refrigerator at 4° until use.

b. Common Antibody Diluent: (BioGenex Laboratory, USA)

Contains Tween-20 in PBS, pH: 7.4 and preserved in 1% BSA plus 0.1% sodium azide-NaN_3 solution. Used for dilution of CD markers.

c. Mounting Media (v/v):

Glycerol 7: Phosphate buffer 3.

v. Culture Media and Solutions Used for Lymphocyte Proliferation

a. Preparation of Antibiotic Solutions (Hudson and Hay, 1989)

It is prepared by dissolving the antibiotics (section 3.3.4) in 100 ml of sterile (DDW) then dispense into sterile 10 ml containers and stored refrigerated at 2-8°C.

b. HEPES Buffer (N-2-hydroxyethyl piperazine-N-2 ethane-sulphonic acid) (Hudson and Hay, 1989)

It is a biological buffer effective in reducing pH flactuation over the pH range 7-8 and to keep minimal cytopathic effects without effecting the normal physiological osmolality range (250-325 mOsm / Kg). The optimal buffer system is 25 mM to keep pH range 7.2-7.4 at 37°C which was prepared by dissolving 0.6 g in 100 ml DDW and then added to tissue culture as 1 / 100 ml of tissue culture medium .

c. Preparation of RPMI-1640 Complete Culture Medium (Work and Burdon, 1980)

It was prepared by measuring out 900 ml DDW at room temperature and while gently stirring the water, 10.4 g RPMI-1640 powdered medium was added with 2 g of analar sodium bicarbonate and stirred until dissolved. The original package was rinsed with small amount of water to remove all traces of powder and was added to solution. Then a 10 ml of antibiotic solution and 10 ml HEPES buffer solution were added and the final volume was brought to 1 liter with DDW then sterilized immediately by filtration using a membrane with a porosity of 0.22 µm. Then 100 ml of filtered heat-inactivated human AB serum (section 3.3.6.5.) was added. Sterilization was checked by incubation of whole medium in incubator at 37°C for 24 hrs, then aseptically dispensed aliquots in sterile containers and stored at -20°C until used.

d. Red Blood Cells Lysis Buffer (Johnstone and Thrope, 1987)

$KHCO_3$	10 mM
NH_4Cl	0.155 M
EDTA	0.1 mM

The gradients were mixed, autoclaved at 115°C for 10 min, stored in 4°C.

e. Concavalin-A (Con-A) Working Solution (10 mg/10ml)

Ten mg of Con-A was dissolved in 5 ml physiological saline. The volume was complete to 10 ml. Filtered by 0.45 millipore and stored at 4°C.

f. MTT Working Solution (5 mg/ml)

MTT powder was dissolved in PBS and filtered by 0.45 millipore to sterilized and remove a small amounts of insoluble residue present in some batches of MTT, then stored at 4°C.

(Note: the MTT solution is prepared just prior to culture application).

g. Acidic Isopropanol

Acidic isopropanol was prepared by supplement absolute isopropanol with 0.04 N HCl.

h. Human AB Serum (Schendel *et al*., 1997)

Human AB serum was obtained from blood bank and centrifuged at 4000 rpm for 20 min. Carefully the serum was removed from the upper layer without disturbing the sediment. Then distributed into sterile 50 ml conical flasks, and incubated for 40 min in a 56°C water bath. The tubes were allowed to cool to room temperature and store at -20°.

vi. Buffers Used for ELISA (Johnstone&Thrope, 1987)

a. Coating Buffer

Na_2CO_3	1.59 g
$NAHCO_3$	2.93 g
D.W.	900 ml

Then complete the volume to 1L, pH was adjusted to 9.6

b. Washing Buffer: pH: 7.4

1. PBS containing 0.1% (v/v) Tween-20.
2. PBS containing 0.05% (v/v) Tween-20.

c. Blocking Buffer

PBS containing 0.1% (w/v) BSA

d. Incubation Buffer

A. PBS containing 0.1% (v/v) Tween-20 and 1% BSA.

B. PBS containing 0.05 % (v/v)Tween-20 and 0.1% BSA.

e. Substrate Buffer; Magnesium Tris Buffer (MTB)

Tris-OH	50 mM
$MgCl_2$	2 mM

pH adjusted to 8.6, store in refrigerator 4°C.

3.4 Methods:

3.4.1 Hematological Tests

3.4.1.i Determination of Glycosylated Hemoglobin HbA1c

- **Principle**

The VARIANT HbA1c program utilizes the principles of ion-exchange high-performance liquid chromatography (HPLC) for the automatic and accurate separation of HbA1c. The separation of HbA1c is performed rapidly and precisely without interference from labile A1c lipemia or temperature fluctuations.

The **VARIANT's** two dual-piston pumps deliver a programmed buffer gradient of increasing ionic strength to the system. Prepared samples are automatically injected into the analytical flow path and applied to the cation exchange column, where the Hb is separated based upon the attraction of the Hb to the column material. The separated Hb then passes through the flow cell of the filter photometer where changes in the absorbance (415 nm) are measured, and back ground variations are corrected by an additional filter at 690 nm.

A chromatogram (graph) of the changes in the absorbance is plotted versus the retention time. Each chromatgram printout is accompanied by a report

identifying each peak detected, plus the relative percent and retention times of each peak.

- **Procedure: "According to the manufacturing instructions"**

A. Preparation of Reagents

1. Allow all reagents, except for the calibrator diluent, to reach room temperature (15-30°C) before performing the assay.
2. Reconstitute the lyophilized HbA1c calibrator with 10 ml of cold calibrator diluent (section 3.3.5.6), allow standing for 5-10 minutes, swirl gently to dissolve.
3. In each run, diabetic and non-diabetic control specimens should be included. Add 0.5 ml of DDW to reconstitute the lyophilized controls, let stand 2-3 min. and then diluted 1:200 prior to analysis.
4. Use a fresh aliquot of whole blood primer at the beginning of each run by adding 1 ml of DDW to the vial, swirl gently and allow to stand for 10 min at 15-30°C.

B. Sample Collection and Preparation

1. The whole blood specimens should be collected in a vacuum collection tube containing EDTA, and should be thoroughly mixed.
2. Prepare two vials for HbA1c calibrator by adding 500 μl of reconstituted calibrator in each one.
3. Use two vials for the Hb control.
4. Add 1 ml of Bio-rad hemolysis reagent to each control and patient sample vials.
5. Remove 5 μl of whole blood of the patient or reconstituted control. Carefully wipe the pipette tip to remove excess sample. Dispense it into the bottom of the patient sample vial or control vial to make the dilution 1:200.

6. Cap or parafilm, each sample and vortex to mix thoroughly.
7. Let samples stand at 18-28°C for at least 15 min. Do not allow samples to stand for more than one hour.
8. Place the sample tray and cover into the sample compartment.
9. Select the HbA1c program.

- **Interpretation of the Result**

The following HbA1c ranges might be used for interpretation of results. However, factors such as duration of diabetes, adherence to therapy and the age of the patients should also be considered in assessing the degree of blood glucose control (Table 3-2).

Table (3-2): HbA1c ranges and the degree of blood glucose control according to American Diabetes Association (ADA), (2004)

HbA1c %	Degree of glucose control
>8	High risk of developing long-term complications such as retinopathy, nephropathy…etc. Action suggested depends on individual patient circumstances.
<7	Target ADA
<6	Non-diabetic level.

3.4.1.ii Determination of C-peptide in Human Serum

- **Principle**

The IRMA of C-peptide is a "sandwich" type assay. Mouse mcAbs directed against two different epitops of C-peptide and hence not competing are used. Serum samples, the controls and calibrators are incubated in tubes coated with the first mcAb in the presence of the second mcAb labeled with iodine 125. After incubation, the contents of the tubes are rinsed so as to remove unbound

^{125}I-labeled antibody. The bound radioactivity is then determined in a gamma counter.

- **Procedure "According to the manufacturing instruction"**

A. Let all the reagents come to room temperature. Reconstituted the contents of the six calibrator vials which contain from 0 to 6400 pm of C-peptide (section 3.3.5.4) with the volume of distilled water indicated on the label, also the two control sera, wait for 10 min and mix gently.

B. Assay procedure:

1. Step 1: Additions

To coated tubes, add 50 µl of calibrator, control or sample and 150 µl of tracer, mix.

Note: Add 150 µl of tracer to 2 additional tubes to obtain total CPM.

2. Step 2: Incubation

Incubate 2 hours at room temperature (18-25°C) with shaking (>280 rpm).

3. Step 3: Counting

Aspirate carefully the contents of tubes (except the 2 tubes "total CPM"). Wash twice with 2 ml of wash solution, and count activity (CPM) for 1 min.

- **Interpretation of the Result**

Results are obtained from the standard curve by interpolation for standard curve. Put the determined radioactivity (CPM std–CPM std0) value on vertical axis and the C-peptide concentration of the calibrators on the horizontal axis (PM) (Figure 3-1).

Note: To convert pmol/L into ng/ml, the results were multiplied by 0.003.

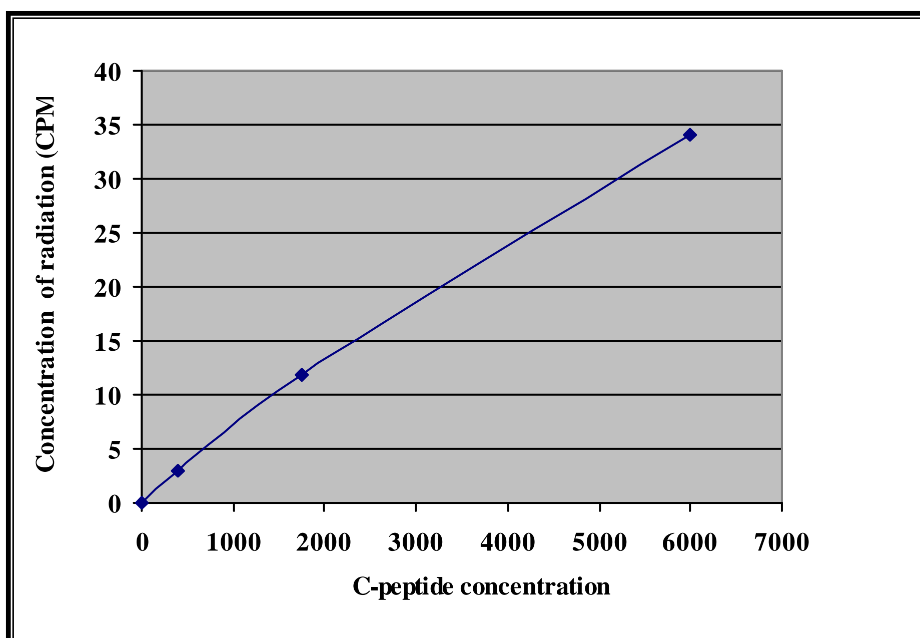

Figure (3-1) Standard curve for serum C-peptide (pmol/L)

3.4.2 Serological Typing of HLA Antigen

This test was carried out in the tissue typing laboratory of the Al-Karama Hospital in Baghdad.

Five ml of venous blood were drawn from each subject (patients, siblings and controls). The collected blood was dispensed into plastic or glass universal tubes containing heparin (10 IU/ml) as anticoagulant. The test microcytotoxicity was established by Terasaki and McClelland, (1964).

♦ **Principles:**

Microlymphocytotoxicity assay is a complement dependent reaction based on the reaction of HLA anti-sera which recognize the correspondent membrane bound antigen on the viable human lymphocytes in the presence of rabbit's complement.

If the antigen under test is on the lymphocytes, the formed antigen-antibody complexes will activate the added complement, results in death of the

reacted cells which permit absorption of an indicator dye (eosin) to score the reaction and determine the HLA phenotypes. Stained lymphocytes = positive reaction, unstained lymphocytes = negative reaction.

- **Setting up Typing Plates**

These were prepared in advance in batches of 50 or 100 plates. The Terasaki plates (60 wells) were filled with neutral oil (liquid paraffin) up to brim. By using a 5o μl of Hamilton multisyring, 1μl of each HLA-antisera was dispensed into each well. Then the plate was labeled and stored at -70°C until used. Positive and negative control sera were included in each plate. The wells contained antisera specific for HLA-class I (A, B and C) and class II (DR and DQ) antigens, which were available in the Histocompatiblity Laboratory at the Al-Karama Hospital (Appendix-II).

- **Isolation of Lymphocytes**

The Ficoll-Isopaque technique originally described by Boyum (1968) that was reported in Schendel *et al*. (1997) has been used for separation of lymphocytes with greatest apparent success. The density gradient centrifugation depends upon the specific gravity of a gradient, made by lymphoprep (a commercially prepared mixture of sodium metrizoate and Ficoll, density 1.077 g/L).

3. Five ml of heparinised blood were mixed with equivalent volume of serum free culture RPMI-1640 medium.
4. By using 10 ml centrifuge tube, 5 ml of diluted blood were carefully layered over 3 ml of lymphoprep taking care not to disturb the interphase. Pipetting blood into or under the lymphoprep solution can be avoided by holding the tube at 45° angle and allowing it to flow slowly down the side of the tube.
5. The centrifuge tubes were capped tightly to assure sterility and carefully balance them so that no shaking occurs during centrifugation.

6. Centrifuged by using temperature controlled centrifuge (18-20°C) for 20-25 min to give relative centrifugal force (rcf) at the interphase of about 700 g (2100 rpm).

5. After centrifugation the lymphocytes were visible as a distinct cloudy band between the serum components in the upper fraction and the lymphoprep solution of the lower fraction. Red cells and other leucocytes are found in the pellet (Figure 3-2).

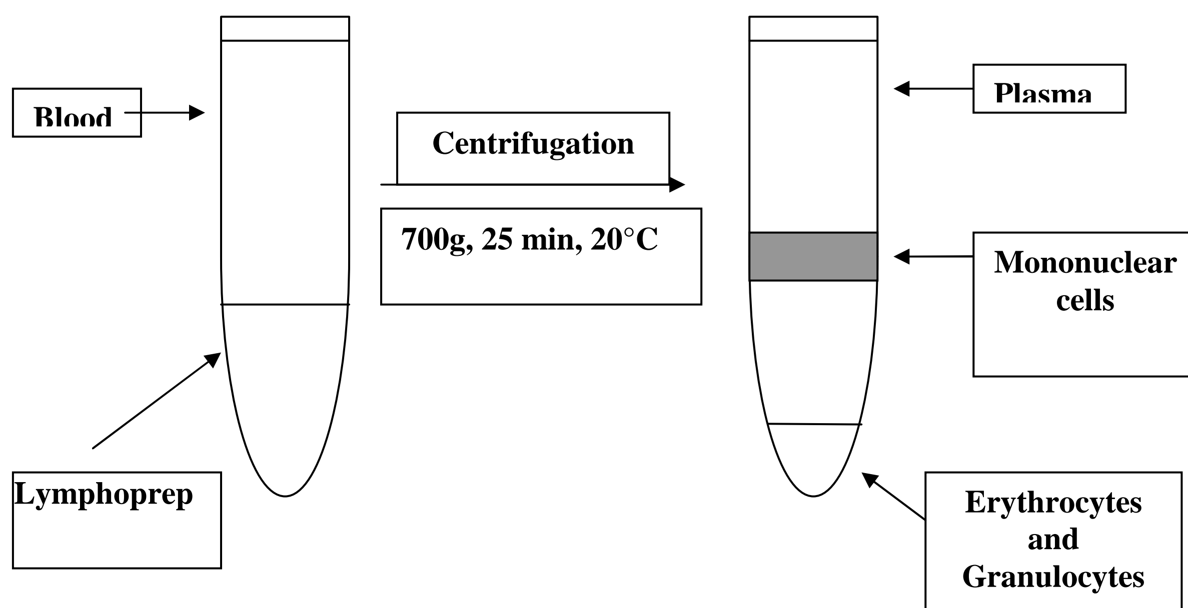

Figure 3-2: Isolation of mononuclear cells on lymphoprep adapted from Schendel *et al*., (1997)

6. The band at the interface was collected by means of a sterile Pasteur pipette and transferred into 10 ml test tube containing at least three times the volume of washing medium (section 3.3.6.iii.c) and suspended evenly. Then lymphocyte suspension was centrifuged for 5 min at 2000 rpm. This step was repeated once again.

7. The supernatant was discarded and the cells were resuspend in washing media. A third wash was also done but at lower speed (1000 rpm) for 10 min. This step is very important; it helps to maintain most platelets in the supernatant.
8. The cells were resuspended in 2 ml warm RPMI-1640 supplemented with 10% heat inactivated human type AB serum.

Note: Separating monocytes from lymphocytes is best done before gradient centrifugation, using whole peripheral blood or leukocyte-rich plasma. The method depends on the adherence of monocytes to glass surfaces (Slides, a flask with flat bottom or petridishes) for 45-60 min at 37°C, which yield lymphocytes preparation of high purity 90% (Rowlands *et al*., 1994).

♦ **Counting of Lymphocytes and Determining Their Viability**

Ten µl of cell suspension was added to 10 µl of trypan blue stain solution (section 3.3.6.iii.a) in a small tube. After 3 min, cells were counted in improved Neubauer counting chamber. Count the number of lymphocytes in five large squares (80 small sequares), for each sample (a minimum of 100 cells).

The final lymphocyte concentration / ml = number in five large squares x 10^5.

Live cells can exclude trypan blue dye; therefore viability was determined according to the following equation (Johnstone and Thrope, 1987):

$$\text{Lymphocyte viability}\,(\%) = \left[\frac{\text{Number of viable cells}}{\text{Sum of viable and dead cells}}\right] x100$$

The viability accepted should be 95% and above. The final lymphocyte concentration was adjusted to 2-3x10^6 cells/ml.

♦ **Red Blood Cells (RBCs) Lysis**

A few individuals have lymphocytes with altered density that do not separate normally on the density gradients. RBCs lysis technique was found necessary because that RBCs are the major contamination with isolated lymphocytes. RBCs lysis buffer (section 3.3.6.v.d) was used for further purification of lymphocytes although the lymphocytes may be damaged to some extent either by the lysis buffer or the massive concentration of hemoglobin that is released (Johnston and Thrope, 1987).

1. The lymphocyte suspension was centrifuged at 300 g for 5 min., the supernatant was discharded and the pellet resuspend in 1 ml of lysis buffer and incubated at room temperature for 5 min.,then centrifuged at 300 g for 10 min.
2. The supernatants was discarded.
3. The cell pellet was washed twice by resuspension in washing media (section 3.3.6.iii.c) and centrifuged at 300 g for 5 min.
4. The procedure was repeated if the red cell contamination is still too high

Note: Contamination by RBCs and granulocytes was normally less than 3% as confirmed by Ad'hiah,(1990).

♦ **Separation of Lymphocytes into B and T Cells**

HLA DR and DQ antigens have a limited tissue distribution, being expressed mainly on the surface of B lymphocytes. Thus B lymphocytes are the best for the serological detection of HLA-DR and DQ antigens, so there is a requirement to have a cell suspension rich in B-cells.Nylon wool is one of the main methods that can be used to separate B and T cells from one another due to surface membrane properties of B-cells, by which it could adhere to the nylon wool fibers (positive selection) whereas T-cells can be easily washed off. This separation method allows B-cells enrichment to 70-80% in the samples prepared. The method is outlined in the following steps as described by Danilous *et al*., 1990).

Two ml disposable plastic syringe was loosely filled with approximately 0.15 g nylon wool. The nylon wool was rinsed with 10 ml physiological saline solution then with 10 ml of warmed (37°C) washing medium (section 3.2.6.iii.c).

1. Both ends of the syringe were sealed immediately with parafilm and left for 30 min in incubator at 37°C.
2. Lymphocyte pellet were resuspended in 1 ml warm medium and immediately poured into prepared syringe and allowed to be absorbed into the nylon wool completely. Both ends of the syringe were sealed with parafilm again and incubated for a further 30 min at 37°C.
3. After incubation; non-adherent T-cells were washed out of the syringe with 10 ml of warm medium and collected in silicon coated glass tube.
4. The nylon-wool adherent B-cells were isolated by adding a warm washing medium (10 ml) to the syringe-barrel and the nylon wool was squeezed by the syringe piston. This step was repeated 5-8 times adding 2 ml of medium to the syringe each time.
5. B and T cells suspension were collected separately and centrifuged twice at 1000 rpm for 10 and 5 min respectively.
6. Cells number was adjusted to 2-3x10^6 cells/ml.

T-cells can now be used in the phenotyping of HLA-class I (A, B and C) antigens and B-cells are used for phenotyping of class II (DR and DQ) antigens .

Microlymphocytotoxicity Assay

The same procedure was used for both HLA-class I and class II typing except a prolonged incubation period that was necessary for class II typing. Before serological typing, 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 (25°C) for 15 min.

1.One µl of lymphocyte suspension (2000-3000 cells) was dispensed in each well using a 50 µl Hamilton syringe attached to repeating dispenser. The plates were incubated at 20-25°C for 30 min for HLA class I and 60 min for HLA calls II antigens.

2.Rabbit complement was thawed rapidly, immediately before use, and held on ice. Five µl of complement were added to each well followed by further incubation at 20-25°C for 60 min and 120 min for HLA class I and class II antigens respectively.

3.Four µl of eosin stain solution 5% (section 3.2.6.3.) were added to each well and left for 5 min.

4.The reaction was fixed by adding 10 µl of formaldehyde solution. This is prepared by adjusting the pH of the stock 37% solution by shaking with sodium bicarbonate powder until the pH is 7.2. A layer of mineral oil was added to prevent contact with air and help stabilize the pH of the solution. The Plate was kept at 4°C, ready to be read within 1-24 hrs using inverted phase contrast microscope.

♦ **Evaluation of the Reactions**

Viable cells were light and shining (negative reaction) while dead cells appear dark and larger (positive reaction). Each well was scored according to the percentage of cell death (percentage of eosin stained cells). The score range was as follows (Stocker and Bernoco, 1979):

Score	Percentage of cell death	Interpretation
1	0-19% dead cells	Negative reaction
2	20-29% dead cells	Doubtful negative reaction
4	30-49% dead cells	Weakly positive reaction
6	50-79% dead cells	Positive reaction
8	80-100% dead cells	Strongly positive reaction
0	Not readable	Invalid test

3.4.3 Lymphocyte Subtyping for CD Markers

Phenotyping of surface antigens of PBL of both patients and controls was done by direct IF technique using specific fluorescein labeled mcAbs (conjugate). Phenotyping was accomplished by two main types:

A. Fixation of PBL

The fixation procedure dissolves and removes some of the lipids, so that all of the cellular proteins are accessible to added antibodies. In addition, fixation is critical for cell adhesion to IF-slides and to be sticky apart from the frequent washing steps later (Thompson, 1978).

B. Labeling of PBL Surface Antigens (CD Markers)

In the present study, numerous CD markers were investigated including: CD_3 (pan T-lymphocytes), CD_4 (Th), CD_8 (Tc), $CD_{45}RA$ (Naive cells), $CD_{45}RO$ (Memory cells), CD_{19} (B-lymphocytes), CD_{56} (NK-cells), DR-antigen and other activated markers like CD_{38}.

The method of IF-labeling of fixed cells was done as described by Wigzell and Anderson (1971).

- **Principle**

The conjugated antibody (mcAb) is applied to the PBL on the slide and allowed to incubate, then the slides are washed well in buffered saline and after being mounted in glycerol is viewed under the microscope with appropriate illumination, as illustrated in figure (3-3).

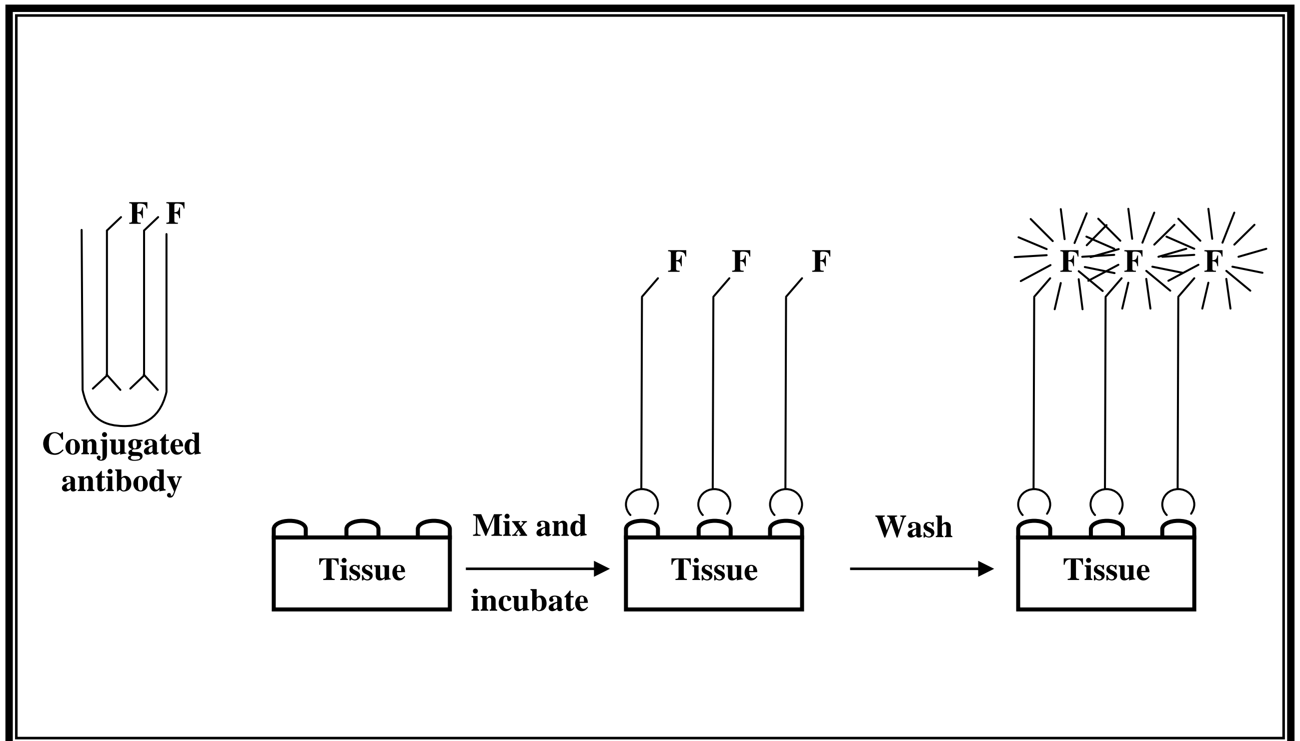

Figure 3-3: Illustrates the principle of the direct IF technique(adapted from Thompson, 1978).

- **Preparation of Slides and Fixation**

1. The isolated lymphocytes prepared in (section 3.4.2.) were adjusted to a cell count of $1x10^6$ cell / ml in RPMI-1640 medium, cells should be >95% viable as determined by trypan blue dye exclusion. Then 10 µl per well on IF-slides was added. Samples were allowed to dry completely at room temperature.
2. Slides were dipped into BFA fixative (section 3.2.6.iv.a) for 1-3 min, left to dry completely at room temperature.

3. Slides then were checked with a microscope for even spread of cells, then covered with aluminum foil and stored at -20°C till assayed.

- **Labeling for Direct IF Test**

1. The IF-slides pre-coated with lymphocytes were removed from freezer, allowed to reach room temperature, unwrapped and washed with PBS prepared in (section 3.3.6.ii) by dipping into PBS-filled jar with stirring for 10 min.
2. Slides were lain flat section side up in humidity chamber, then 10 µl of a specific anti-CD marker antibody (FITC labeled mcAb) at 1:5 dilution with common antibody diluent (section 3.3.6.iv.b) were added to each well. The chamber was covered , and the slides were left undisturbed in incubator at 37°C for 2 hrs (37°C increases sensitivity without increasing background staining) (Serotec Data Sheet, 1999).
3. Slides, then were transferred to staining jar filled with PBS at room temperature and PBS was replaced twice at 5 min intervals.
4. One to two drops of mounting media (section 3.3.6.iv.c) was added to each well to enhance fluorescence, prevent dehydration and prevent fading of mcAbs on exposure to UV-light. Then cover slips were lowered into place slowly to avoid bubbles. Slides were ready for examination with IF-microscope at 490 nm immediately or up to 3 days as a maximal duration.
5. Slides were viewed first by objective lense 40X, and the number of lymphocytes was counted in this field, then switched to the UV light and the number of the only stained cells was counted. This maneuver was repeated till 200 cells had been counted. Positive cells give green-apple color.

Calculation:

$$\text{Percentage of positively stained cells} = [\frac{\text{Number of labeled cells}}{\text{Total number of cells}}]x100$$

Note: The natural decomposition that occurs with inadequate storage and formalin fixation does not only destroy the antigen but also causes antibodies and similar complex proteins to adhere non-specifically throughout the tissue.

3.4.4 Lymphocyte Transformation Assessment by MTT Assay

- **Principle**

It is a rapid colorimetric assay for cellular growth and survival *in vitro*. The micorculture tetrazolium assay (MTT) was originally developed by Mosmann, (1983) to measure the conversion of soluble 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) into a blue or purple coloured formazan precipitate by the mitochondrial enzyme succinate-dehydrogenase (Denizot and Lang, 1986). The assay detects living, but not dead cells. Depending on this principle, the more active viable cells, the more dehydrogenase enzyme is produced and the more tetrazolium formazan is formed. This method can therefore be used to measure the cytotoxicity of cytotoxic drugs on living cells which are exposed to cytotoxic agents and can also measure the proliferative activity of living cells when exposed to mitogenic substances (Mosmann, 1983; Shimoyama *et al*., 1989).

- **Objective of MTT Assay in this Study**

1. To measure the cell mediated immune (CMI) activity of PBL of diabetic patients in comparison with healthy controls when incubated with concavalin-A (con-A) (an extract of the jack bean) as a mitogens.
2. To study whether the different viral antigens including CVB5, poliovirus and Adenovirus have any association with proposed CMI activation or not after incubation with PBL of TIDM patients and healthy controls.

- **Procedure**

The test was done completely under sterile conditions. After preparation of PBL suspension (section 3.4.2) in complete RPMI-1640 medium supplemented with 10% heat inactivated human AB serum (section 3.3.6.v.h) which is suitable for cell culture, the final lymphocyte concentration was adjusted to 1-2x10^6 cells / ml.

1. One hundred µl of PBL suspension was added to each well of 96 flat-bottom microculture plates for each TIDM patient and healthy control and as following:
   - Triplicate wells for control negative.
   - Triplicate wells for Con-A.
   - Triplicate wells for CVB5.
   - Triplicate wells for poliovirus Trivalent vaccine.
   - Triplicate wells for adenovirus.
2. Incubated for 24 hrs at 37°C in a humidified 5% CO_2 incubator.
3. After incubation, 10 µl of each of the following mitogen and antigens were added:
   - Con-A (100 µg/ml) per well prepared from the working solution (section 3.3.6.v.e).
   - CVB5 antigen solution (1:5 dilution) per well.
   - Poliovirus Trivalent Vaccine (1:5 dilution) per well.
   - Adenovirus type 3,4,7 solution (1:10 dilution) per well.

Note: The final concentration or dilution for the last three viral antigens was achieved according to the result of MTT serial dilution run of these antigens which revealed that 1:5 dilution is the best for CVB5 and poliovirus trivalent vaccine, while 1:10 dilution is the best for adenovirus to exert its antigenic ability.

4. Incubated for 3 days at 37°C in a humidified 5% CO_2 incubator with once or twice addition of complete RPMI medium to the wells during the period of incubation to avoid evaporation.
5. At the end of incubation period 20 µl of MTT working solution (section 3.3.6.v.f) was added to each culture well and the culture were incubated for 4 hrs at 37°C.
6. Finally the cultured medium was removed from each well and the converted dye was solubilized by adding 100 µl of acidic isopropanol (section 3.3.6.v.g). The absorbency of each well was measured by microculture plate reader using a test wavelength of 570 nm (Mosmann, 1983) or 540 nm (Mizutani *et al*., 1994). Plates were normally read within 1 hr of adding isopropanol.

Note: Isopropanol is preferred than ethanol because of a reduced evaporation rate.

- **Interpretation of Readings**

The percent of proliferative response of lymphocytes due to exposure to mitogens or antigens was calculated by the following formula:

$$\%\ \text{Proliferation} = \left[\frac{\text{Absorbancy of experimental wells}}{\text{Absorbancy of control wells}} - 1\right] x100$$

MTT assay can measure the viability of cells and the cytotoxicity of cytotoxic agents on living cells (Mizutani *et al*., 1994).

The percent cytotoxicity was calculated by the following formula:

$$\%\ \text{Cytotoxicity} = \left[1 - \frac{\text{Absorbancy of experimental wells}}{\text{Absorbancy of control wells}}\right] x100$$

3.4.5 Serum Cytokines Assessment

3.3.5.i Detection of Serum IFN-γ by ELISA

♦ **Principle (The same principle for hIL10 and hIL-6)**

It is two immunological step sandwich type assays. In the first step, the IFN-γ is captured by a monoclonal antibody bound to the wells of a microtiter plate. In the second step a biotinylated antibody binds to the solid phase antibody-antigen complex and in turn binds the conjugate. After incubation, the wells are washed and the antigen complex bound to the well detected by addition of a chromogenic substrate. The intensity of coloration is proportional to the IFN-γ concentration in the sample or standard (Figure 3-4).

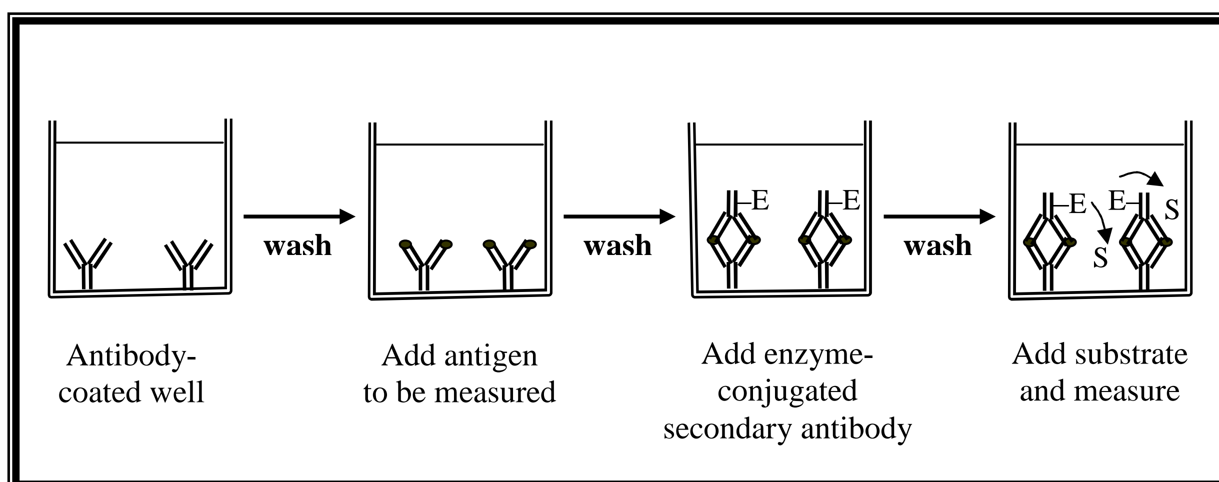

Figure 3-4: Illustrates the principle of the Sandwich ELISA technique which used for the determination of antigen qualitatively or quantitatively (adapted from Goldsby *et al.*, 2000).

♦ **Procedure "According to manufacturing instruction"**

Let the component of the kit (section 3.3.5.i) equilibrate 30 min at room temperature. Then reconstitute the lyophilized standard with 1 ml distilled water. This will result in a 250 IU/ml IFN-γ solution, with appropriate diluant, prepare a fresh dilution series of IFN-γ standard according to the instruction of manufacturer (25, 6.25, 1.56, 0.39 and 0 IU/ml).

1. Add 50 µl of each diluted standard and sample to the corresponding wells of micorculture plate coated by hIFN-γ. Incubated 2 hrs at 18-25°C while shaking.
2. Wash the wells three times with diluted washing solution using a microtiter plate washer.
3. Add 50 µl of biotinylated antibody and 100 µl of streptavidin-HRP conjugate, incubate 30 min at 18-25°C while shaking.
4. Wash the wells three times with diluted washing solution.
5. Add 100 µl of substrate chromogen solution, incubated 20 min at 18-25°C while shaking. Avoid exposing the substrate to direct sunlight.
6. The reaction was stopped by adding 50 µl of stopping solution in each well and the absorbance was read at 450 nm, using microculture plate reader.

♦ **Interpretation of the Results**

The sample results are calculated by interpolation from a standard curve that is performed in the same assay as that of the sample. The curve is drawn, plotting on the horizontal axis the IFN-γ concentration of the standard and on the vertical axis the corresponding absorbance (Figure 3-5). Locate the absorbance of each sample is located on the vertical axis and read off the corresponding IFN-γ concentration on the horizontal axis.

3.4.5.ii EILSA for Quantitative Determination of hIL-10

♦ **Procedure "According to the manufacturing instructions"**

1. Coat the 96-well micorculture plate with hIL-10 mcAb 9D7 (section 3.3.5.ii), diluted to 2 µg/ml in PBS, pH 7.4 (section 3.3.6.ii) by adding 100 µl/well. Incubate overnight at 4-8°C.
2. Wash twice with PBS.

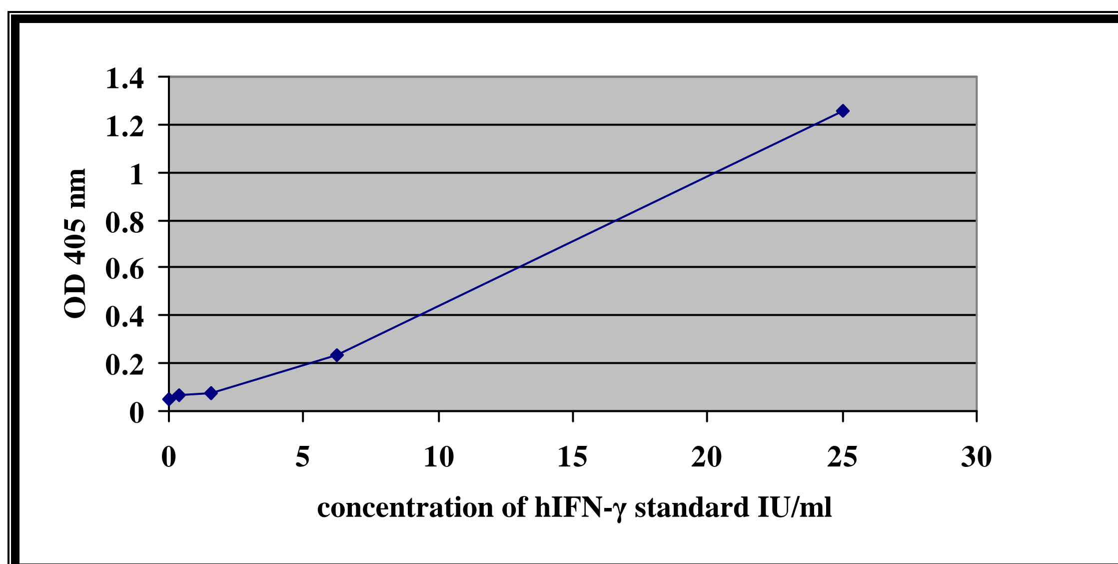

Figure 3-5: hIFN-γ standard curve.

3. Block plates by adding 200 µl/well of blocking buffer (section 3.3.6.vi.c) to block the unspecific sites. Incubate for 1 hr at room temperature.
4. Wash the wells five times with PBS containing 0.05% Tween (section 3.3.6.vi.b) using a microtiter plate washer.
5. Prepare hIL-10 standard by reconstituting contents of vial in 20 µl of 5 mM Tris, pH 8.2 to a concentration of 0.1 mg/ml. Dilute in PBS with 0.1% BSA section (3.3.6.vi.c) to make up a stock solution of 10 µg/ml. for the test prepare dilutions of the stock using the standard range (1000, 100, 10, 1 and 0.1 pg/ml) in incubation buffer (section 3.3.6.vi.d).
6. Add 100 µl/well of patient and control serum or standards for each dilution and incubate for 2 hrs at room temperature.
7. Wash as in step 4.
8. Add 100 µl/well of biotinylated mcAb 12G8 at 1 µg/ml in incubation buffer. Incubate for 1 hr at room temperature.
9. Wash as in step 4.

10. Add 100 µl/well of streptavidin-ALP diluted 1:1000 in incubation buffer. Incubate for 1 hr at room temperature.
11. Wash as in step 4.
12. Add 100 µl/well of substrate solution (PNPP), which is prepared by dissolving 1 tablet of (PNPP) in 5 ml of MTB substrate buffer, pH 8.6 (section 3.3.6.vi.e).
13. Measure the optical density (OD) at 405 nm for PNPP in an ELISA reader after suitable developing (usually within one hour).

♦ **Interpretation of the Results**

As mentioned in detection of serum hIFN-γ (section 3.4.5.i.). Figure (3-6) represents the standard curve of hIL-10.

According to the manufacturing instruction, standard range is 0.5-300 pg/ml and the limit of detection is 0.5 pg/ml.

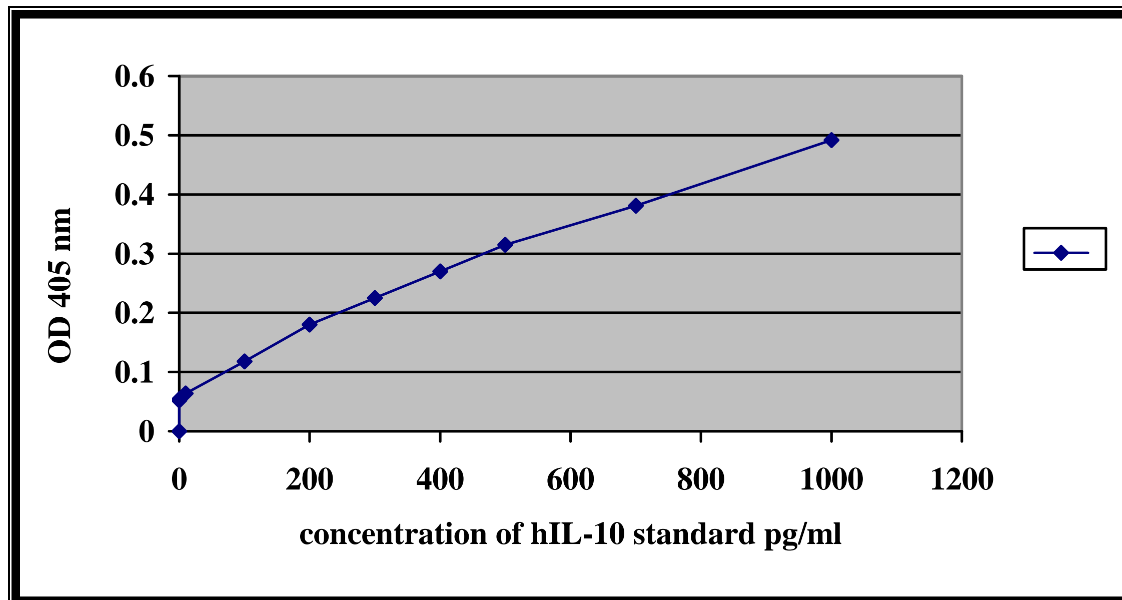

Figure 3-6: hIL-10 standard curve.

3.4.5.iii ELISA for Quantitative Determination of hIL-6

♦ **Procedure: "According to the manufacturing instructions"**

1. Coat the 96 microtiter plate with hIL-6 mcAb 13A5 (section 3.3.5.iii) diluted to 2 μg/ml in PBS pH 7.4 by adding 100 μl/well. Incubate overnight at 4-8°C.
2. Wash twice with PBS (200 μl/well).
3. Block plates by adding 200 μl/well of Incubation buffer (section 3.3.6.vi.d). Incubate for 1 hr at room temperature.
4. Wash the wells five times using washing buffer (section 3.3.6.vi.b).
5. Prepare hIL-6 standard by reconstituting contents of vial in 50 μl acetic acid (10 mM) to a concentration of 0.1 mg/ml. Dilute in solution (section 3.3.6.vi.d) to make up a stock solution of 10 μg/ml. For the test, prepare dilutions of the stock using the standard range (10000, 1000, 100, 10, 1 and 0.1 pg/ml) in incubation buffer.

The next steps were the same as mentioned in hIL-10 assessment (section 3.3.7.ii.).

♦ **Interpretation of the Results**

As listed in assessment of IFN-γ (section 3.4.5.i), Figure (3-7) represents the standard curve of hIL-6.

According to the manufacturing instructions, standard range is 13-1300 pg/ml and the limit of detection is 7 pg/ml.

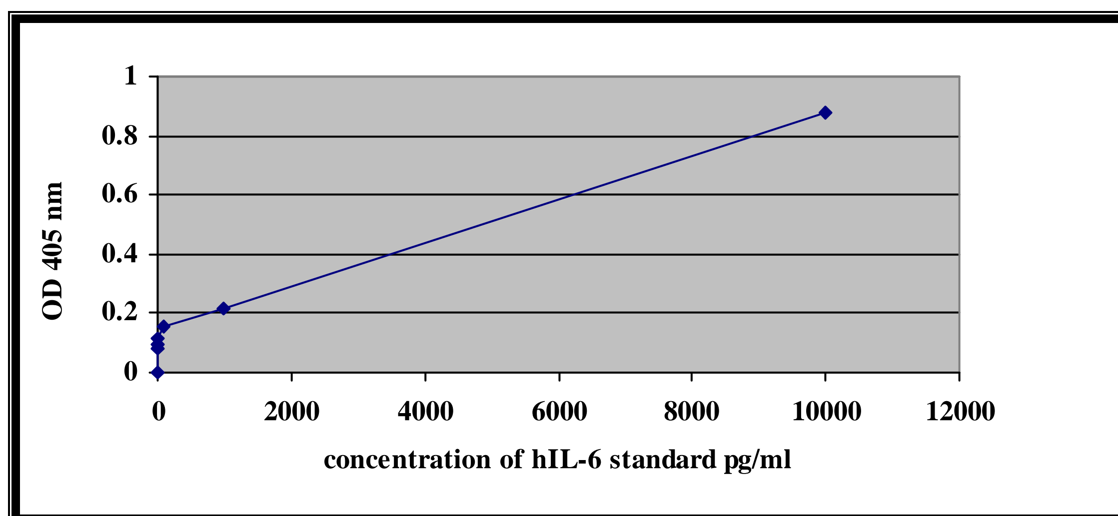

Figure 3-7: hIL-6 standard curve.

3.4.5 Assessment of Specific Humoral Immune Response

3.4.6.i Estimation of Total Serum Immunoglobulins (IgG, IgM and IgA)

- **Principle**

The concentrations of Igs were measured by single (SRID) method. The procedure consists in an immunoprecipitation in agarose between an antigen (sample being assayed) and it's homologus antibody (monospecific antisera). The sample diffuses radialy through the gel and the substance being assayed from a precipitin ring with the monospecific antisera.

- **Procedure "According to the manufacturing instruction"**

1. Endoplate and references were removed from refrigerator, equilibrated reagents to room temperature, mixing reference sera, controls and patents sample in their own containers thoroughly.
2. Five µl of reference sera, control and patents sample were added each into appropriate wells, then lid were firmly replaced and incubated at room temperature (18-25°C) on a level surface for 48 hrs incase of IgA, IgG and for 72 hrs in case of IgM.

3. Immuno-precipitin ring diameter was measured to the nearest 0.1 mm using specific viewer device.

♦ **Interpretation of the Result**

The reference curve is constructed on graph paper (standard Igs concentration vs squares of the rings diameter). The curve was prepared by plotting the results of human reference sera of high, medium and low concentration as shown in figure (3-8). Unknown concentrations were determined from the reference curve and expressed as mg/dl.

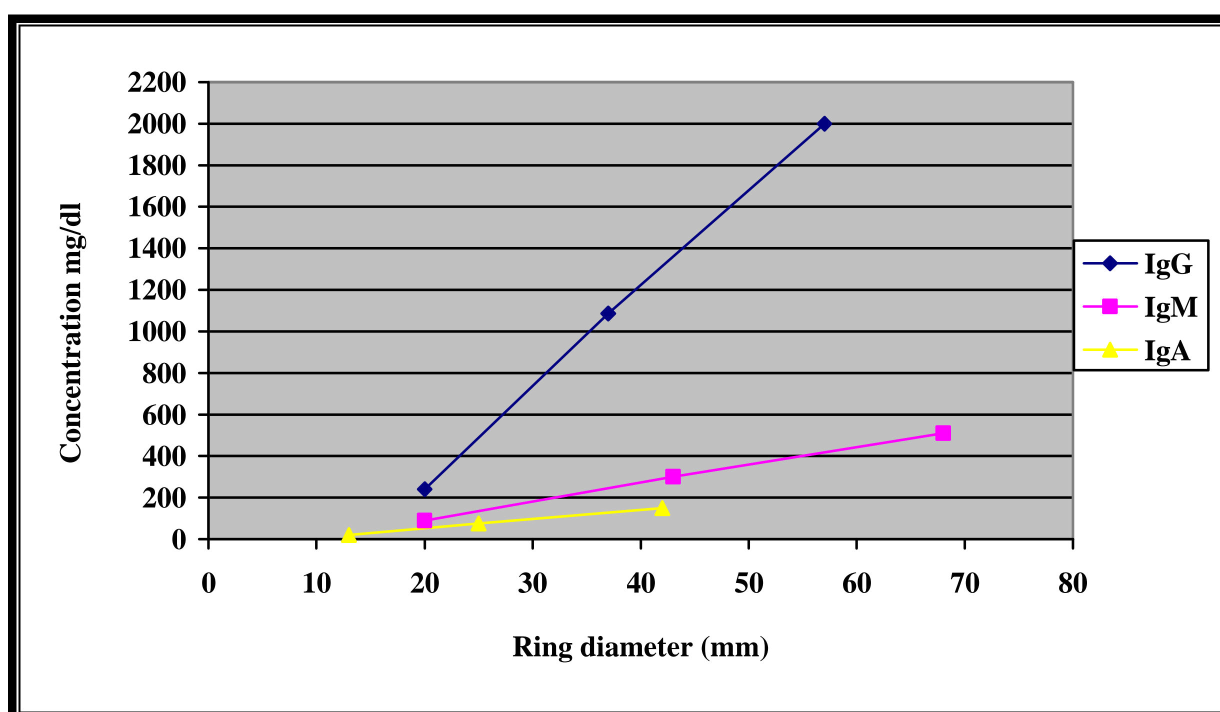

Figure 3-8: Standard curves of immunoglobulins (IgG, IgM and IgA)

Reference values for serum immunoglobulins

Age	IgA mg/dl	IgG mg/dl	IgM mg/dl
3-9 years	30-240	610-1380	20-134
9-15 years	60-300	630-1400	30-148

3.4.6.iiAssessment of Complement components C_3 and C_4

Non-specific humoral immune response mediated by complement and other acute phase proteins. Assessment of such immune response done by estimation of each complement component concentration.

SRID was used for quantitative determination of C3 and C4 using RID-Endoplate kits,and the reference curve is constructed on graph paper (as mentioned previously) (Figure 3-9).

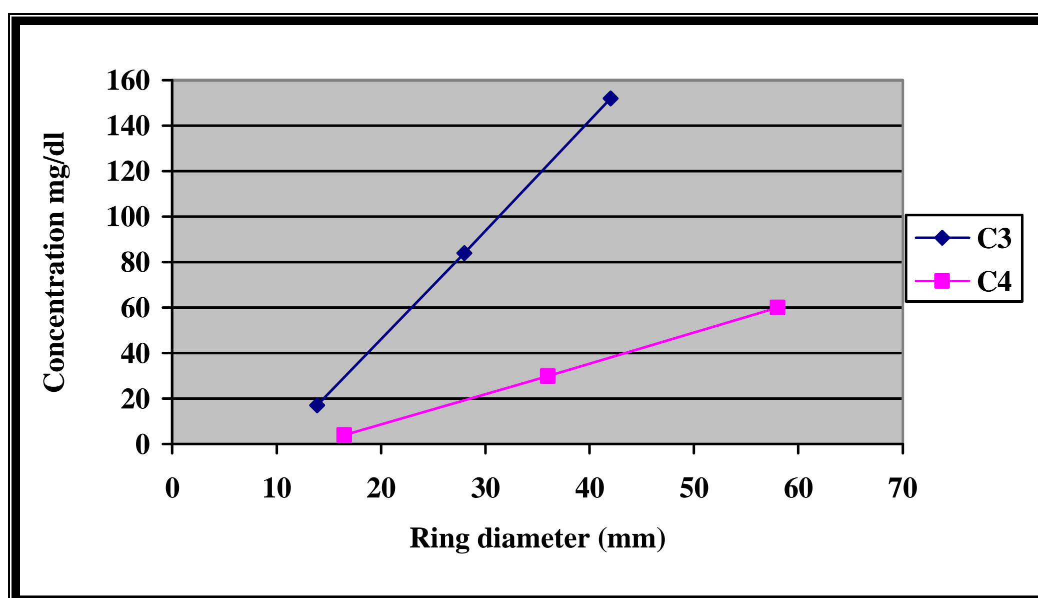

Figure 3-9: Standard curves of complement components (C3 and C4).

3.4.6.iii ELISA for Detection of Serum CVB_5, Polio and Adeno Virus IgG Antibodies

- **Principle**

Serum IgG against $CVB_{5,}$ polio and adenovirus antigens can be detected quantitatively with an indirect ELISA, Figure (3-10).

Serum containing primary antibody (Ab1) is added to an antigen coated microtiter well and allowed to react with the antigen attached to the well. Then the enzyme-conjugated anti-human IgG, the secondary antibody (Ab2) was

added which binds to the Ab1. After incubation, the wells are washed and a substrate for the enzyme is added. The amount of colored reaction product that forms is measured by microtiter plate reader.

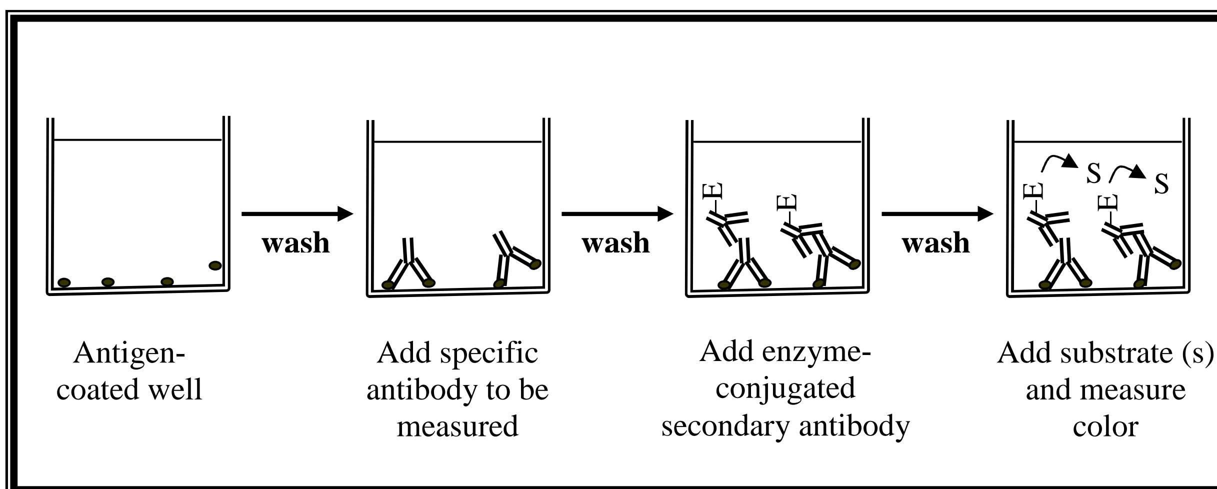

Figure 3-10: Illustrate the principle of indirect ELISA used for the qualitative or quantitative determination of antigen(adapted from Goldsby *et al*., **2000).**

- **Standardization Procedure for ELISA Test**

  Standardization was carried out for the following variables:

1. Viral antigens include: CVB_5, oral poliovirus vaccine and adenovirus type 3, 4 and 7.
2. Antisera includes:
   - Positive human CVB_5 sera.
   - Negative human CVB_5 sera.
   - Positive human adenovirus sera.
   - Negative human adenovirus sera.

   Positive human poliovirus sera obtained from vaccinated children.

   Pooled positive antisera was obtained from 10 individual samples of vaccinated children (5 vac^+) with a period not less than 3-4 weeks from

the last vaccine given to the child. The pool antisera was then divided into small amount and stored at -20°C.

- Negative human poliovirus sera obtained from old person (more than 60 years old),(Adul-Karim, 2000).

3. Anti-human IgG conjugate.

Checker board test was done for the determination of optimal concentration of antigens, optimal antisera dilution and optimal anti-human conjugate dilution to be used in the ELISA (Abdul-Karim, 2000).

Procedure:

(This procedure is used for each virus antigen in private)

1. Coating the microtiter plates was done by using double folds serial dilutions of coating buffer-diluted CVB_5 antigen solution per column, starting form 100 µg/ml to 12.5 µg/ml. Added the coating buffer alone as a negative antigen control in B wells (Blank).
2. The positive (P) control CVB_5 sera were added as serial dilutions (1:2, 1:4, 1:8 and 1:16) and each dilution was applied on two successive columns. The CVB_5 negative (N) control sera were applied on two columns.
3. The third layer was the standardization of the conjugate anti-human IgG which added in two serial dilutions 1:500 and 1:1000.
4. The substrate chromogen was added and then read by ELISA reader to select the best reading which is of moderate (OD) value to avoid over-reading or under-reading and specifying the antigens serum and conjugate concentrations and dilutions to be our standard levels for later on work on ELISA. The plan of ELISA standardization was illustrated in figure (3-11).

Serum serial dilutions		1	2	3	4	5	6	7	8	9	10	11	12
1:2	A	P	N	P	N					P	N	P	N
	B	B	A+C	B	A+C					B	A+C	B	A+C
1:4	C	P	N	P	N					P	N	P	N
	D	B	A+C	B	A+C					B	A+C	B	A+C
1:8	E	P	N	P	N					P	N	P	N
	F	B	A+C	B	A+C					B	A+C	B	A+C
1:16	G	P	N	P	N					P	N	P	N
	H	B	A+C	B	A+C					B	A+C	B	A+C

CVB_5 Ag 100 µg/ml (columns 1–4): Anti-human Conj-1/500 (columns 1–2); Anti-human Conj 1/1000 (columns 3–4)

CVB_5 Ag 50 µg/ml (columns 9–12): Anti-human Conj-1/500 (columns 9–10); Anti-human Conj 1/1000 (columns 11–12)

Serum serial dilutions		1	2	3	4	5	6	7	8	9	10	11	12
1:2	A	P	N	P	N					P	N	P	N
	B	B	A+C	B	A+C					B	A+C	B	A+C
1:4	C	P	N	P	N					P	N	P	N
	D	B	A+C	B	A+C					B	A+C	B	A+C
1:8	E	P	N	P	N					P	N	P	N
	F	B	A+C	B	A+C					B	A+C	B	A+C
1:16	G	P	N	P	N					P	N	P	N
	H	B	A+C	B	A+C					B	A+C	B	A+C

CVB_5 Ag 25 µg/ml (columns 1–4): Anti-human Conj-1/500 (columns 1–2); Anti-human Conj 1/1000 (columns 3–4)

CVB_5 Ag 12.5 µg/ml (columns 9–12): Anti-human Conj-1/500 (columns 9–10); Anti-human Conj 1/1000 (columns 11–12)

Figure 3-11: The plan of ELISA standardization. B=Blank; Coating buffer only, P=human positive control sera, N=human negative control sera, A+C=antigen + conjugate only.

The following concentrations and dilutions were specified by standardization procedure:

- Optimal concentration for CVB_5 antigens = 50 μg/ml.
- For adenovirus 3, 4, 7 antigens = 12.5 μg/ml.
- For oral poliovirus vaccine concentration = 1:2.
- Optimal serum dilution for CVB_5, adenovirus and oral poliovirus vaccine =1:2.
- Optimal anti-human IgG conjugate dilution = 1/1000.

♦ **Procedure of ELISA: "As described by Davidkin *et al*. (1998) and Harkonen *et al*. (2003)"**

2. The microtiter plates were coated by 50 μl (50 μg/ml) of CVB_5 antigen solution in carbonate buffer pH 9.6 (section 3.3.6.vi.a). The plates were incubated overnight at 4°C.
3. Washed the plates twice with PBS supplemented with 0.1% Tween-20.
4. Blocked with 275 μl of PBS containing 0.1% BSA to prevent non-specific binding. Incubated at room temperature for 30 min.
5. Washed 3 times with PBS containing 0.1% Tween-20.
6. Fifty μl (1:2 dilution) in incubation buffer (PBS supplemented with 0.1% Tween and 1% BSA (section 3.3.6.vi.d) of each TIDM patient and healthy control sera was added to each well. Incubated at 37°C for 1 or 2 hrs.
7. Washed as in step 4.
8. Fifty μl of (1:1000) HRP-conjugated anti-human IgG was added to each well. Incubated at 37°C for 1 or 2 hrs.
9. Washed as in step 4.
10. Fifty μl /well of the substrate-chromogen (TMB/H_2O_2) was added. Incubated for 25 min at room temperature in dark place, then added 50 μl of 1M HCl as stopping agent.

11. Finally the optical density (OD) was read by microtiter plate reader at 492 nm.

Note: For each run of ELISA test, we used both CVB_5 antibodies positive and negative controls, each one of them was added to 8 wells. They were used in calculation of cutoff value, which was a limit threshold that below it any reading must be considered as a negative and equal or above it to be considered as positive. They were all used in the estimation of specificity and sensitivity of the test.

- **Interpretation of the Result**

Cutoff value at each run was calculated by getting the mean of OD reading for the 8 wells that contains CVB_5 antibodies control negative plus 2 standard deviation (SD).

Sample value lie below the cutoff value (mean negative + 2 SD) were considered negative. Those who were equal or greater than cutoff value were considered positive (Voller *et al*., 1980).

Note: The same procedure was conducted for detection of serum adenovirus and poliovirus IgG antibodies, except for the later one, the incubation buffer (section 3.3.6.vi.d) and stopping agent 12.5% H_2SO_4 were used.

3.4.6.iv Assessment of Serum Anti-GAD Autoantibodies

- **Principle**

IRMA of anti-GAD autoantibodies is a sandwich type assay. Samples or standards are incubated with ^{125}I-labeled human recombinant GAD_{65}, by adding of protein A to precipitate any ^{125}I-GAD/anti-GAD complex which has been formed after centrifugation the precipitates are counted for ^{125}I. Values are calculated by interpolation from the standard curve. The radioactivity is directly proportional to the concentration of anti-GAD autoantibodies in the sample.

- **Procedure "According to the manufacturing instruction"**

A. Preparation of Protein A Suspension and Tracer:

The content of the protein A and the tracer vials (section 3.3.5.v) were reconstituted with 2.6 ml of assay buffer. Waited for at least 10 min. following reconstitution and mixed gently to avoid foaming before dispensing.

B. Assay Procedure

1. Step 1: Addition 1

Add to plastic tubes 20 µl of standard (7 tubes); controls (2 tubes) or sample. Then add 50 µl of tracer. Mix and incubate 2 hrs at room temperature (18-25°C).

Note: Add 50 µl of tracer to 2 additional tubes to obtain total CPM.

2. Step 2: Addition 2

Add 50 µl of protein A suspension, mix and incubate 1 hr at room temperature.

3. Step 3: Addition 3

Add 1 ml of ice cold assay buffer (2-8°C) and mix.

4. Step 4: Separation and Counting

Centrifuge 20 min at 1500 g at 2-8°C. Remove the supernatants by aspiration or decantation (except the 2 tubes "total CPM"). Then count bound CPM (B) and total CPM (T) for 1 min.

- **Interpretation of the Result**

Results were obtained from the standard curve by interpolation, using B/T (%) or B/B max (%) on vertical axis and the anti-GAD concentration of the standards on the horizontal axis (U/ml). For each sample locateed the B/T (%) or

B/B max (%) on the vertical axis and read of the corresponding anti-GAD autoantibody concentration on the horizontal axis in U/ml.

Values below 1 U/ml were considered normal, whereas values above 1 U/ml should be therefore considered as pathological.

3.4.7 Statistical Analysis

3.4.7.i Regarding of HLA and disease association the frequency distribution for selected variables was done first. The strength of disease association with particular HLA antigen was determined by calculating the relative risk (RR) stated as the chance of individuals with disease association HLA antigen has developing the disease compared to individuals lacking it. A RR value can ragne from less than one (negative association) to more than one (positive association) while RR value of 1 indicates no differences in disease susceptibility. If the association is negative, it indicates a protective effect, therefore, the preventive fraction (PF) was calculated, while if it is positive, it indicates increased susceptibility to that specific disease, therefore the etiological fraction (EF) was calculated. The significance of such association was assessed by either Chi-Square test or Fisheri exact probability. The latter test was more preferred, because allows for the correction of probability and not affected by small numbers (less than 5). The mathematical formulas of such parameters were as the following:

$$RR = \left[\frac{P^{+} x\, C^{-}}{P^{-} x\, C^{+}}\right] \text{ or } \left[\frac{a\ x\ d}{b\ x\ c}\right]$$

$$PF\left[\frac{1 - RR\left[\frac{a}{a+b}\right]}{RR\left[1 - \frac{a}{a+b}\right] + \left[\frac{a}{a+b}\right]}\right]$$

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

P^+ or a: number of patients possessing the disease associated HLA molecule.
P^- or b: number of patients lacking that particular HLA molecule.
C^+ or c: number of controls possessing that HLA molecule.
C^- or d: number of controls lacking that particular HLA molecule.

3.4.7.ii Differences between Means

The tests which have been used for statistical analysis were:

a- Quantitative (parametric) tests

1- Student t-test was used to measure the differences of certain variables between two means. The results were expressed as means ± standard error (SE).
2- Single Factor ANOVA (F-test) was used in this study to find out whether the difference among more than two groups of samples is significant or not.

b- Qualitative (Non Parametric) tests:

1- Chi Square Tests, were used for the measurements of correlation and dependency among different variable observations, mainly two groups.
2- Pearson Correlation (R), which measures to what degree the two variable observations are correlated to each other, and the type of this correlation whether direct, inverse, or no correlation at all.

Results

4.1 Clinical Findings

Patients population consisted of 60 TIDM patients, who were early onset of the disease (diagnosis less than five months). The patients were subdivided according to their ages into two groups in order to compare their immune responses, 36 diabetic children were equal or less than 10 years. This group consisted of 13 males and 23 females with mean age of 6.74 ± 1.12 years. The second group comprised 24 diabetic children who were more than 10 years, and consisted of 15 males and 9 females with mean age of 13.11 ± 2.68 years .The patients population were matched with 50 apparently healthy individuals as control group for age and sex.

4.1.1 Biochemical and Hematological Characteristics of TIDM Patients

As shown in table (4-1) the mean fasting plasma glucose (FPG) values were 223.11 ± 19.69 mg/dl and 221.6 ± 22.16 mg/dl in patients ≤10 years and >10 years old respectively comparing to nearly 91.23 mg/dl in control group. The criteria for the diagnosis of DM as listed in the report of the expert committee on the diagnosis and classification of diabetes mellitus (2003), estimated that FPG ≥126 mg/dl and 2 hours plasma glucose (PG) ≥ 200 mg/dl during OGTT confirms the diagnosis.

Glycosylated hemoglobin (HbA_{1C}) is an indirect measurement of mean blood glucose concentration. Each 1% change in HbA_{1C} value reflects a large change in mean plasma glucose (25-35 mg/dl), (National Diabetes Data Group, 1984). So HbA1c range can be used for assessing the degree of blood glucose control. The level of HbA1c% can be classified into three categories; <6% in normal (non-diabetic level); <7% which indicates good glycemic control and the

third class >8% which means poor glycemic control with high risk of developing long-term complications of diabetes.

The mean HbA_{1C}% in patient group ≤10 years old was 9.70 ± 0.36% vs 4.74 ± 0.12 % in healthy individuals. Out of 36 patients, one patient had HbA_{1C} <6% and another one <7%, while the other 34 patients were within the >8% category. The mean HbA_{1C}% in patient group >10 years old was (10.24 ± 0.46% vs 4.84 ± 0.08 in control group. Out of 24 patients, only one patient had HbA_{1C} value <7%.

The mean fasting serum C-peptide value was 0.54 ± 0.05 ng/ml and 2.08 ± 0.13 ng/ml in patients and controls ≤ 10 years old respectively. In TIDM patients >10 years old the mean serum C-peptide value was 0.58 ± 0.11 ng/ml in comparison with 2.54 ± 0.15 ng/ml in healthy individuals. The normal value of fasting serum C-peptide is 0.071-4.37 ng/ml.

All these three parameters were apparently highly significant between the patients and controls in both age groups.

The recent results indicate that age has no impact on the degree of metabolic decomposition at the clinical presentation of TIDM . As shown in table (4-1), there was no statistically significant differences in mean values of FPG (P2 = 0.86); HbA_{1C} (P2=0.36) and fasting serum C-peptide (P2=0.70) between patients ≤10 years and those >10 years old.

Table 4-1: t-test comparison between TIDM patients in both age groups and healthy controls regarding their mean values of some biochemical and hematological characteristics of TIDM.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
Fasting Plasma glucose (mg/dl)	Controls	21	91.32	1.16	80	99	0.000 (HS)	Controls	29	91.15	4.41	85	97	0.000 HS)	0.86 (NS)
	TIDM	36	223.11	19.69	66	500		TIDM	24	221.6	22.16	90	400		
HbA_{1C} %	Controls	21	4.74	0.12	4.20	5.9	0.000 (HS)	Controls	29	4.84	0.08	4.10	5.50	0.000 (HS)	0.36 (NS)
	TIDM	36	9.70	0.36	5.1	16.2		TIDM	24	10.24	0.46	6.80	15.80		
Fasting Serum C-peptide (ng/ml)	Controls	21	2.08	0.13	1.09	3.00	0.000 (HS)	Controls	29	2.54	0.15	1.06	3.60	0.000 (HS)	0.70 (NS)
	TIDM	36	0.54	0.05	0.01	1.41		TIDM	24	0.58	0.11	0.03	1.78		

P_1 : Patients vs. controls

P_2 : Patients ≤10 years vs. patients >10 years.

NS: not significant

HS: High significant

4.2 HLA Antigen Association

This test was conducted on the following main groups:

Sixty TIDM patients, eighty healthy controls and fifty siblings of TIDM patients.

The frequencies of HLA antigens (-A; -B; -C; -DR and -DQ) were compared between TIDM patients and controls, siblings and controls and between TIDM patients and siblings.

4.2.1 HLA Association with TIDM

The distribution of HLA-A; -B, -C, -DR and -DQ antigens with their frequencies in TIDM patients and controls are presented in table (4-2), while antigens showing significant variations between patients and controls are given in table (4-3).

The results of comparing TIDM patients to controls showed several antigen deviations in their frequencies.

At HLA-A locus, the antigen A9 showed significant deviation.The antigen A9 was significantly increased (P=0.004) in the patients and such difference were associated with RR value of 2.88 and EF value of 0.261. This positive association remained significant after correction (Pc=0.032).

At HLA-B locus three antigens (B8, B12 and B15) were significantly increased in the TIDM patients (P=0.002, 0.032 and 0.018 respectively) in comparison to controls. The frequencies of these antigens were (28.33 vs 8.75% ; 11.66 vs 2.50% and 11.66 vs 2.0% respectively) and such differences associated with RR values of (4.122, 5.150 and 9.113 respectively) and EF values of (0.214, 0.093 and 0.103 respectively). However, one positive association remained significant after correction (Pc=0.032) and this was with B8 while both B12 and B15 return to non significancy (Pc=0.512 and 0.288 respectively).

Table 4-2: HLA antigen frequencies in control, T_1DM patients and sibling groups.

HLA-antigens	Control (Number = 80)		TIDM patients (Number =60)		Siblings (Number = 50)	
HLA-A locus	**No.**	**%**	**No.**	**%**	**No.**	**%**
A1	15	18.75	12	20.00	5	10.0
A2	30	37.50	26	43.33	11	22.0
A3	7	8.75	5	8.33	2	4.00
A9	15	18.75	24	40.00	10	20.0
A10	10	12.50	12	20.00	5	10.0
A11	16	20.00	0	ND	0	ND
A19	32	40.00	16	26.66	11	22.0
A28	8	10.00	7	11.66	3	6.00
HLA-B locus						
B7	6	7.50	6	10.00	7	14.00
B8	٧	8.75	17	28.33	5	10.00
B12	٢	2.50	7	11.66	6	12.00
B13	٢	2.50	2	3.33	1	2.00
B14	٤	5.00	0	ND	0	ND
B15	١	2.00	7	11.66	1	2.00
B16	٢	2.50	6	10.00	1	2.00
B17	١	1.25	0	ND	1	2.00
B18	٤	5.00	2	3.33	5	10.00
B27	٥	6.25	2	3.33	2	4.00
B35	١١	13.75	2	3.33	2	4.00
B37	٤	5.00	7	11.66	1	2.00
B40	٢	2.50	3	5.00	3	6.00
B41	8	10.00	5	8.33	2	4.00
B51	23	28.75	9	15.00	6	12.00
B73	2	2.50	2	3.33	4	8.00
HLA-C_W locus						
Cw2	3	٣,٧٥	٣	٥,٠٠	٣	٦,٠٠
Cw4	15	١٨,٧٥	٤	٦,٦٦	٨	١٦,٠٠

Cw5	2	٢,٥٠	2	3.33	0	ND
Cw7	13	16.25	19	31.66	10.	20.00
HLA-antigens	**Control (Number = 80)**		**TIDM patients (Number =60)**		**Siblings (Number = 50)**	
HLA-DR locus	**No.**	**%**	**No.**	**%**	**No.**	**%**
DR1	18	22.50	21	35.00	12	24.00
DR2	20	25.00	4	6.66	7	14.00
DR3	21	26.25	32	53.33	18	36.00
DR4	10	12.50	30	50.00	17.0	34.00
DR5	1	11.25	2	3.33	1	2.00
DR6	3	3.75	6	10.00	3	6.00
DR7	12	15.00	14	23.33	10	20.00
DR8	8	10.00	11	18.33	6	12.00
DR10	0	ND	4	6.66	6	12.00
HLA-DQ locus						
DQ1	18	22.5	4	6.66	11	22.00
DQ2	12	15.00	20	33.33	11	22.00
DQ3	16	20.00	24	40.00	15	30.00

Table 4-3: Antigens of HLA-class I and class II regions showing significant variations between TIDM patients, siblings and controls.

HLA	TIDM vs control					Siblings vs control					TIDM vs siblings	
	RR	EF	PF	P	PC	RR	EF	PF	P	PC	P	PC
A2	–	–	–	–	–	0.470	–	0.198	0.047	NS	0.014	NS
A9	2.88	0.261		0.004	0.032	–	–	–	–	–	0.019	NS
B8	4.122	0.214	–	0.002	0.032	–	–	–	–	–	0.014	NS
B12	5.150	0.093	–	0.032	NS	5.318	0.097	–	0.036	NS	–	–
B15	9.113	0.103	–	0.018	NS	–	–	–	–	–	–	–
B35	0.216	–	0.107	0.031	NS	–	–	–	–	–	–	–
B51	0.437	–	0.162	0.041	NS	0.337	–	0.191	0.019	NS	–	–
Cw4	0.309	–	0.128	0.031	NS	–	–	–	–	–	–	–
Cw7	2.388	0.183	–	0.026	NS	–	–	–	–	–	–	–
DR2	0.214	–	0.195	0.003	0.027	–	–	–	–	–	–	–
DR3	3.210	0.366	–	$9.7x10^{-3}$	0.008	–	–	–	–	–	0.051	NS
DR4	7.00	0.428	–	$1x10^{-5}$	$9x10^{-5}$	2.428	0.176	–	0.003	0.027	0.026	NS
DR5	–	–	–	–	–	0.160	–	0.095	0.049	NS	–	–
DQ1	0.246	–	0.168	0.008	0.024	–	–	–	–	–	0.019	NS
DQ2	2.833	0.215	–	0.009	0.027	–	–	–	–	–	–	–
DQ3	2.666	0.249	–	0.008	0.024	–	–	–	–	–	–	–

RR: relative risk; EF: Etiological fraction; PF: Preventive fraction; P: Fisher exact probability; PC: Corrected probability

In contrast, the B35 and B51 antigens significantly decreased in the patients compared with controls (3.33 vs 13.75 and 15.0 vs 28.75 respectively), but such negative association also failed to remain at a significant level after correction (Pc=0.496 and 0.656 respectively).

At HLA-C locus, Cw7 antigen significantly increased in the TIDM patients (31.66 vs 16.25%, P=0.026, RR=2.388 and EF=0.183). Such positive association was felt after correction (PC=0.104). In other hand, the Cw4 antigen significantly decreased in the patients than in controls (6.66 vs 18.75%, P=0.031), but the negative association failed again to retain a significant level after correction (PC=0.124).

At HLA-class II region (DR-loci), three antigens showed different frequencies in patients and controls, these were DR2, DR3 and DR4. Increased frequencies of DR3 (53.33 vs 26.25%) and of DR4 (50.0 vs 12.5%) were observed in the patients. The positive association RR values were of 3.210 and 7.00 respectively and EF values of 0.366 and 0.428 respectively. Such positive association was highly significant ($P=9.7x10^{-3}$ and $1x10^{-5}$ respectively) and remained highly significant after correction (PC=0.008 and $9x10^{-5}$ respectively). In contrast DR2 antigen significantly decreased in the patients (6.66 vs 25.0%). Such negative association was significant (P=0.003) and remain significant after correction (PC=0.027).

At HLA-DQ loci, two antigens DQ2 and DQ3 were significantly increased in the patients compared with controls (33.33 vs 15.0%, P=0.009, RR=2.833, EF=0.215) for DQ2 while (40.0 vs 20.0%, P=0.008, RR=2.666 and EF=0.249) for DQ3. This positive association remained significant after correction (PC=0.027 and 0.024 respectively). The antigen DQ1 was significantly decreased in TIDM patients (6.66%) vs (22.5%) in controls, such negative association (P=0.008) remained significant after correction (PC=0.024).

4.2.2 HLA Association with TIDM in Siblings

The distribution of HLA-A; -B; -C; -DR and -DQ antigens in siblings and controls are given in table (4-2), while antigens showing significant variations between siblings and controls are listed in table (4-3).

At HLA-A locus, the antigen A2 showed decreased frequency in siblings of TIDM patients. The antigen A2 had a frequency of 22.0% in the siblings, while in the controls was 37.5%. This negative association was significant (P=0.047) before correction, and failed to reach significance level after correction (PC=0.376).

At HLA-B locus, the antigen B12 showed significant (P=0.036), increased frequency (12.0 vs 2.5%) with RR value of 5.318 and EF value of

0.097. Correcting of probability of this antigen failed to reach significancy (PC=0.576). In contrast, the B51 antigen showed negative association with the disease in the siblings. The antigen had a frequency of 12.0% in the siblings, while in the controls, the frequency was 28.75%. Although the association was significant (P=0.019), the corrected probability failed again to attain a significant level (PC=0.304).

At HLA-class II region (DR loci), increased frequency of antigen DR4 (34.0 vs 12.5%, P=0.003) was observed in the siblings. The RR value of such positive association was 2.428, and the EF value was 0.176. This association was significant (P=0.003) before correction, and after correction (PC=0.027). On the other hand DR5 antigen showed decreased frequency in the siblings as compared to controls (2.0 vs 11.25 respectively). Such negative association was significant before correction (P=0.049) but not after (PC=0.441).

4.2.3 TIDM Patients vs Siblings

As listed in table (4-3), both the TIDM patients and their siblings shared the HLA-A2 and DQ1 as protective antigens, while A9, B8, DR3 and DR4 were susceptible one. No other antigen in the present study was found to be common between the patients and their siblings. Such association was significant before correction but not after (PC=0.112, 0.152, 0.224, 0.459, 0.228 and 0.057 respectively).

4.3 Phenotypic Characteristic of Peripheral Blood T-Lymphocytes

PBL phenotyping can give an idea of the immunological status in patients with TIDM and it can be considered as a mirror image of the immunity.

The isolated PBL were tested for some surface markers by using mcAbs and counted from yielding fluorescent cells with an intense yellow-green color.

4.3.1 Total T-Cells (CD_3^+), T-helper Cells (CD_4^+) and T-cytotoxic/ suppressor Cells (CD_8^+)

As shown in table (4-4) (Figure 4-1) TIDM patients ≤10 years old have shown CD_3^+ cells percentage (66.03%) which was significantly lower than the control group (73.76%) (P_1=0.0001). On other hand, the same result was obtained among patients group >10 years old in which CD_3^+ cells percentage decreased significantly (64.75%) in comparison with control group (75.31%) (P_1=0.0001).

Decreased percentage means of CD_4^+ cells were observed in patients (40.39%) as compared to controls (42.67%) in the age group ≤10 years old and the same decreased percentage means were observed also in patients (37.88%) than controls (41.17%) in age group >10 years. These differences were not significant (P_1= 0.12; 0.098 respectively).

There was a highly significant decrease in mean percentage of CD_8^+ cells in patients compared to controls (23.5 vs 28.43% respectively, P_1= 0.000) in age group ≤10 years old, and the same significant decrease was shown among patients >10 years old 23.92% than controls 29.62%, P= 0.0001 (Table 4-4).

No statistically differences was shown in the mean percentage of CD_3^+ (P_2=0.44); CD_4^+ (P_2= 0.2) and CD_8^+ (P_2= 0.71) between patients in both age groups.

Table 4-4: The differences in the mean percentage of peripheral CD_3^+, CD_4^+ and CD_8^+ lymphocytes between control and TIDM patients groups.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
CD_3^+	Controls	21	73.76	0.95	60	79	0.000 (HS)	Controls	29	75.31	1.17	63	89	0.000 (HS)	0.44 (NS)
	TIDM	36	66.03	1.13	49	80		TIDM	24	64.75	1.44	49	78		
CD_4^+	Controls	21	42.67	0.78	33	48	0.12 (NS)	Controls	29	41.17	1.24	29	56	0.098 (NS)	0.2 (NS)
	TIDM	36	40.39	1.19	29	51		TIDM	24	37.88	1.51	27	49		
CD_8^+	Controls	21	28.43	0.79	21	35	0.000 (HS)	Controls	29	29.62	0.87	20	37	0.000 (HS)	0.71 (NS)
	TIDM	36	23.50	0.67	17	31		TIDM	24	23.92	0.87	17	35		

P_1 : Patients vs. controls

P_2 : Patients ≤10 years vs. patients >10 years.

NS: Not significant

HS: High significant

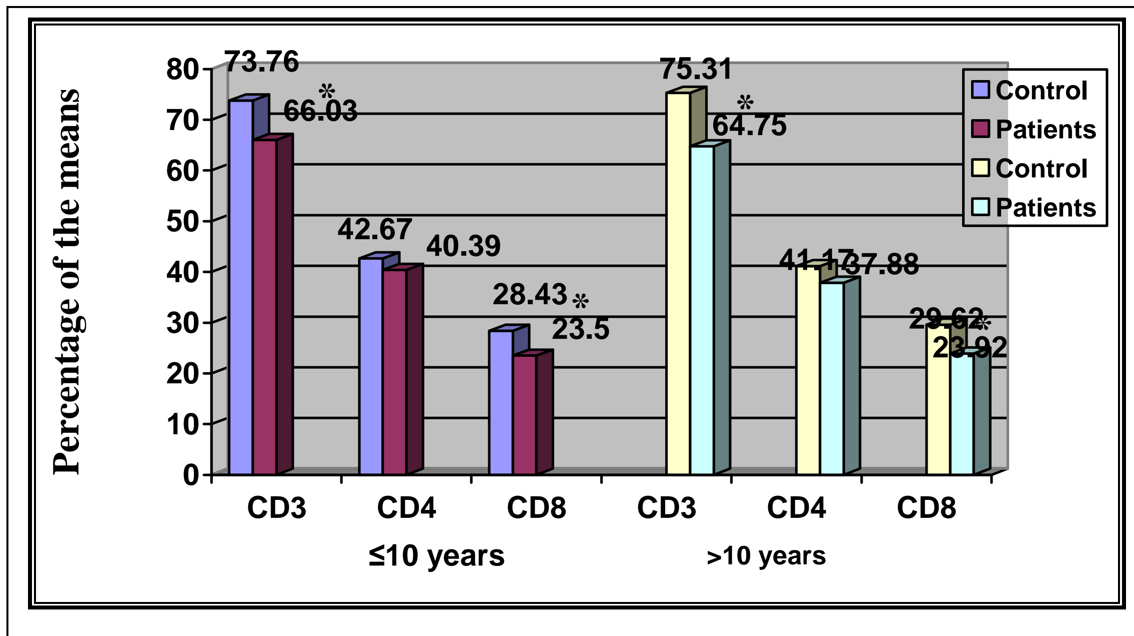

Figure 4-1: Bar chart of the mean percentage of CD_3^+, CD_4^+ and CD_8^+ cell populations for the healthy controls and TIDM patients

4.3.2 CD_4^+/CD_8^+ Ratio

CD_4^+/CD_8^+ ratio is important because it represents an index that refers to immunological balance between T-helper cells and T-cytotoxic cells in the immune system in that the higher CD_4^+/CD_8^+ ratio, the nearer balance point would be to T-helper cells, which means lower cytotoxic activity and higher other forms of CMI and humoral immunity. The CD_4^+/CD_8^+ ratio was significantly higher among patients in ≤10 years old group than controls (1.78 vs 1.52, P_1= 0.015), table (4-5). In other hand, a significant difference was also found between patients and controls in >10 years old group concerning the CD_4^+/CD_8^+ ratio (1.64 vs 1.42) respectively (P_1= 0.034) (Figure 4-2). No significant differences were shown in CD_4^+/CD_8^+ ratio between the patients in both age groups (P_2= 0.30).

Table 4-5: The difference in mean peripheral CD_4^+ / CD_8^+ lymphocyte ratio between control and diabetic patients.

Age	Groups	No.	CD4/CD8 ratio				P_1	P_2
			Mean	SE	Mean	SE		
≤10 years	Controls	21	1.52	0.05	1.08	2.14	0.015 (S)	0.30 (NS)
	TIDM	36	1.78	0.9	1.1	2.83		
>10 years	Controls	29	1.42	0.07	1.05	2.55	0.034 (S)	
	TIDM	24	1.64	0.10	1.11	2.76		

P_1 : Patients vs. controls

P_2 : Patients ≤10 years vs. patients >10 years.

NS: Not significant

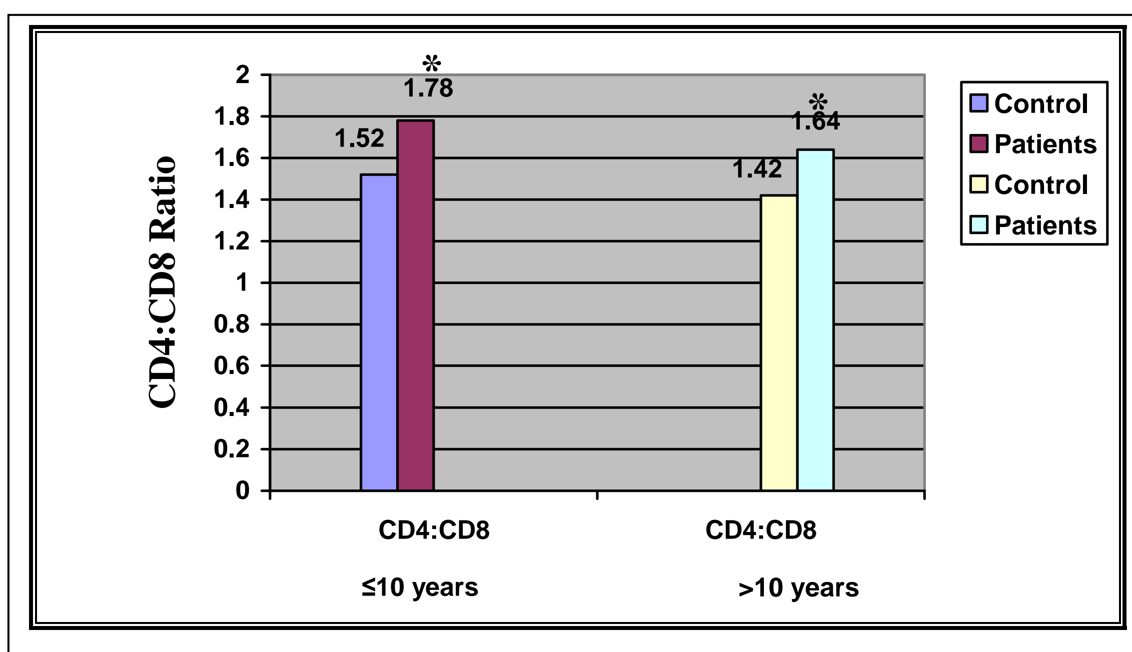

Figure 4-2: Bar chart of the CD_4^+:CD_8^+ ratio of healthy control and TIDM patients.

4.3.3 $CD_4^+$and CD_8^+ Cell Populations was the main Determinant of CD_4^+/CD_8^+ Ratio in TIDM Patients

CD_4^+/CD_8^+ ratio is governed by two cell population namely CD_4^+ cells and CD_8^+ cells. So it is important to know which one of these determinants is the master key for the determination of CD_4^+/CD_8^+ ratio in TIDM patients. By applying the pearson correlation and linear regression equation it found that both CD_4^+ cell population and CD_8^+ cells population were correlated with CD_4^+/CD_8^+ ratio and dynamically do control the ratio. In TIDM patients CD_4^+ cell subsets showed a significant direct positive correlation with CD_4^+/CD_8^+ ratio (r= 0.83, P=0.001) (Figure 4-3), on the other hand CD_8^+ cells showed a highly significant negative correlation with CD_4^+/ CD_8^+ ratio (r= -0.79, P=0.0001), (Figure 4-4).

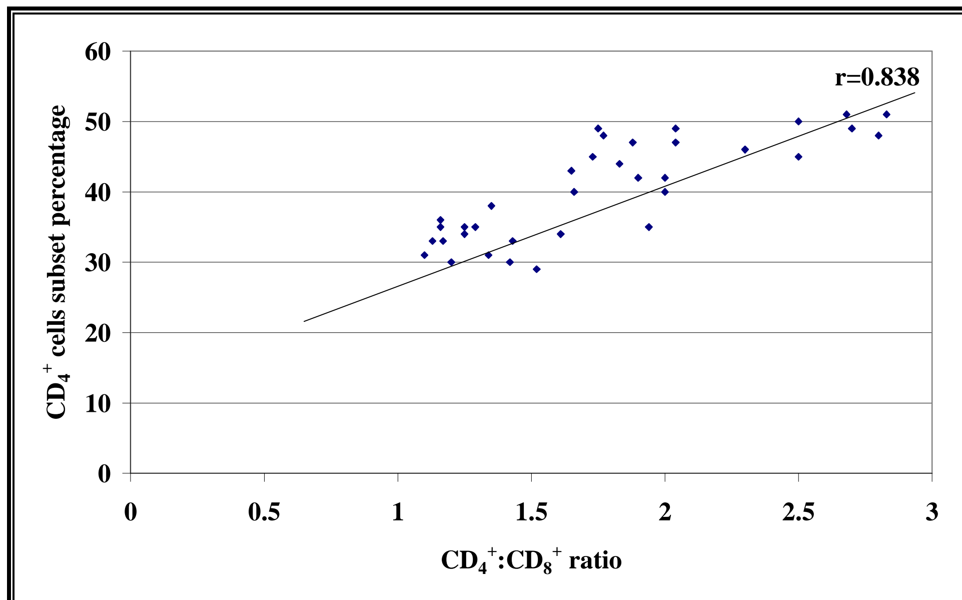

Figure 4-3: Direct linear regression and correlation between CD_4^+ cells and CD_4^+: CD_8^+ ratio in TIDM patients.

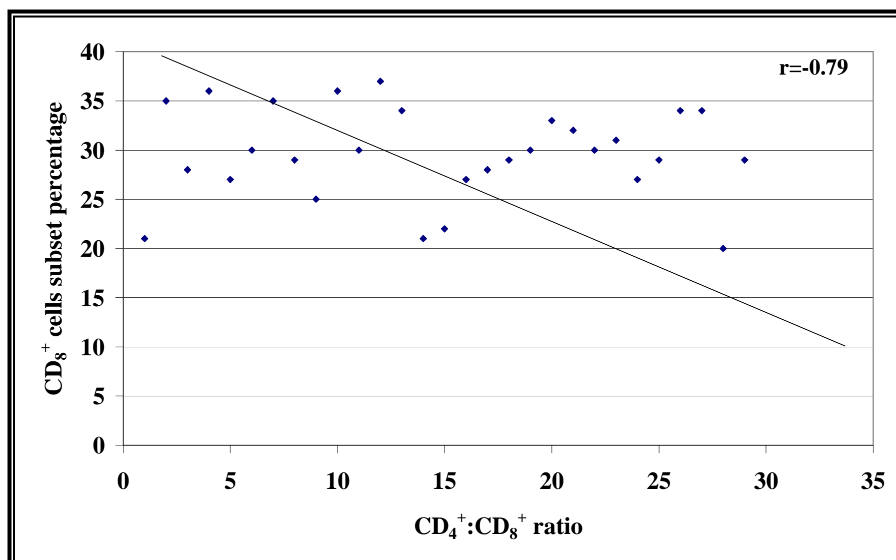

Figure 4-4: Inverse linear regression and correlation between CD_8^+ and CD_4^+: CD_8^+ ratio in TIDM patients.

4.3.4 $CD_{45}RA^+$ and $CD_{45}RO^+$ Cells

Table (4-6) and figure (4-5) showed that there was highly significant decrease in mean percentage of $CD_{45}RA^+$ (naive, unprimed) lymphocytes in patient group ≤10 years old (64.33 % compared to control group (72.67%) (P_1= 0.001). This highly statistical decrease was shown also in patients >10 years old than controls (53.08 vs 61.14% respectively, P_1= 0.001).

The $CD_{45}RO^+$ (memory, primed) cells were statistically high among diabetic patients in comparison with healthy individuals (34.75 vs 25.05% respectively, P_1= 0.0001) in age group ≤10 years old. This statistical increase was also demonstrated among patients in age group >10 years than controls (46.75 vs 38.14% respectively, P_1= 0.0001) (table 4-6).

The results indicated highly significant increase of the mean percentage of activation $CD_{45}RO^+$ cell subset among patients >10 years old (46.75%) than patients ≤10 years old (34.75%) (P_2= 0.0001) and this significant level reflected on the mean percentage of $CD_{45}RA^+$ cells in patients ≤10 years old 64.33% vs 53.08% in >10 years old patient (P_2=0.0001).

Table 4-6: The differences in mean peripheral $CD_{45}RA^+$ and $CD_{45}RO^+$ lymphocyte % between control and TIDM patients groups.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
$CD_{45}RA^+$	Controls	21	72.67	0.95	65.00	80.0	0.001 (HS)	Controls	29	61.14	1.11	51.0	71.0	0.001 (HS)	0.0001 (HS)
	TIDM	36	64.33	1.38	52.0	77.0		TIDM	24	53.08	1.35	42.0	64.0		
$CD_{45}RO^+$	Controls	21	25.05	1.32	17.0	35.0	0.0001 (HS)	Controls	29	38.14	1.04	30.0	48.0	0.0001 (HS)	0.0001 (HS)
	TIDM	36	34.75	1.39	22.0	47.0		TIDM	24	46.75	1.29	37.0	57.0		

P_1 : TIDM Patients vs. controls; P_2 : TIDM Patients ≤10 years vs. patients >10 years; HS: High significant

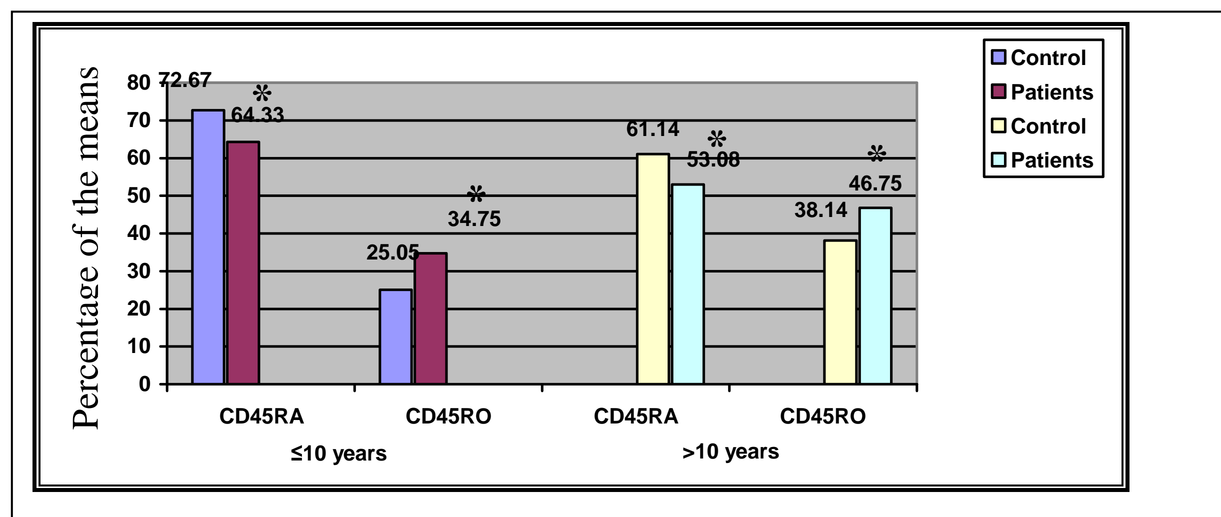

Figure 4-5: Bar chart of mean percentage of $CD_{45}RA^+$ and $CD_{45}RO^+$ cell populations for the healthy control and TIDM patients.

4.3.5 Correlation of CD_3^+ (Pan Cells) with the Naïve $CD_{45}RA^+$ and Memory $CD_{45}RO^+$ Cell Subsets

There was negative correlation between the percentage of CD_3^+ and $CD_{45}RA^+$ cells subsets in patients (r= -0.57, P=0.0001) as shown in figure (4-6) whereas significant direct positive correlation was demonstrated between the percentage of CD_3^+ and $CD_{45}RO^+$ cells subsets (r= 0.57, P=0.0001) figure (4-7).

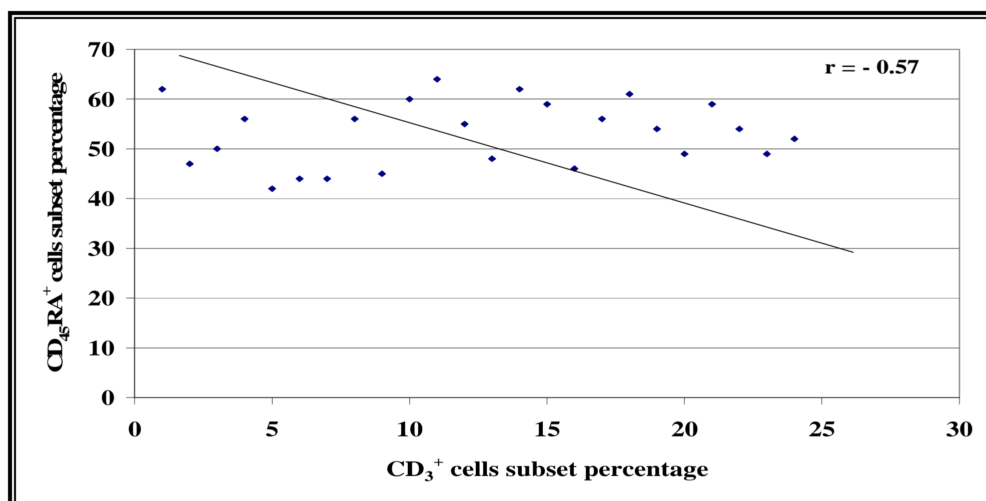

Figure 4-6: Inverse linear regression and correlation between percentage of CD_3+ and $CD_{45}RA^+$ cell subsets.

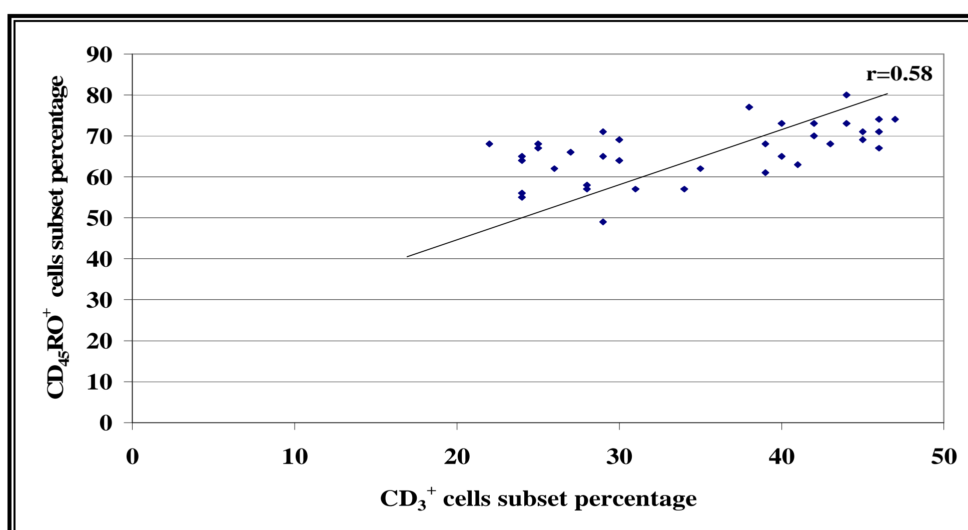

Figure 4-7: Direct linear regression and correlation between percentage of CD_3^+ and $CD_{45}RO^+$ cell subsets.

4.3.6 CD_{38}^{+} Lymphocytes

Increased percentage of activation marker CD_{38}^{+} cells were observed in TIDM patients (24.72%, 23.83%) as compared with the control group (16.86%, 15.97%) in the age group ≤10 years and >10 years old respectively. These differences were highly significant (P_1=0.0001) between the patients and healthy individuals, but failed to reach a significant level (P_2= 0.44) between the patients in both age groups (table 4-7), figure (4-8).

Table 4-7: The differences in mean peripheral CD_{38}^{+} lymphocyte % between control and TIDM patients groups.

Age	Groups	No.	CD_{38}^{+} lymphocyte %				P1	P_2
			Mean	SE	Min.	Max.		
≤10 years	**Controls**	21	16.86	0.76	13.00	23.00	0.0001 (HS)	0.44 (NS)
	TIDM	36	24.72	0.81	15.00	38.00		
>10 years	**Controls**	29	15.97	0.63	12.00	23.00	0.0001 (HS)	
	TIDM	24	23.83	0.82	15.00	31.00		

P_1 : TIDM Patients vs. controls

P_2 : TIDM Patients ≤10 years vs. patients >10 years.

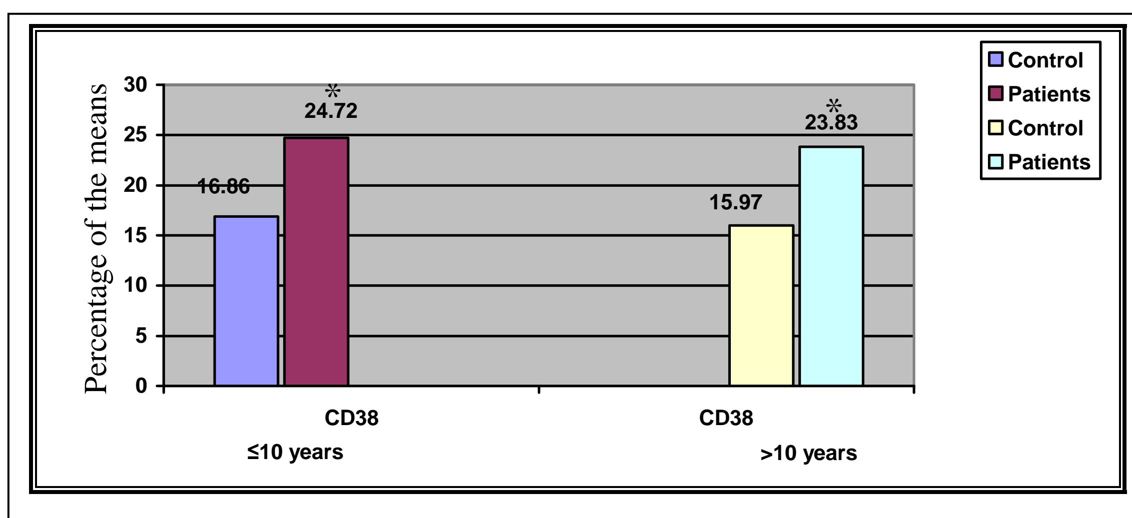

Figure 4-8: Bar chart of mean percentage of CD_{38}^{+} cell populations for the healthy control and TIDM patients.

There was strongly direct positive correlation between the mean percentage of CD_{38}^{+} cells and CD_4^{+} cells (r= 0.808) (Figure 4-9) CD_{19}^{+} cells (r= 0.602) (Figure 4-10) .

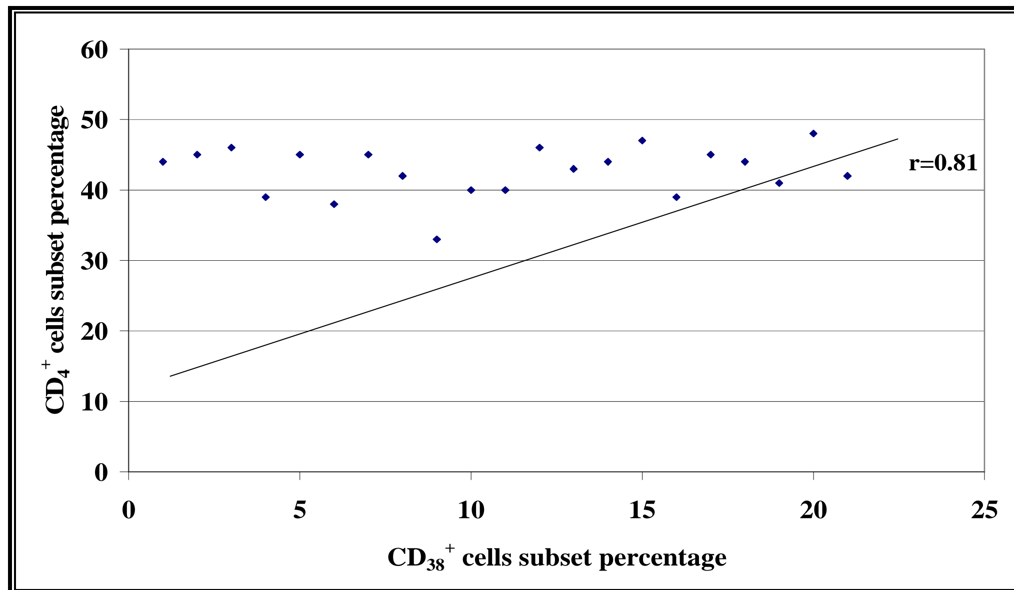

Figure 4-9: Positive linear regression and correlation between CD_{38}^{+} and CD_4^{+} cell subsets percentage.

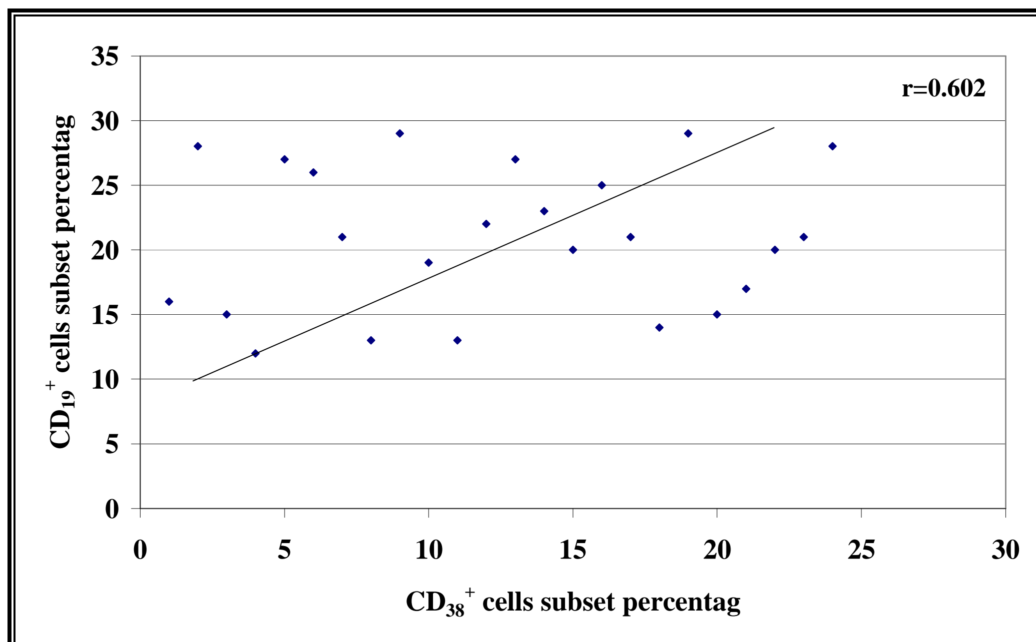

Figure 4-10: Positive linear regression and correlation between CD_{38}^{+} and CD_{19}^{+} cell subsets percentage.

4.3.7 HLA-DR^{+} Lymphocytes

TIDM patients ≤10 years old and >10 years showed increased percentage means of HLA-DR^{+} cells (32.09% and 31.38% respectively) as compared to the control groups (28.47% and 28.08% respectively). Both differences were significant (P_1 value = 0.005 and 0.038 respectively). But the differences were not significant (P_2 = 0.75) between the patients in both age groups (Table 4-8), (Figure 4-11).There was direct positive correlation between the mean percentage of CD38 Cells and HLA-DR+ cells population (r=0.581).

Table 4-8: The differences in mean peripheral HLA-DR^{+} lymphocyte % between control and TIDM patient groups.

Age	Groups	No.	HLA-DR^{+}				P_1	P_2
			Mean	SE	Min.	Max.		
≤10 years	Controls	21	28.47	0.86	20.00	37.00	0.005 (S)	0.79 (NS)
	TIDM	36	32.09	0.89	25.00	39.00		
>10 years	Controls	29	28.08	1.17	20.00	38.00	0.038 (S)	
	TIDM	24	31.38	1.01	23.00	40.00		

P_1: TIDM patients vs. control
P_2: TIDM patients ≤10 years vs. patients >10 years old.

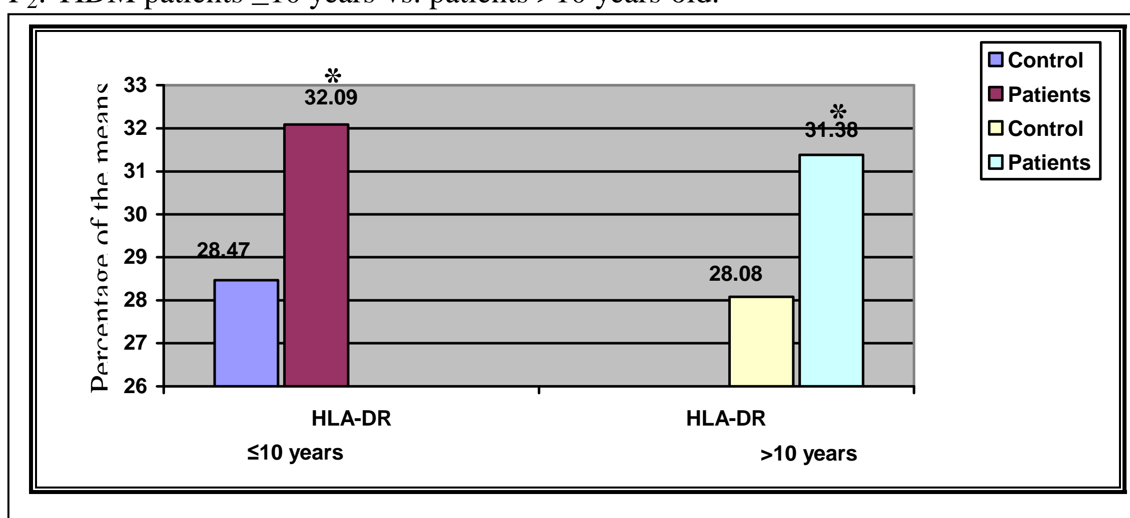

Figure 4-11: Bar chart of the mean percentage of HLA-DR^{+} cell population in healthy control and TIDM patients.

4.3.8 CD_{56}^{+} Lymphocytes

Significant decreased percentage means of CD_{56}^{+} cells were observed in patients ≤10 years old as compared with controls (8.17 vs 10.67% respectively, P_1 = 0.001), whereas a highly significant decreased percentage means of CD_{56}^{+} cells were observed among patients >10 years old than controls (9.21 vs 13.07%, respectively, P_1 = 0.001). This deviation was not significant between patients in both age groups (P_2=0.13), (Table 4-9).

Table 4-9: The differences in mean peripheral CD_{56}^{+} cells (NK) % between control and T_IMD patient group.

Age	Groups	No.	CD_{56}^{+} cells (NK) %				P_1	P_2
			Mean	SE	Min.	Max.		
≤10 years	Controls	21	10.67	0.55	7.00	17.00	0.001 (S)	0.13 (NS)
	TIDM	36	8.17	0.47	4.00	13.00		
>10 years	Controls	29	13.07	0.52	7.00	19.00	0.000 (HS)	
	TIDM	24	9.21	0.48	5.00	14.00		

P_1: TIDM patients vs. control
P_2: TIDM patients ≤10 years vs. patients >10 years old.

4.3.9 Peripheral Blood B-Lymphocytes (CD_{19}^{+})

B-lymphocytes were tested and counted as in T-lymphocytes. As demonstrated in table (4-10) (Figure 4-12), increased percentage means of CD_{19}^{+} cells were observed in patients ≤10 years old (20.28%) and in patients >10 years old (20.88%) as compared to controls (14.95% and 14.72% respectively). Both differences were significant (P_1 value =0.003 and 0.0001 respectively), but the difference failed to reach a significant level (P_2 = 0.681) between patients in both age groups.

Table 4-10: The differences in mean peripheral CD_{19}^{+} lymphocyte % between control and TIDM patients group.

Age	Groups	No.	CD_{19}^{+} Mean	SE	Min.	Max.	P_1	P_2
≤10 years	Controls	21	14.95	1.03	6.00	25.00	0.003 (S)	0.68 (N)
	TIDM	36	20.28	0.90	11.00	29.00		
>10 years	Controls	29	14.72	0.61	8.00	20.00	0.0001 (HS)	
	TIDM	24	20.88	1.14	12.0	29.0		

P_1: TIDM patients vs. control
P_2: TIDM patients ≤10 years vs. patients >10 years old.

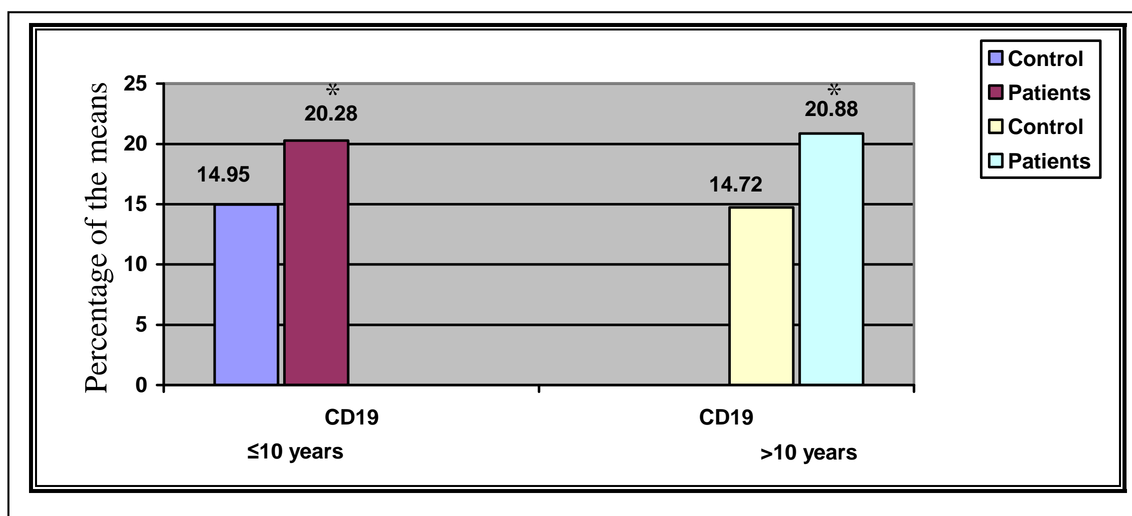

Figure 4-12: Bar chart of the mean percentage of CD_{19}^{+} cell population in the healthy control and TIDM patients groups.

4.3.10 Correlation of CD_{19}^{+} Cells and HLA-DR^{+} Cells

The statistical analysis revealed that there was a strong direct positive correlation between CD_{19}^{+} cells and HLA-DR^{+} activation marker cells subsets ($r = 0.92$, $P = 0.0001$) (Figure 4-12).

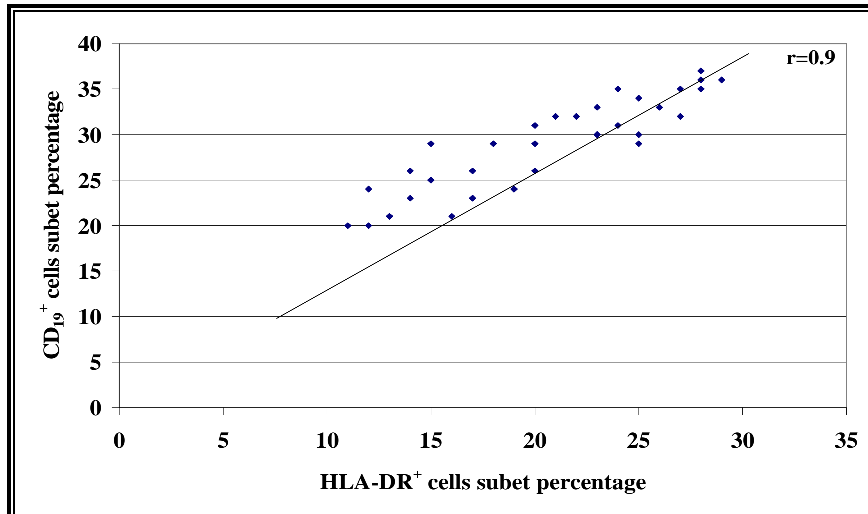

Figure 4-13: Direct linear regression and correlation between CD_{19}^+ and $HLA\text{-}DR^+$ cell subsets in TIDM patients.

4.3.11 Correlation of CD_{19}^+ Cells with $CD_{45}RA^+$ Cells and $CD_{45}RO^+$ Cells

CD_{19}^+ cell population were found to be correlated negatively and significantly with the $CD_{45}RA^+$ cell subset in TIDM patients (r = -0.62, P= 0.0001) (Figure 4-14).

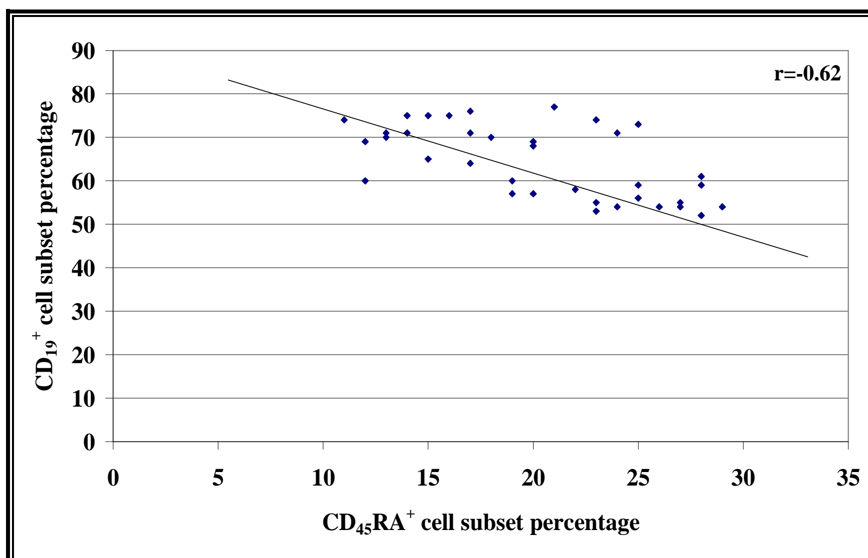

Figure 4-14: Inverse linear regression and correlation between CD_{19}^+ and $CD_{45}RA^+$ cell subsets in TIDM patients.

Moreover the present finding also revealed significant direct positive correlation between CD_{19}^{+} cells and activated $CD_{45}RO^{+}$ cells subset in TIDM patient (r = 0.63, P = 0.0001), (Figure 4-15) and with activated CD_{38}^{+} cell subsets (r = 0.602).

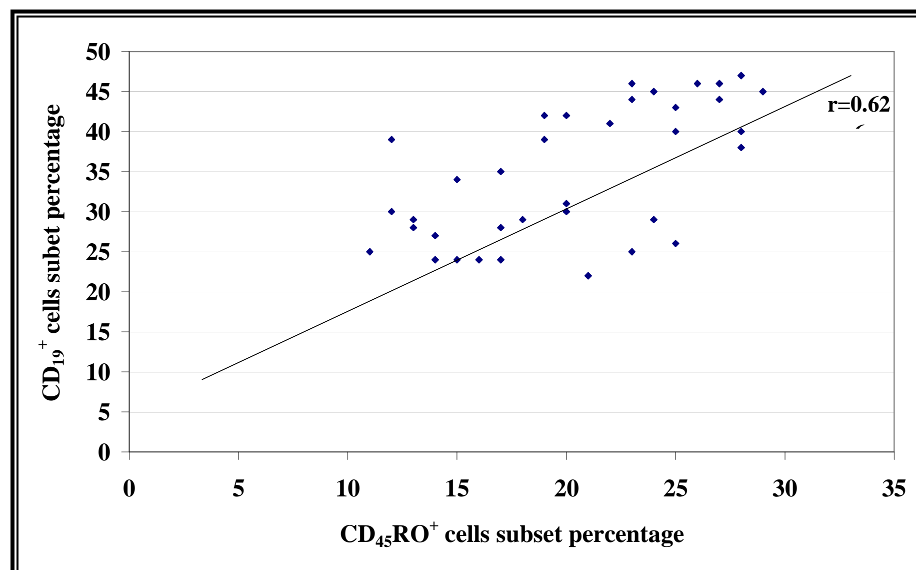

Figure 4-15: Direct linear regression and correlation between CD_{19}^{+} and $CD_{45}RO^{+}$ cell subsets in TIDM patients.

4.4 Functional Activity of PBL

-Lymphocyte Proliferation by Using MTT Assay

The objective of using MTT assay was the evaluation of CMI level in TIDM patients and healthy control groups by the potential of PBL to be stimulated by mitogen and by using specific viral antigens (CVB_5, Poliovaccine and Adenovirus) as biological materials that could enhance the immunological state. The best index to be used for the evaluation of CMI activity in MTT stimuli-based mitogenesis is to use the proliferative percentage.

$$\text{Proliferative \%} = \left[\frac{\text{Control positive (Con - A)}}{\text{Control Negative (-ve)}}\right] - 1x100$$

So the higher the proliferative percentage, the higher level of CMI activity would be definitely expected and vice versa.

The results of mean proliferative percentage in response to Con-A were represented in table (4-11), Figure (4-16).

A similar mean lymphocyte proliferation percentage in response to Con-A mitogen was seen among patients and control groups, but newly diagnosed TIDM patients tended to have a lower non significant proliferative percentage than control subjects ≤10 years old (83.33 vs 85.93% respectively, P_1=0.82) and in >10 years old group (86.04 vs 92.7% respectively, P_1= 0.62).

Table 4-11: t-test between controls and TIDM patient groups regarding comparison of MTT proliferation percentage in response to Con-A.

Mitogen	≤10 years					>10 years					P_2
	Groups	No.	Mean	SE	P_1	Groups	No.	Mean	SE	P_1	
Con-A	Controls	21	85.93	10.60	0.82 (NS)	Controls	29	92.70	10.2	0.62 (NS)	0.57 (NS)
	TIDM	36	83.33	5.60		TIDM	24	86.04	8.27		

P_1: TIDM patients vs. control
P_2: TIDM patients ≤10 years vs. patients >10 years old.

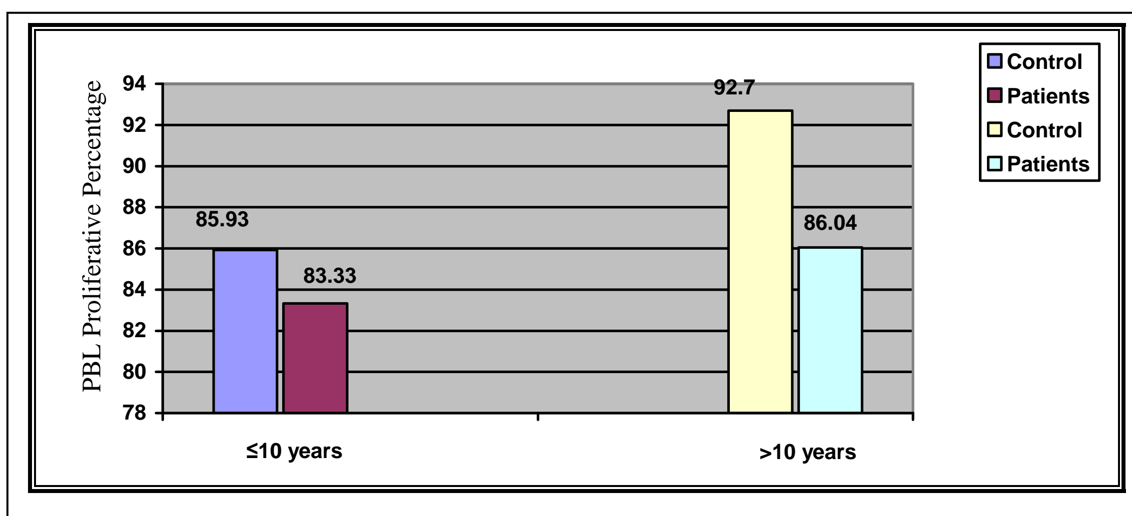

Figure 4-16: Bar chart of the mean MTT proliferative percentage in response to Con-A in healthy controls and TIDM patients.

4.4.1 Role of Viral Antigens in Functional Activation of PBL

Considering the response to different viral antigen, a lower mean proliferative percentage was seen among patients ≤10 years old in response to CVB_5 compared to controls (36.67 vs 49.16% respectively) and among patients >10 years old than controls (38.87 vs 51.20% respectively). This differences failed to reach significant levels in both age groups (P_1=0.061, P_1= 0.14 respectively), table (4-12), Figure (4-17).

Significant decline of proliferative response against poliovaccine was seen in TIDM patients (34.44%) than controls (47.38%) (P_1= 0.045) in ≤10 years old group and (28.30 vs 40.86% respectively, P_1= 0.004 in >10 years old group (Table 4-12), figure (4-17).

Table 4-12: Comparison of mean proliferation percentage of PBL between controls and TIDM patients in response to CVB_5, poliovaccine and adenovirus.

Viral antigens	**≤10 years**					**>10 years**					**P_2**
	Groups	**No.**	**Mean**	**SE**	**P_1**	**Groups**	**No.**	**Mean**	**SE**	**P_1**	
CVB_5	**Controls**	21	49.16	5.88	0.061 (NS)	**Controls**	29	51.20	5.97	0.14 (NS)	0.57 (NS)
	TIDM	36	36.67	3.08		**TIDM**	24	38.87	5.08		
Polio vaccine	**Controls**	21	47.38	5.83	0.045 (S)	**Controls**	29	40.86	3.28	0.004 (S)	0.14 (NS)
	TIDM	36	34.44	2.79		**TIDM**	24	28.30	3.28		
Adeno-virus	**Controls**	21	20.67	2.24	0.82 (NS)	**Controls**	29	28.61	3.73	0.23 (NS)	0.35 (NS)
	TIDM	36	19.97	1.61		**TIDM**	24	23.02	3.27		

P_1: TIDM patients vs. control

P_2: TIDM patients ≤10 years vs. patients >10 years old.

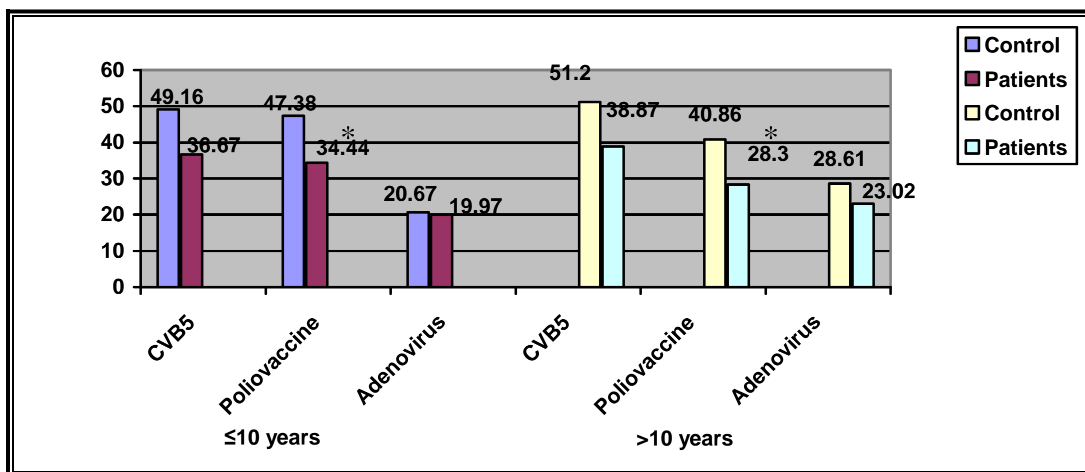

Figure 4-17: Bar chart of the mean MTT proliferative percentage in response to specific viral antigens (CVB_5, poliovaccine and Adenovirus) in the healthy control and TIDM patients.

*

A non significant proliferative percentage decline in response to adenovirus was observed in patients (19.97%) and controls (20.67%) (P_1= 0.82) in ≤10 years old group and also in patients >10 years old (23.02%) in comparison with controls (28.61%) (P_1=0.23).

No statistical differences appeared in the mean lymphocyte proliferative percentage between patients in both age groups against CVB_5 (P_2=0.57), polivaccine (P_2=0.14) and Adenovirus (P_2=0.57).

4.4.2 Mitogenic Properties of Tested Viral Antigens *In Vitro*

To confirm the immunostimulatory effect of CVB_5, polio and adenovirus, we compared them with Con-A, mitogen as a control positive for PBL mitogenesis and with control negative (Table 4-13). Statistical analysis, had shown that CVB_5 had a mitogenic potential in vitro. By comparing the mean of MTT OD value of CVB_5 (0.369) with control negative (0.270) in patient group ≤10 years old, it was found that control negative is significantly lower than CVB_5 mean of MTT reading (P_1= 0.045). The same statistical difference was seen among patients >10 years old between OD value of CVB_5 and control

negative (0.368 vs 0.265, P_2=0.037). This indicates that CVB5 may have a role in inducing the disease in those patients. The mean MTT reading of CVB_5 was significantly lower than Con-A mean MTT reading in patients ≤10 years old (0.369 vs 0.495 respectively, P_1=0.045) and (0.368 vs 0.493 respectively, P_1= 0.045) in patients >10 years old. This means that CVB_5 has a good mitogenic potential, but does not reach the high level of Con-A.

Table 4-13: Paired t-test among CVB_5, polio vaccine, adenovirus, Con-A and control negative MTT reading means for the comparison among TIDM patients (OD) reading.

≤ 10 years (n= 36)		**P_1**	**>10 years (n= 24)**		**P_2**
CVB_5	C-ve	0.045 (S)	CVB_5	C-ve	0.037 (S)
0.369	0.270		0.368	0.265	
CVB_5	Con-A	0.045 (S)	CVB_5	Con-A	0.045 (S)
0.369	0.495		0.368	0.493	
Polio	C-ve	0.054 (S)	Polio	C-ve	0.074 (NS)
0.363	0.270		0.340	0.265	
Polio	Con-A	0.037 (S)	Polio	Con-A	0.017(S)
0.363	0.495		0.340	0.493	
Adeno	C-ve	0.21 (NS)	Adeno	C-ve	0.17 (NS)
0.324	0.270		0.326	0.265	
Adeno	Con-A	0.006 (S)	Adeno	Con-A	0.007(S)
0.324	0.495		0.326	0.493	

Concerning the poliovaccine and adenovirus, it was found that control negative mean of MTT OD value (0.270) was lower than poliovaccine (0.369) and adenovirus (0.324) mean of MTT readings in patients group ≤10 years old. These means were weakly significant among poliovaccine (P_1= 0.054) and not statistically different among adenovirus. On the other hand,the same results were demonstrated among patients >10 years old, (0.265 vs 0.340, P_2= 0.074) for poliovaccine and (0.265 vs 0.326 P_2= 0.17) for adenovirus. This indicates that poliovaccine and adenovirus do not have a valuable mitogenic potential *in vitro*

compared with that of CVB_5, although poliovaccine had a weak mitogenic potential in patients group ≤10 years old and might have weakly immunostimulatory activity *in vivo* (Table 4-13).

Lymphocyte proliferation percent against both poliovaccine and adenovirus showed significant positive correlation with lymphocyte proliferative percent in response to CVB_5 (r =0.38, r = 0.25 respectively, P<0.05), nevertheless a positive correlation between poliovaccine and adenovirus (r = 0.45, P<0.05).

4.5 Serum Levels of Cytokines

4.5.1 Serum Level of hIFN-γ

This humoral mediator has been measured by using ELILSA technique. Similar means of serum levels of IFN-γ were observed in the investigated patients ≤10 years and >10 years old who showed higher means (75.60 and 70.78 pg/ml respectively) than controls (42.66 and 40.39 pg/ml respectively). Out of 29 healthy controls in the age group >10 years old, only one of them had serum IFN-γ less than standard level (0.095 pg/ml). The statistical analysis revealed a significant difference between patients and controls (P_1= 0.005 and 0.006 respectively), while between patients nonstatistical difference appears (P_2= 0.73) table (4-14), figure (4-18).

4.5.2 Serum Level of hIL-10

Table (4-15) figure (4-18), demonstrated the mean serum levels of IL-10 in the studied groups. The mean value of serum IL-10 for patients group ≤10 years old was significantly higher than controls (104.92 vs 57.01 pg/ml respectively, P_1= 0.003). Patients >10 years old showed also significant elevation in IL-10 serum levels (84.22 pg/ml) compared with controls (59.50

pg/ml) (P_1=0.037). A statistically difference of mean IL-10 concentration appears between patients in both age groups (P_2= 0.04).

4.5.3 Serum Level of hIL-6

The estimated levels of IL-6 in sera of the patients were higher than control group (147.6 vs 80.4 pg/ml respectively, P_1= 0.036) in ≤10 years old group table (4-16), figure (4-18). Out of 36 patients, 4 patients had serum levels of IL-6 out of standard level; three were less (0.097, 0.098 and 0.098 pg/ml), while the fourth one was high (2224.29 pg/ml) than the standard. In the same age group, out of 21 controls, 2 individuals had serum IL-6 levels less than standard (0.082 pg/ml).

The mean levels of serum IL-6 were also significantly elevated in the patients >10 years old compared to controls (171.8 vs 81.6 pg/ml respectively, P_1= 0.04). Out of 24 patients, 2 patients had serum IL-6 concentration less than standard (0.082 and 0.092 pg/ml) and another 2 patients had high levels (1410.86 and 1654.20 pg/ml) than standard. Concerning the healthy controls, 12 individuals had serum IL-6 level less than the standard levels. No significant difference appear in the serum IL-6 concentration between the two age groups of patients (P_2=0.70).

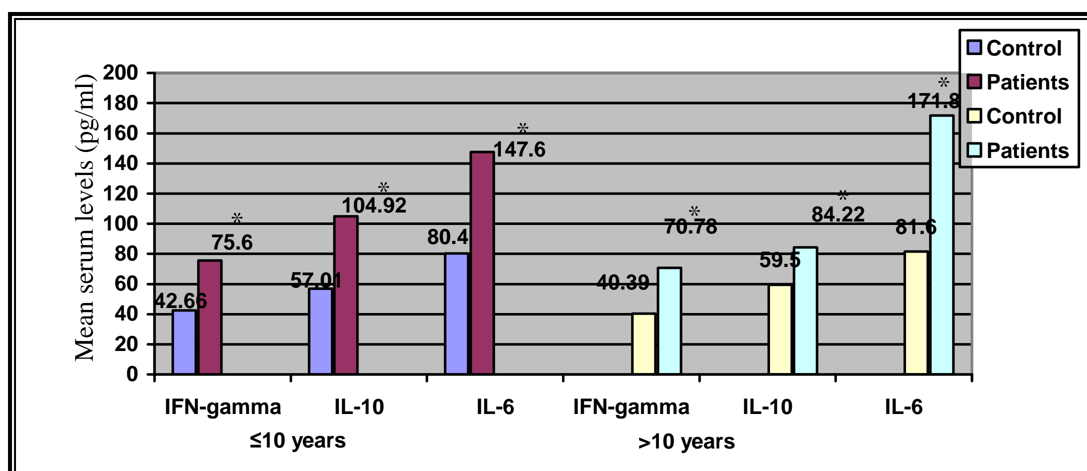

Figure 4-18: Bar chart of mean serum levels of hIFN-γ, IL-10 and IL-6 in the healthy controls and TIDM patients.

Table 4-14: Mean concentration of serum hIFN-γ in healthy subjects and TIDM patients groups.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
hIFN-γ (pg/ml)	Controls	21	42.66	3.95	12.67	81.33	0.005 (s)	Controls	28	40.39	1.19	10.83	90.37	0.006 (S)	0.73 (NS)
	TIDM	36	75.60	10.3	30.1	345.5		TIDM	24	70.78	9.78	30.94	203.93		

P_1: TIDM patients vs. control
P_2: TIDM patients ≤10 years vs. patients >10 years old.

Table 4-15: Mean concentration of serum hIL-10 in control and TIDM patients group.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
hIL-10 (pg/ml)	Controls	21	57.01	9.92	20.97	97.62	0.003 (s)	Controls	29	59.50	12.6	20.43	81.37	0.037 (S)	0.04 (S)
	TIDM	36	104.92	8.81	57.63	360.0		TIDM	24	84.22	4.67	61.86	141.36		

P_1: TIDM patients vs. control
P_2: TIDM patients ≤10 years vs. patients >10 years old.

Table 4-16: Mean concentration of serum hIL-6 in control and TIDM patients groups.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
hIL-6 (pg/ml)	Controls	19	80.4	56.4	0.1	524.10	0.036 (s)	Controls	17	81.6	69.0	0.1	499.61	0.04 (S)	0.70 (NS)
	TIDM	32	147.6	45.0	0.1	1018.70		TIDM	20	171.8	80.2	0.1	981.30		

P_1: TIDM patients vs. control
P_2: TIDM patients ≤10 years vs. patients >10 years old.

4.5.4 Correlation between Lymphocyte Proliferation by MTT and Serum Cytokines Levels in TIDM Patients

It has been found that CMI level presented by proliferative percentage in response to CVB_5 was directly positively correlated with the serum IFN-γ level (r =0.332, P<0.05) figure (4-19) and inversely correlated with the serum IL-6 levels (r = -0.326, P<0.05) figure (4-20, while a weak inversely correlation has been gotten with IL-10 level (r = -0.18). Concerning the proliferative percentage in response to poliovaccine, it has been found that there was a direct positive correlation with the serum IFN-γ and IL-10 levels (r =0.332 figure (4-21), r = 0.619 figure (4-22) respectively, P<0.05), while a weak inversely correlation was found with the IL-6 serum level (r = -0.134). The proliferative percentage in response to adenovirus was also found in direct positive correlation with the serum IFN-γ level (r = 0.54, P<0.05) and with IL-10 level (r = 0.25) and inversely correlated with the IL-6 levels (r = -0.27).

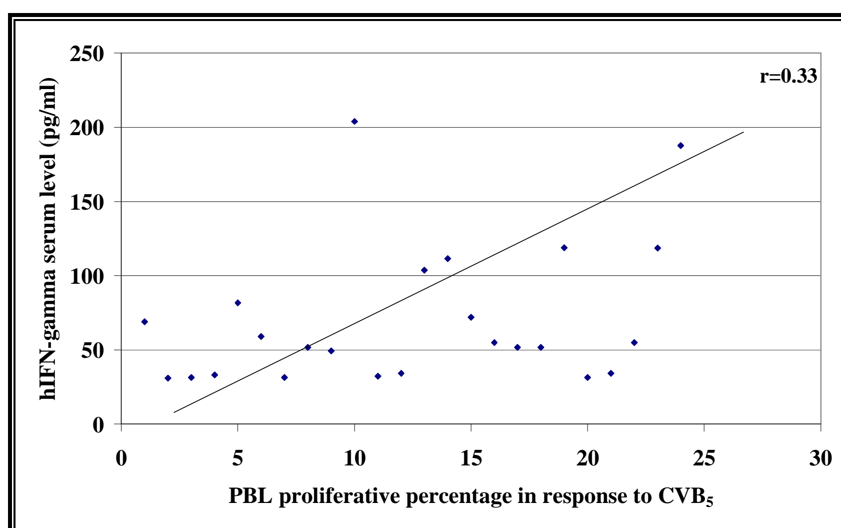

Figure 4-19: Direct linear regression and correlation between proliferative percentage of PBL in response to CVB_5 and serum level of IFN-γ.

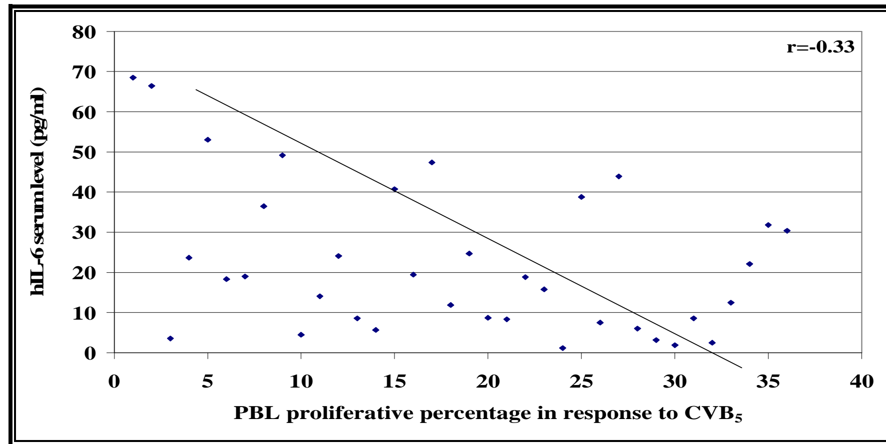

Figure 4-20: Inverse linear regression and correlation between PBL proliferative percentage in response to CVB_5 and serum level of IL-6.

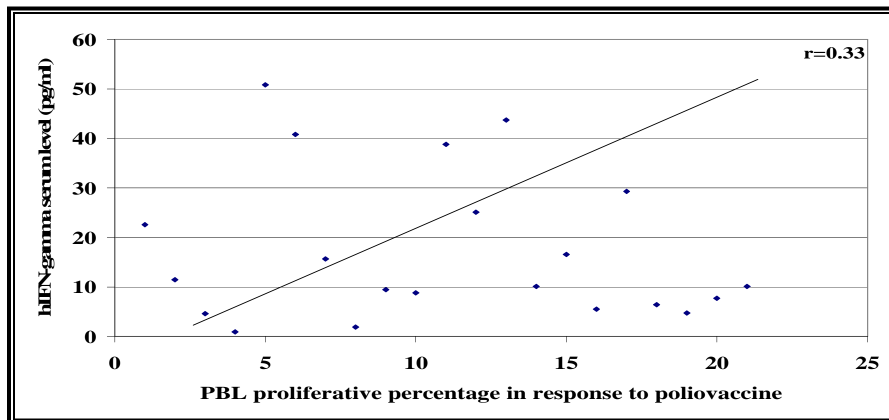

Figure 4-21: Direct positive linear regression and correlation between PBL proliferative percentage in response to Poliovaccine and serum level of IFN-γ.

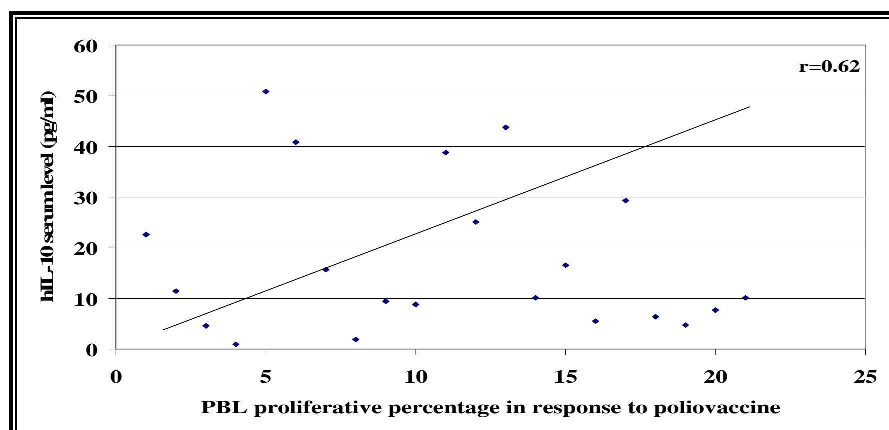

Figure 4-22: Direct linear regression and correlation between PBL proliferativ percentage in response to poliovaccine and serum level of IL-10.

4.5.5 Correlation between PBL CD Markers and Serum Cytokine Levels in TIDM Patients

By using Pearson correlation test to detect any relation between PBL CD markers and serum cytokines, it was found that serum level of IFN-γ was positively correlated with activation markers on PBL including $HLA\text{-}DR^+$ cells (r = 0.21), $CD_{45}RO^+$ cells (r = 0.20), ($P<0.05$) while no correlation was detected between IFN-γ level and CD_{38}^+ cells.

Serum IL-10 levels was positively correlated with $HLA\text{-}DR^+$ cells (r = 0.27), $CD_{45}RO^+$ cells (r = 0.23) and with CD_{38}^+ cell subsets (r = 0.22), ($P<0.05$).

An inversely linear correlation was found between serum IL-6 level and $HLA\text{-}DR^+$ cells (r = -0.34), $CD_{45}RO^+$ cells (r = -0.25), CD_{38}^+ cells (r = -0.22) as well as CD_{56}^+ cell subsets (r = -0.43).

4.6 Immunoglobulins (Igs) and Complement Profile

4.6.1 Total Serum Igs

In this study, the total serum levels of Igs (M, G and A) were evaluated to determine their differences between TIDM patients and healthy controls.

The results of total serum Igs were determined in table (4-17) figure (4-23). They showed significant differences in both age studied groups, between the patients and healthy controls. The mean total serum IgM in patients ≤10 years and >10 years (106.16 and 107.53 mg/dl respectively) was both significantly higher than controls (82.25 and 81.94 mg/dl respectively) (P_1= 0.005, 0.005).

Mean serum IgG levels for the patients were significantly higher than controls (1081.2 vs 910.3 mg/dl respectively, P_1= 0.013) in ≤10 years old group and (1246.0 vs 1021.3 mg/dl respectively, P_1= 0.04) in >10 years old group.

Table 4-17: Differences of mean values (mg/dL) of tatal Igs between control and TIDM patients

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
IgM	Controls	21	82.25	6.66	25.90	150.30	0.005 (s)	Controls	29	81.94	5.28	20.70	153.80	0.005 (S)	0.970 (NS)
	TIDM	36	106.16	6.13	47.20	231.6		TIDM	24	107.53	6.68	35.20	171.60		
IgG	Controls	21	910.3	50.8	649.0	1407.9	0.013 (S)	Controls	29	1021.3	40.7	692.9	1679.7	0.040 (S)	0.187 (NS)
	TIDM	36	1081.2	42.7	672.2	2109.1		TIDM	24	1246.0	103.0	224	2938		
IgA	Controls	21	116.43	9.3	57.4	197.60	0.0001 (HS)	Controls	29	145.7	10.4	72.6	278.9	0.0001 (HS)	0.436 (NS)
	TIDM	36	354.6	22.5	130.8	644.0		TIDM	24	355.4	39.2	97.4	647.0		

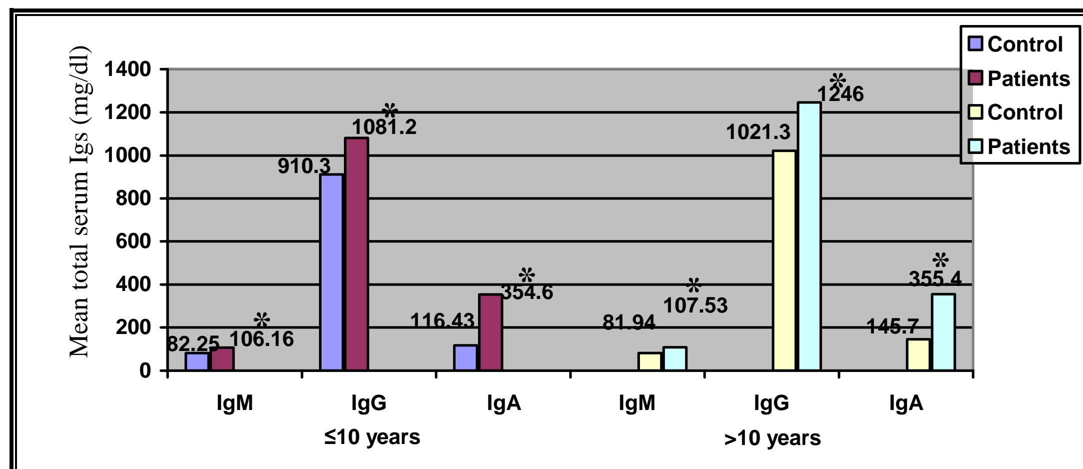

Figure 4-23: Bar chart of mean serum total Igs levels in healthy controls and TIDM patients.

The mean total serum IgA level was (354.6 mg/dl) for patients ≤10 years old which was significantly higher than controls (116.43 mg/dl) (P_1= 0.0001). The same significant elevation was observed in mean serum IgA level for patients group >10 years old compared with controls (355.4 vs 145.7 mg/dl respectively, P_1= 0.0001).

No statistical differences were observed for total serum Igs between patients in two age groups.

4.6.2 Serum Complement Components (C_3 and C_4) Levels

The results of mean serum levels of C_3 and C_4 are illustrated in table (4-18), figure (4-24).

The serum levels of C_3 were significantly lowered in patients ≤10 years old compared to controls (98.53 vs 114. 48 mg/dl respectively, P= 0.037) and (106.87 vs 125.11 mg/dl respectively P=0.041) in >10 years old group.

The level of serum C_3 in patients >10 years old slightly increased but failed to reach statistically significance when compared with the serum C_3 level of patients ≤10 years old (P_2= 0.218).

Statistical analysis of serum C_4 levels results revealed significant differences in both age groups; mean values of C_4 serum level decreased in patients (23.21 mg/dl) compared to controls (29.38 mg/dl), P_1= 0.003 in age group ≤10 years, and the same decline was demonstrated among patients >10 years old (26.50 mg/dl) in comparison to controls (31.04 mg/dl) P_1= 0.037.

The results rendered were not significant between the patients in both age groups (P_2= 0.12).

Table 4-18: Differences of mean values (mg/dL) of complement C_3 and C_4 between control and TIDM patient groups.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
C_3	Controls	21	114.48	5.30	33.5	162.5	0.037 (s)	Controls	29	125.11	5.3	64.00	195.30	0.041 (S)	0.218 (NS)
	TIDM	36	98.53	4.03	39.70	168.90		TIDM	24	106.87	6.87	37.50	181.90		
C_4	Controls	21	29.38	1.38	20.50	41.60	0.003 (S)	Controls	29	31.04	1.16	16.00	42.30	0.037 (S)	0.12 (NS)
	TIDM	36	23.21	1.37	6.20	46.20		TIDM	24	26.50	1.77	11.10	43.10		

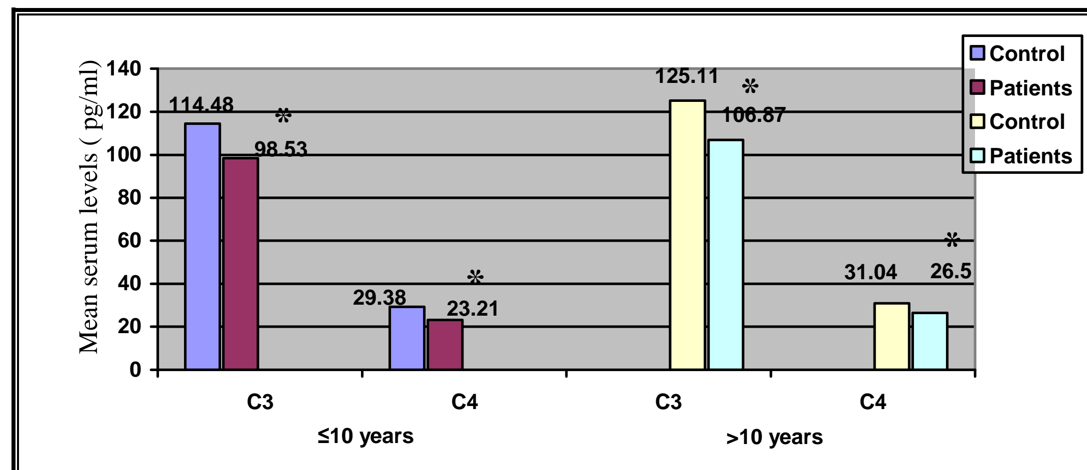

Figure 4-24: Bar chart of mean serum complement component C_3 and C_4 levels in healthy controls and TIMD patients.

4.6.3 GAD Autoantibodies

GADA were detected in 30 of Iraqi children with newly diagnosed TIDM (50%). A higher significant proportion of the girls tested positive for GADA (5/9; 55.6%) were observed in age group >10 years old than of girls ≤10 years old (11/23; 47.8%), (P_2= 0.049); while the proportion of boys tested positive for GADA was higher in age group ≤10 years old than >10 years old (7/13; 53.8% vs 7/15; 46.7%), but this difference fails to be significant (P_2= 0.804), table (4-19). No statistical differences were observed between males and females in each age group (P_1= 0.729 and 0.673 respectively). The proportion of index cases positive for the both age groups in comparison with controls were shown in table (4-20). A higher significant proportion of the patients was positive to GADA in both age groups (18/36, 50% and 12/24, 50% respectively) as compared to control groups (1/21; 4.76% and 2/29; 6.9% respectively). This differences were highly significant P_1=0.0001.

Table 4-19: Differences of sero positive / negative of GADA between control and TIDM males and females patients.

Parameter	≤10 years (n=36)					>10 years (n=24)					
	GADA+		GADA−		P_1	GADA+		GADA−		P_1	P_2
	No.	%	No.	%		No.	%	No.	%		
Males	7	53.8	6	46.1	Chi 0.729 (NS)	7	46.7	8	53.3	Chi 0.673 (NS)	Chi 0.804 (NS)
Females	11	47.8	12	52.2		5	55.6	4	44.4		Chi 0.049 (S)

Table 4-20: Differences of sero positive / negative of GADA between control and TIDM patient groups.

<table>
<tr><th rowspan="2">Age</th><th rowspan="2">Groups</th><th rowspan="2">No.</th><th colspan="2">Sero positive</th><th colspan="2">Sero negative</th><th rowspan="2">P_1</th><th rowspan="2">P_2</th></tr>
<tr><th>No.</th><th>%</th><th>No.</th><th>%</th></tr>
<tr><td rowspan="2">≤10 years</td><td>Controls</td><td>21</td><td>1</td><td>4.76</td><td>20</td><td>95.24</td><td rowspan="2">Chi 0.0001 (HS)</td><td rowspan="4">Chi 1.00 (NS)</td></tr>
<tr><td>TIDM</td><td>36</td><td>18</td><td>50.00</td><td>18</td><td>50.00</td></tr>
<tr><td rowspan="2">>10 years</td><td>Controls</td><td>29</td><td>2</td><td>6.90</td><td>27</td><td>93.10</td><td rowspan="2">Chi 0.0001 (HS)</td></tr>
<tr><td>TIDM</td><td>24</td><td>12</td><td>50.00</td><td>12</td><td>50.00</td></tr>
</table>

4.6.4 Serological Finding for Anti-Viral IgG

Viruses have been indicated to be associated with the onset of TIDM in many epidemiological and serological studies (Dahlquist, 1997).Many viruses espically enteroviruses including (CVB and Poliovirus) and Adenovirus have been implicated. So TIDM children were serologically studied for the presence of anti-CVB_5; anti-polio and anti-adeno IgG in Iraqi children and compared the results with the control group.

4.6.4.i Anti-CVB_5 IgG in TIDM Patients

Serum IgG of CVB_5 have been measured by using ELISA technique which measures the optical density (OD) of patients and controls sera. The value which is equal or higher than cutoff OD value (=0.138) considered as sero positive.

Only 12 patients out of 60 were sero-positive (20%) compared to 4 healthy individuals out of 50 (8%) who were sero-positive for anti-CVB_5 IgG. These differences were statistically significant (P=0.048) (table 4-21).

Table 4-21: Prevalence of sero positive / negative IgG against CVB_5 in control and TIDM patient groups.

Virus	Groups	No.	Sero positive		Sero negative		P_1
			No.	%	No.	%	
CVB_5	Controls	50	4	8.0	46	92.0	0.048 (S)
	TIDM	60	12	20.0	48	80.	

Chi= 2.994

4.6.4.ii Anti-Polio IgG

As shown in table (4-22), out of 60 patients 19 (31.67%) were sero-positive for anti-polio-IgG compared to 13 (26%) healthy controls, so no difference appeared between both groups (P = 0.649). The value which is equal or more than cutoff OD value (0.178) is considered as sero positive.

Table 4-22: Prevalence of sero positive / negative IgG against poliovirus in control and TIDM patient groups

Virus	Groups	No.	Sero positive		Sero negative		P_1
			No.	%	No.	%	
Polio	Controls	50	13	26.0	37	74.00	0.649 (NS)
	TIDM	60	19	31.67	41	68.33	

Chi = 0.207

4.6.4.iii Anti-Adeno IgG

As shown in table (4-23), only 4 patients were sero positive for anti-adeno IgG (6.67%) compared with the control group who were all sero-negative. The values which were equal or higher than 0.2 cutoff (OD) value are considered as sero-positive. This differences were highly significant between the two groups (P= 0.000).

Table 4-23: Prevalence of sero positive / negative IgG against Adenovirus in control and TIDM patients groups

Virus	Groups	No.	Sero positive		Sero negative		P_1
			No.	%	No.	%	
Adeno	Controls	50	0	0	50	100.00	0.000 (HS)
	TIDM	60	4	6.67	56	93.33	

4.7 Relation between Mean Lymphocyte Proliferation Percentage and Anti-Viral IgG in TIDM Patients

CMI level, checked by MTT viral antigens-based stimulation of PBL, is tested for relation with the presence of anti-viral IgG in the sera of TIDM patients to detect any relation that can clarify if the PBL was primed previously by the same viral antigen.

Results represented in table (4-24) showed a significant increase of mean proliferative percentage in response to CVB_5 in the patients who were sero-positive for anti-CVB_5-IgG compared with the sero-negative patients (50.58 vs 22.99%) (P=0.048).

The mean proliferative percentage for sero-positive and sero negative anti-polio-IgG patients were illustrated in table (4-25). It was found that the patients who were sero-positive for anti-polio IgG had higher proliferative percentage reading in response to polio-vaccine (31.48%) than those patients who were sero-negative (20.61%) and these differences were significant at the level (P=0.039).

The study also demonstrated increased mean proliferative percentage of PBL in response to adenovirus in sero-positive anti-adeno IgG patients in comparison to sero-negative anti-adeno IgG patients (30.10 vs 14.16%) and again these differences reach the significant level (P=0.042) table (4-26).

Table 4-24: Relation of mean PBL proliferative percentage in response to CVB_5 with the anti- CVB_5 IgG.

CVB_5		No.	Proliferation percentage	SE	P
Anti-CVB_5 IgG	+ve	12	50.58	10.09	0.048 (S)
	–ve	48	22.99	3.27	

t = 2.62

Table 4-25: Relation of mean PBL proliferative percentage in response to poliovaccine with the anti-polio IgG.

Poliovaccin		No.	Proliferation percentage	SE	P
Anti-Polio IgG	+ve	19	31.48	5.83	0.039 (S)
	–ve	41	20.61	2.92	

t = 3.85

Table 4-26: Relation of mean PBL proliferative percentage in response to adenovirus with the anti-adeno IgG.

Adenovirus		No.	Proliferation percentage	SE	P
Anti-Adeno IgG	+ve	4	30.10	6.45	0.042 (S)
	–ve	56	14.16	2.22	

t = 2.66

Moreover, the present findings also revealed a significant positive correlation between the PBL proliferative percentage in response to CVB_5 and anti-CVB_5-IgG ($r = 0.412$). Strong negative correlation was also detected between proliferative percent in response to adenovirus and anti-adeno-IgG ($r = -0.635$) while the correlation found with the anti-polio-IgG was weakly positive ($r = 0.101$).

4.8 Relation of HLA Class II Alleles with the PBL Proliferation Percentage in TIDM Patients

To find out any relation between the HLA-class II risky alleles (genetic factors) and proliferative percentage of MTT (CMI level), ANOVA test was applied to compare the proliferative percentage in patients with HLA-DR risky alleles (DR3; DR4 and DR3/DR4) with those patients who had other alleles. Results represented in table (4-27) showed that the mean PBL proliferative percentage in response to different tested viral antigens were significantly higher in the patients with DR4, DR3 and DR3/DR4 serotypes compared with the children carrying other alleles. The significant levels scored (P= 0.021) in response to CVB_5, (P=0.031) in response to poliovaccine, and (P= 0.041) in response to adenovirus. Moreover, the mean proliferative percentage were significantly higher in patients carrying DR4 allele than those in patients with DR3 alleles in response to CVB_5 (62.67 vs 43.32%, P=0.038), to poliovaccine (59.86 vs 38.40%, P=0.031) and to adenovirus (46.02 vs 22.48%, P= 0.046).

Concerning the HLA-DQ risky alleles DQ2, DQ3, DQ2/DQ3), our results represented in table (4-28) showed a significant increase of proliferative percentage in patients carrying different HLA-DQ risky alleles compared with the patients who lack these alleles. The results scored as significant levels of (P= 0.032) in response to CVB_5, (P= 0.038) in response to poliovaccine, and (P= 0.042) in response to adenovirus.

As detected in table (4-28), the proliferative percentages were significantly higher in patients with DQ3 alleles than in patients with DQ2 alleles in response to all tested viral antigens.

Table 4-27: Relation of mean lymphocyte proliferation percentage in response to different viral antigens with the HLA-DR risky alleles in TIDM patients.

Viruses	DR3/DR4 (n=25)	DR3 (n=7)	DR4 (n=5)	Others (n=23)	ANOVA F-test	P
CVB_5	40.37	43.32	62.27	29.73	8.585	0.021 (S)
Polio vaccine	34.42	38.4	59.86	25.27	7.689	0.031 (S)
Adenovirus	29.44	22.48	46.02	26.14	5.704	0.041 (S)

Table 4-28: Relation of mean lymphocyte proliferation percentage in response to different viral antigens with the HLA-DQ risky alleles in TIDM patients.

Viruses	DQ2/DQ3 (n=9)	DQ3 (n=15)	DQ2 (n=11)	Others (n=25)	ANOVA F-test	P
CVB_5	42.84	60.90	26.41	33.63	7.975	0.032 (S)
Polio vaccine	39.31	48.09	23.21	27.29	6.695	0.038 (S)
Adenovirus	22.26	37.41	31.37	26.74	5.684	0.042 (S)

4.9 Relation of HLA-DR, -DQ Risky Alleles with Sero-Positive GADA in TIDM Patients

Table (4-29) represented the distribution of sero-positive GADA in patients with HLA-DR risky alleles, and in those with other different alleles.

The proportion of sero-positive GADA in patients with HLA-DR risky alleles were significantly higher (P= 0.001) than those who had other alleles. The DR3/DR4 combination seemed to have the high prevalence (53.33%) compared to DR4 (10.0%) and DR3 (6.67%).

Table 4-29: Distribution of sero-positive GADA in TIDM patients and relation with HLA-DR risky alleles.

Parameter	No.	DR3/DR4 No. (%)	DR3 No. (%)	DR4 No. (%)	Others No. (%)	P
GADA +ve	30	16 (53.33)	2 (6.67)	3 (10.0)	9 (30.0)	0.001 (S)

Chi = 16.523

The results represented in table (4-30) indicate a high proportion of $GADA^+$ in patients carrying DQ3 risky allele (43.33%) comprised to DQ2/DQ3 (23.33%) and DQ_2 (10%). By using chi-square test, the statistical analysis showed a significant differences of sero-positive GADA in patients with DQ risky alleles than those carrying other alleles (P= 0.016).

Table 4-30: Distribution of sero-positive GADA in TIDM patients and relation with HLA-DQ risky alleles.

Parameter	No.	DQ2/DQ3 No. (%)	DQ3 No. (%)	DQ2 No. (%)	Others No. (%)	P
GADA +ve	30	7 (23.33)	13 (43.33)	3 (10.00)	7 (23.33)	0.016 (S)

Chi = 5.059

Discussions

The identification of genetic factors, environmental triggers, the timing of initial events, the progressiveness and persistence of beta cell autoimmunity once initiated, and the role of factors promoting or preventing progression to clinical disease are some of the open issues, in order to develop specific therapies to prevent or delay the onset of TIDM.

5.1 Biochemical and Hematological Characteristics

5.1.1 $HbA1_C$

Since the level of glycosylated hemoglobin has been shown to provide an index of glycemic control during the previous 2-3 months period, it has been used increasingly in the clinical management of diabetes (National Diabetes Data Group, 1984). The results indicated that age has no impact on the degree of metabolic decomposition at the clinical presentation of TIDM.

5.1.2 Serum C-Peptide

Determination of serum C-peptide is useful in diabetic patients. It can distinguish insulin secreted endogenously from insulin administrated exogenously and can quantify the former when anti-insulin antibodies preclude the direct measurement of insulin (Granner, 1996). The level of serum C-peptide is correlated with the level of insulin secreted by the pancreas, so mean C-peptide values seem to be lower in TIDM patients due to a decline in insulin release by the pancreas after beta-cells destruction, in addition to the negative feed back of exogenous insulin (Ganong, 1997). The results showed that there was no statistically difference on beta-cells survival in TIDM patients among the two age groups. However other reports indicated that age was an established factor affecting endogenous insulin secretion in children with recent-onset

diabetes, with lower serum C-peptide concentration recorded in younger children than in older one (Schiffrin *et al*., 1988).

5.2 HLA Association Alleles

Type I diabetes mellitus can be considered as an organ-specific autoimmune disease. It is known that TIDM has been transferred from prediabetic subjects to an HLA identical sibling as a consequence of bone marrow transplantation (Lampeter *et al*., 1993). The present study detected that immunogenetic predisposition may be considered as an important factor for the development of TIDM in association with the HLA antigens in which markers of human HLA showed different distributions in patients, siblings and controls. These markers are HLA class I and class II loci on chromosome 6, in which a highly polymorphic status is recognized (Goldsby *et al*., 2000).

At HLA class I region, significant increased frequencies of antigen A9 and B8 were observed in the patients (about one third for each). Such observation scored RR values of 2.88 and 4.12 respectively. Other HLA TIDM association studies carried out in other world population revealed an association with other HLA-class I antigens B15 in Canadian population (Singal and Blajchman, 1973), B8 and B15 in Finnish population (Nerup *et al*., 1974), in addition to A1, A2, B56, B62, Cw3 and Cw7 (Tuomilehto-Wolf *et al*., 1989), A24 in Japanese (Tanaka *et al*., 2002). Such differences can be explained in the ground of racial differences, especially if we consider that HLA antigens show different frequencies in different populations including Iraqis. Mezal (1988) found HLA-A1 and B8 to be associated with TIDM in Basrah population while Al-Samarrai (2001) found a very high significant association of HLA-A24, B8 and B15 with TIDM in her study conducted in Baghdad. As suggested by the statisticians the RR indicates the frequency of a disease in persons with the HLA marker as compared with persons without the marker. A positive association (when the HLA marker which is more frequent in persons with the disease than

in those without it) is indicates by the RR of more than 1.0, a negative association by a RR less than 1.0, and no association by a RR 1.0 (Klein and Sato, 2000). The EF value can range from 0 (no association) to 1 (maximum association). That means a value of 1 for an antigen is interpreted that this antigen is fully responsible for the development of the disease other wise if the value is in between 0 and 1, it indicates that this marker is partially involved in the disease development (Ad'hiah, 1990), and other factors like environment factors can be involved. The EF value of A9 (0.261) and B8(0.214) support the previous hypothesis and so other factors in association with these antigens are contributed the rest percentage required in the development of TIDM. Other positive association were observed in the tested TIDM patients (B12, B15 and Cw7) but the significance was lost when the probability was corrected for the number of antigens tested 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).

At HLA-class II region, further antigens had positive associations with TIDM. These were DR3 (RR= 3.210, EF= 0.366), DR4 (RR= 7.0, EF= 0.428), DQ2 (RR= 2.833, EF= 0.215) and DQ3 (RR= 2.666, EF= 0.249). The polymorphism of HLA-class II loci has gained much interest in the HLA disease association studies, because both α and β chains are highly polymorphic especially at HLA-DR and DQ (Chapel *et al*., 1999). However, multiple studies have reported association between HLA-DR and DQ phenotypes and TIDM. DQ2.DR3 and DQ3.DR4 haplotypes reported as high risk alleles in Caucasians (Kawasaki *et al*., 1998), DR4, DQ4 but not DR3 were found to be dominant in Japanese (Kawabata *et al*., 2002), while DR3,DR4, DR9 and DQ2 were found the only alleles positively associated with TIDM in Koreans (Park *et al*., 1998). In Finland, DQ2/DQ3 genotype was found to be associated with genetic susceptibility and was more frequent in children diagnosed <5 years of age (Komulainen *et al*., 1999), and in diabetes-associated autoantibodies emerged in

children with predisposing HLA-DQ alleles after 3 months of age (Kupila *et al.*, 2002). In Lebanese 77% and 40% of TIDM patients were positive for DQ2 and DQ3 respectively (Zalloua *et al.*, 2002). Al-Samarrai (2001) reported high significant association of HLA-DR3, DR4, DQ2 and DQ3 with TIDM in Iraqi patients. Studies of HLA genes at the molecular levels showed that this association with HLA-DR is secondary to a stronger link with certain HLA-DQ variants.

The critical factor is the amino acid at position 57 in the HLA-DQB chain. Genetic variants of DQB which encode the amino acid aspartate at this position seem to confer protection against TIDM, whereas variants encoding other amino acids increase the risk. Hence the HLA-DR3 and DR4 association arises because these DR-alleles are linked to DQB alleles which do not encode aspartate (Chapel *et al.*, 1999). It is worthy to note that amino acid 57 in HLA-DQB lies in the "antigen binding groove". It was reported that class I HLA-24 gene promotes pancreatic β-cells destruction in an additive manner in the patients with TIDM-susceptible HLA-class II genes (Nakanishi *et al.*, 1993).

Antigens B35, B51, Cw4, DR2 and DQ1 showed a negative association with the disease, but after correction only the DR2 and DQ1 antigens remain significant. These antigens may have protective effect especially if we consider PF values to be 0.195 for DR2 and 0.163 for DQ1 antigens.

In siblings, a significant increased frequency of antigen B12 and DR4 was observed in comparison with control subjects. But this positive association remains significant only for DR4 antigen after correction with RR value of 2.428 and EF value of 0.176. Concerning other world population studies, HLA-DR4 was found to be associated with the presence of ICA (7%) in siblings of TIDM Mexican-American patients (Zeidler *et al.*, 2001). This locus is known to be associated with TIDM risk particularly with in type 1 diabetes families (Kukreja and Maclaren, 1999). Thus it may be much more useful for predicting TIDM in affected families than in population. Sheehy *et al.* (1989) detected a

highly diabetogenic subset of DR4 haplotypes among TIDM patient's sibling and he suggested that DR typing is 6-10 times less powerful as predictor of TIDM in the population than among patients siblings.

Clearly, the structural differences seen between the predisposing and protective HLA molecules will affect their ability to bind or interact with diabetogenic antigens and the TCRs of autoreactive β-cell specific T-cells (Kelly *et al*., 2003). Several mechanisms have been proposed to explain how this might influence the risk of developing autoimmune TIDM:

1. Predisopsing HLA molecules may bind well to the diabetogenic antigens in the periphery and hence activate an autoimmune T-cell response, whereas protective HLA-molecules may not. Alternatively, the protective moleuceles may bind to the autoantigens with a higher affinity, thus competing with the predisposing molecules. So the threshold of binding required for T-cell activation, restricted by the predisposing molecules, may not be reached (Nepom and Kowk, 1998).
2. Protective HLA molecules may form stable complexes with self antigens in the thymus, leading to efficient deletion of potentially autoreactive T cells (negative selection). In contrast, the less stable complexes formed by the predisposing HLA-molecules may result in inefficient T-cell removal and the release of autoreactive T cells into the periphery (Lowe, 1998).
3. Predisposing and protective HLA molecules may interact differently with the TCRs of autoreactive T cells, affecting the phenotype of the T cells (proinflammatory versus regulatory) (Kelly *et al*., 2003) or their activation status (Proliferative versus anergised) (Serreze *et al*., 2004). This immunomodulatory hypothesis is supported by the observation that DQ1 can protect against the development of diabetes, even after the onset of β-cell autoimmunity (Puglies *et al*., 1995).

The association of the HLA-Class II with TIDM has limited diagnostic utility, but it provides insight into the pathological basis of the disease (Williams, 2001).

5.3 Phenotypic Characteristics of PBL

5.3.1 CD_3^+, CD_4^+, CD_8^+ Cell Subsets, CD_4^+/ CD_8^+ Ratio

This study provides evidence that abnormalities of T-cells regulation are detectable in patients with TIDM. Thus, in newly diagnosed patients, the main alteration found was decrease in the pan T-cells (CD_3^+), Cytotoxic/suppressor T-cells (CD_8^+) accompanied by non significant decrease of helper/inducer T-cells (CD_4^+) subsets. CD_4^+/ CD_8^+ ratio is considered as an index of immune activation or suppression. In TIDM patients CD_4^+/ CD_8^+ ratio was higher than normal controls, although the patients had a lower CD_4^+ cell population than controls. This did not mean that TIDM patients had a good immune activity, but the reverse, their high CD_4^+/ CD_8^+ ratio was due to a lower extent of CD_8^+ cells and also to low CD_4^+ cell population. Thus, in general CD_4^+/CD_8^+ was mainly determined by both CD_4^+ T-cells and CD_8^+ T-cells which was statistically confirmed in this study by revealing a clear direct liner relationship with CD_4^+ cells ($r = 0.83$) and inverse linear relationship with CD_8^+ cells ($r = 0.79$). These results come in agreement with the study conducted by Al-Samarrai, (2001) on TIDM Iraqi patients and also with other reported findings (Buschard *et al.*, 1983; Galluzzo *et al.*, 1984; Quiniou-Debrie *et al.*, 1985; Michalkova *et al.*, 2000). The reduction in the amount of suppressor / cytotoxic cells and inducer / helper could theoretically be due to the metabolic dearrangement of the patients at the diagnosis of TIDM and the examination was done under insulin treatment which affect circulating PBL leading to normalizing the T-cell defect (Roider *et al.*, 1984). This hypothesis is confirmed by Buschard and his team (1990) who found a low percentage of CD_8^+ cells at the diagnosis of TIDM, followed by

normalization in the remission period, and may reflect decreased pathogenetic activity as indicated by constant level of C-peptide.

The most pronounced outcome of the present study is the reduction of the cytotoxic / suppressor phenotype CD_8^+ cells at the onset of the disease. This agrees with the classical theory of pathogenesis of autoimmune disease as the depressed immunological suppressive functions trigger the autoaggressive processes (Roitt *et al.*, 1998). This result is in disagreement with other studies which reported increase in CD_8^+ cells percentage and lowering CD_4^+ cell subsets (Ilonen *et al.*, 1984), or lowering CD_8^+ cells and elevating CD_4^+ cell subsets in TIDM patients (Scheinin *et al.*, 1988; Faustman *et al.*, 1989).

5.3.2 $CD_{45}RA^+$ and $CD_{45}RO^+$ Cell subsets

In the present study, a significant decrease in $CD_{45}RA^+$ cells percentage (naïve/resting) and increase the percentage of $CD_{45}RO^+$ cell subset (memory/activated) were found among TIDM patients. CD_{45} family represents a family of surface protein tyrosin phosphatase, which is present in all human leukocytes. $CD_{45}RA$ antigen present in approximately 50% of CD_4^+ cells, 78% of CD_8^+ cells and essentially on all B-lymphocytes and NK cells (Protein reviews on the Web, 2003). A selective loss of $CD_{45}RA^+$ has been seen in autoimmune disease and viral disease (Malavas and Ferrero, 1999).

$CD_{45}RO^+$ activated marker is present on about 40% of PBL including CD_4^+ and CD_8^+ T-cells population. It is present early in T-cell maturation cycle, but upon activation by mitogen or alloantigens, naïve T-cells loss the $CD_{45}RA^+$ and reciprocally acquire the $CD_{45}RO^+$ antigen (Goldsby *et al.*, 2000). The reduction in the amount of (naive/resting) $CD_{45}RA^+$ cells and increase the proportion of (memory/activated) $CD_{45}RO^+$ cell subsets in TIDM patients may be due to vigorously responds of these cells to recall antigens. A statistical analysis in this study confirmed an inverse linear relationship of $CD_{45}RA^+$ cells with CD_4^+ cells subset (r = -0.601) and direct linear relationship of $CD_{45}RO^+$ cell

with the CD_4^+ (r = 0.621), while a very weak correlation was observed with the CD_8^+ T-cells (r = -0.163). The results in other reports confirmed these finding (Pozzilli *et al*., 1983; Michalkova *et al*., 2000). Persistent elevation in cells of suppressor network (CD_8^+ $CD_{45}RA^+$) and depression in cells of helper network CD_4^+ were found among siblings in prediabetic period (Faustman *et al*., 1989), but render to have normal percentage with elevation of $CD_{45}RO^+$ cells subsets in early onset of the disease. This suggests that abnormal numbers of suppressor cells may represent the primary disease process, and the prediabetic individuals may be susceptible to some process like viral infection that would, then, cause beta-cell destruction.

5.3.3 CD_{38}^+ Cell Subsets

The results detected a very high significant elevated percentage of activation CD_{38}^+ antigen in PBL of TIDM patients. CD_{38} is (ADP/ ribosyl cyclase/ ADP ribose hydrolase) an integral membrane glycoprotein. Human CD_{38} is highly expressed on early T-cell precursors migrating to the thymus and on CD_4^+ CD_8^+ double positive thymocytes. During the process of negative selection, CD_{38}^+ expression is decreased and mature single positive T-cells express low levels of CD_{38} (Mehta and Malavas, 2000). It is present on approximately all pre-B-lymphocytes, in 18% of Th and some Tc cells (Malavasi and Ferro, 1999), and in tissues such as human pancreatic islets (Fernandez *et al*., 1998). In pancreatic beta-cells, this enzyme appears to play a role in glucose induce insulin release via a mechanism involves its cyclase activity which leading to increase cytoplasmic Ca^{+2} concentration and insulin release (Pupilli *et al*., 1999). Mature T-cells isolated from peripheral blood can acquire CD_{38}^+ cell surface expression during antigen activation (Mehta and Malavas, 2000).

A strong positive linear relationship is found between CD_{38}^+ cells and CD_4^+ cells (r = 0.808), with CD_{19}^+ cells (r = 0.602) and with HLA-DR$^+$ cell

population (r = 0.581). CD_{38}^{+} act as positive and negative regulator of cell activation and proliferation depending on cellular environment. Thus, mature B-cells proliferate wheras the opposite occurs in immature B-cells in the bone marrow. The CD_{38} signaling pathway in this environment blocks B-lymophopoiesis, mostly by inducing apoptosis (Funaro *et al.*, 1997). In mature T and B cells, CD_{38} signaling depends on cross talk with the Ag-receptor signaling complexes and rely on the cytoplasmic effector molecules associated with those receptors to transduce CD_{38} signal (Morra *et al.*, 1998). No effect of CD_{38} ligation has been observed on cytotoxi T-lymphocytes, while binding of anti-CD_{38} mcAb to human NK cells induce killing (Sconocchra *et al.*, 1997).

IFN-γ is a strong up modulators of CD_{38}, expression in activated B-cells from either healthy individuals or patients with B-chronic lymphocytic leukemia (Bauvois *et al.*, 1999).

CD_{38} involved in adhesion between human lymphocytes and endothelial cells. CD_{38} ligation by specific mcAb reduced the IFN-γ dependent enhancement of monocyte dynamic adhesion to endothelial layer (Mussa *et al.*, 2001). Presence of autoantibodies with anti- CD_{38} specifity in patients with type I and type II diabetes has been reported to down regulate CD_{38} expression in lymphoid cells (Pupilli *et al.*, 1999).

5.3.4 HLA-DR^{+} Cells

High significant elevation of mean percentage of HLA-DR^{+} activation marker was demonstrated in our patients. It has been suggested that one of the important immunoregulatory abnormalities in TIDM is related to HLA-DR^{+} cells and activation of lymphocytes by different stimuli increases their expression of surface markers (Goldsby *e al.*, 2000). This fact is confirmed in our finding that there were strong positive linear correlation between HLA-DR^{+} and CD_{19}^{+} cells (r = 0.90), CD_{4}^{+} cells (r = 0.78) and inverse negative correlation with CD_{8}^{+} cells (r =- 0.39). Many studies confirm these facts (Drell and Notkins,

1987; Hehmke *et al.*, 1995). Tun *et al.* (1994) found elevation of activated T-cells, HLA-DR^+ in prediabetic twins. Buschard *et al.* (1990) also found higher percentage of HLA-DR^+ cells in TIDM patients at diagnosis and after one month, but their percentage decline after 7 months. In contrast, Al-Samarrai (2001) estimated a decline of HLA-DR^+ cells percentage in type I and type II Iraqi diabetic patients diagnosed within two years of onset.

5.3.5 CD_{56}^+ Cell Subsets

Other lineage of cell subsets is NK cells. Most surface antigen detectable on NK cells by mcAbs are shared with T-cells bearing (CD_{56}) or macrophage (CD_{16}) (Goldsby *et al.*, 2000). In the present study the percentage of these cells bearing CD_{56}^+ antigen significantly decreased in TIDM patients. Although NK cells have powerful antitumor effects, mediating their cytotoxicity by an NK-like effector mechanism that is IL-12 dependent (Takeda *et al.*, 1996). They also serve as regulators for the speed of immune responses by secreting IL-4, IL-13 and pro-Th_2 factor that inhibit Th_1 mediated cytotoxic T-lymphocyte (CTL) responses (Symth and Godfrey, 2000). However, their reduced percentage could lead to the genesis of autoimmunity through a deficiency in Th_2 cell function (Kukreja and Maclaren, 1999). This finding was in agreement with other studies (Hehmke *et al.*, 1995; Michalkova *et al.*, 2000; Kukreja *et al.*, 2002).

5.3.6 CD_{19}^+ Cell subsets

The present finding reported a significant elevation of CD_{19}^+ cell subsets in the patients. TIDM involves the interaction of different subsets of lymphocytes and APCs, (presented by B-cells, macrophages and dendritic cells). We found a strong positive linear correlation is found between CD_{19}^+ cells and cells bearing activated markers, HLA-DR^+ (r = 0.9) CD_{38}^+ (r = 0.602) and $CD_{45}RO^+$ cell subsets (r = 0.60).

The question of whether antigen presentation or production of autoantibodies by B-cells is important in diabetes development. One of the important aspects of the function of B-cells as APCs is the ability to concentrate the soluble antigen many folds by the virtue of the antigen specificity of the B-cell receptor (BCR) (Lanzavecchia, 1990). This will allow antigen-specific T-cells to expand, if appropriately stimulated by relevant B-cell populations. This finding was strengthened by other study conducted by Wong *et al*. (2004) who confirmed that the expression of membrane Ig transgene increased insulitis in NOD mice, and the ability of B-cells to produce antibodies is not necessary for B-cells to have some effect on the development of diabetes. Many potential human studies focus on treatment or prevent early diabetes via depletion of B-cells with anti-CD_{20} treatment (Fong, 2002; NIDDK-American diabetes association Clinical trails, 2006). We presume that such therapy has a much greater effect on B-cells as antigen presentation rather than it does on antibody levels.

According to these results and the results of other investigators listed earlier, in our opinion, the reasons of the variable results could be due to the age of the patients, years of autoimmunity, different in time between the day of diagnosis and examination between days and one years: the insulin treatment, potential effect of hyperglycemia, acid-base disturbances and stress associated with diabetes mellitus on lymphoid subsets.

5.4 Functional Activity of PBL

The use of lymphocyte proliferation is one of the more frequently used "*in vitro*" techniques for the study of the specific and non-specific stimulation capability of lymphocytes. The technique is based on the capability of the lymphocytes for responding to an antigen (specific response) which has induced memory lymphocyte, either by vaccination or by natural infection. These lymphocytes, when they are repeatedly contacted with antigens, have a

blastogenic transformation. This lymphoplastogenicity can also be induced in a non-specific way, due to the lymphocyte-capability to reacting to different mitogens (Chapel *et al*., 1999).

MTT has been used in the measurement of proliferative percentage of PBL which has been found lower in TIDM patients than in healthy controls in response to Con-A. Considering the responses to viral antigens, proliferative responses against CVB_5 and adenovirus were tended to have a lower percentage in TIDM patients than controls, but these values were not statistically different, while the proliferative responses against poliovaccine was significantly lower in patients especially in >10 years old group than controls. The low proliferative responses against CVB_5 antigen at disease onset is in agreement with other studies showing reduced T-cell proliferation against CVB_4 (Varela-Calvino *et al*., 2002), while the same investigators found in previous study, no differences in T-cell proliferation against CVB_4-infected lysate between diabetic patients and healthy-non diabetic individuals (Varela-Calvino *et al*., 2000). Another report conducted by Juhela *et al*., (2000) found that PBL of the children at onset of TIDM had significant weaker responses to purified CVB_4 and non-significant decrease in response to poliovirus type 1 and 3 than healthy children, while the responses to adenoviruse did not differ between patients and controls. Temporary decline in T-cell responsiveness at diabetes onset has also described in GAD peptide that contains the homology region to the CVB_4 2C protein (Schloot *et al*., 1997b).

These studies with the present study results are open to several interpretations. One explanation is that, decreased responses of PBL are due to redistribution of virus-specific T-cells, with virus-responder cells presumed to have homed to the pancreas and therefore unavailable for detection in peripheral blood (Varela-Calvino and Peakman, 2003), and so T-cell responses to various viral antigens may be suppressed at the onset of the disease. On the other hand, Varela-Calvino *et al*. (2002) in his study indicates abundance of circulating

primed CVB_4 specific responder T-cells that secretes IFN-γ in TIDM patients with relative lack of proliferation. These finding have been related to two broadly defined phenotypes of memory T-cells characterized by Sallusto and Lanzavecchia (2001). Primed (memory) T-cells with the capacity to proliferate termed as "central memory" TCM cells. These cells lack immediate effector function and predominantly produce IL-2, the major T-cell growth factor to support proliferation and express CCR_7, a chemokine receptor, that direct homing to lymph nodes. In contrast the primed memory cell subsets that produces the proinflammatory cytokines IFN-γ during an immune response termed "effector memory" subset TEM, those cells donot express CCR_7, present in the circulation at sites of infection or tissue inflammation and release cytokines.

5.5 Cytokines Profile

Cytokines are important to the outcome of an autoimmune disease, TIDM is the result of T-cell mediated destruction of pancreatic β-cell. There are three major mechanisms by which β-cell destruction may occur (Almawi *et al*., 1999):

1. Perforin and granzymes released from the granules of cytotoxic T-cell.
2. Cell death receptors, such as CD_{95} and TNF R1.
3. Inlfammatory cytokines including IL-1, IFN-γ and TNF-α.

5.5.1 IFN-γ

The present data demonstrated that serum IFN-γ concentration were higher in patients with TIDM compared to its concentration in the healthy controls. These data were in common with other studies which stated that proinflammatory cytokines IFN-γ may play an important role in the pathogensis of TIDM, and its concentration was higher in TIDM patients (Varela-Calvino *et al*., 2002; Ozer *et al*., 2003; Al-Zaidi, 2005). Many studies largely support the concept that β-cell destructive insulitis is associated with increased expression of

proinflammatory cytokines (IL-1, TNF-α and IFN-α) (Hussian *et al*., 1996) and Th_1 cytokines (IFN-γ, TNF-β, IL-2) and IL-12 (Kukreja and Maclaren, 1999).

Mechanically, proinflammatory and Th_1 cytokines including IFN-γ induced and accelerated β-cell destruction through direct and indirect mechanisms, directly by:

1. Th_1 cytokines including IFN-γ exerted their effects primarily at the level of macrophages, enhancing infiltration of these cells in the islet, thus accelerating β-cells destruction through the release of performed de novo synthesized cytotixc mediators (nitric oxide, oxygen radicals … etc.) (Karlsen *et al*., 2000; Thomas *et al*., 2002). These radicals can inactivate mitochondrial and cystolic enzymes, leading to decreased ATP levels and impaired insulin secretion.
2. Induced T-cells infiltrate the islets (MHC class I restricted CD_8^+ T-cells) because IFN-γ and TNF-up regulate expression of MHC class I, which in conjugation with autoreactive T-cells could bring about extensive tissue damage on rodents and human β-cells (Kukreja and Maclaren, 1999; Seewaldt *et al*., 2000; Boic, 2004).
3. IFN-γ may render β-cells susceptible to T-cell mediated killing via induction of Fas (CD_{95}) receptor on their surface. Ligation of Fas receptors on β-cells by Fas ligand (CD_{95}L) on CD_4^+ and/or CD_8^+ T-cells has been postulated to be a mechanism of β-cell death by apoptosis in TIDM patients (De-Blasio *et al*., 1999; Amrani *et al*., 2000).

Indirectly by several mechanisms as a result of their capacity to inhibit the production of Th_2 cytokines and Th_2 cell activity:

1. Induced activation and expansion of bystander autoreactive T-cell, resulting in an increase in their overall proportion (Lehmann *et al*., 1993).
2. Inhibited the production of soluble cytokines antagonists including the IL-1 receptor antagonist, which resulted in stimulation of IL-1 production by the macrophages, and in conjugation with continued autoantigenic

stimulation, significant augmentation in the expression of IFN-γ and other Th_1 cytokines (Faust *et al.*, 1996).

A direct linear correlation was found between IFN-γ and PBL proliferative percentage in response to CVB_5 (r = 0.33), poliovaccine (r = 0.33) and adenovirus (r = 0.54). This might indicate a previous exposure of lymphocytes to tested viruses and might enhance the release of IFN-γ by effector memory subsets in response to viruses. Heitmeir *et al.* (2001) proposed a model for the effects of dsRNA (the viral replicative intermediate) plus IFN-γ induced beta-cell damage (Figure 5-1). In the course of viral infection, dsRNA, the active component that activates the antiviral response, stimulates IL-1β expression by beta-cells and IL-1α and IL-1β expression by macrophages. IL-1β requires proteolytic processing for activation, an event that appears to be mediated by IFN-γ induced ICE (IL-1β-converting enzyme) activation in beta-cell. Active ICE cleaves inactive pro-IL-1β to the active mature cytokine resulting in the release of IL-1β by beta-cells followed by autocrine or paracrine stimulation of adjacent beta-cells to express iNOS (nitros oxide sysnthase) and produce nitric oxide (NO). The local release of IL-1 by resident macrophages has also contributed to the IFN-γ induced iNOS expression by the islet results in a potential inhibition of insulin secretion and islet degeneration. However, in the presence of inflammatory T-cells capable of producing IFN-γ, viral infection would be predicted to induce islet cell necrosis in addition to apoptosis, and the necrotic events may elicit two responses: 1- increase islet inflammation because the necrosis of B-cells and 2- release of beta-cell antigens and induction of autoimmunity directed against remaining beta-cells (Scarim *et al.*, 2001).

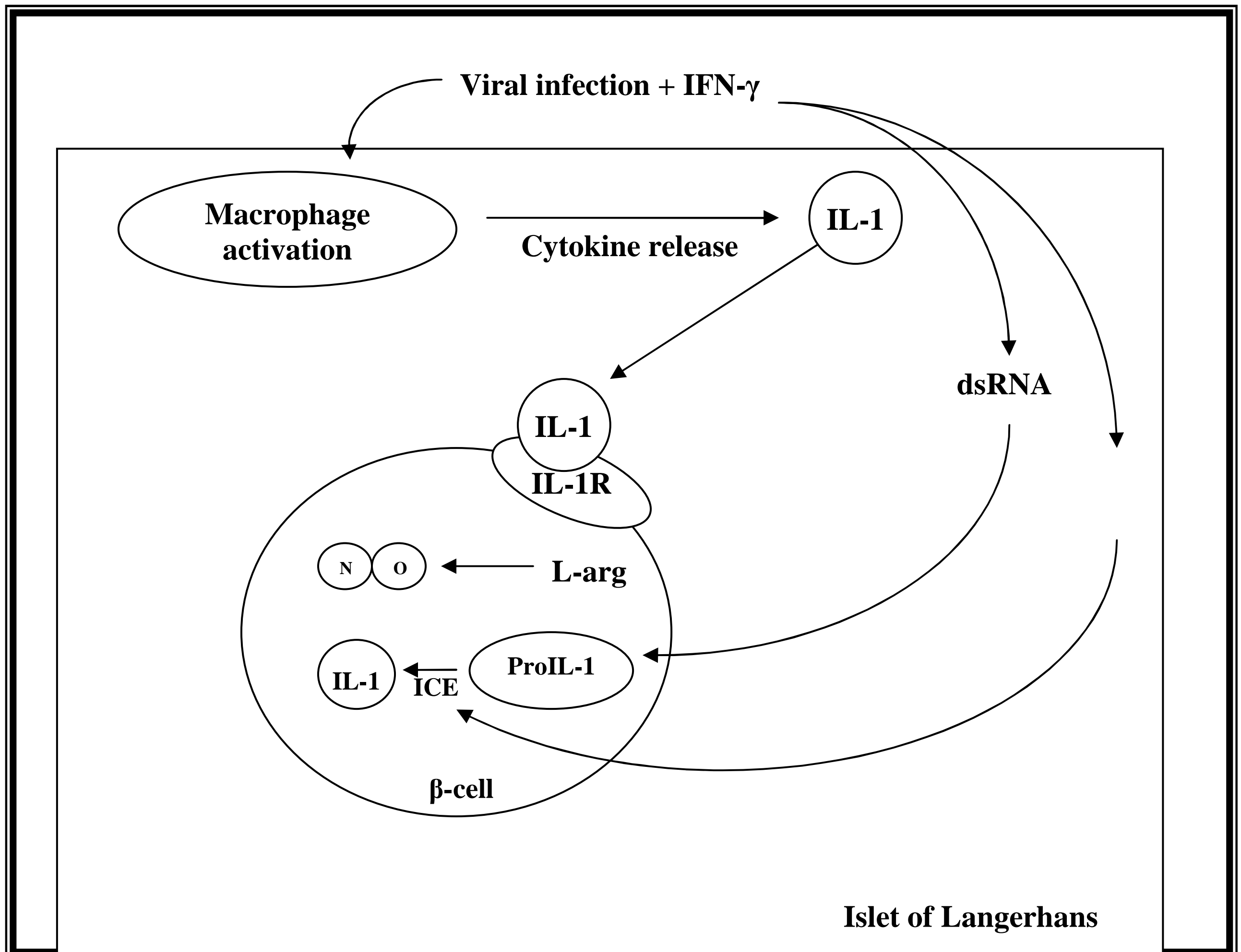

Figure 5-1: Schematic model of viral infection + IFN-γ induced beta-cell damage (Heitmeier *et al*., 2001).

5.5.2 IL-10

TIDM is associated with altered humoral and cellular immunity exemplified by altered production and response to macrophage and T-cell derived cytokines and T-helper cell differentiation (Kukreja and Maclaren, 1999).

The results of this study indicated a high level of serum IL-10 in TIDM patients compared to healthy controls. This result was encountered to many reports which found that TIDM could be prevented by induction of Th_2 cells or

by treatment with Th_2 cytokines which in turn blocked the production of Th_1 cytokines (Kirsten *et al.*, 1998). In contrast, other reports pointed against the anti-inflammatory action of Th_2 cytokines. Th_2 cytokines (IL-10 but not IL-4) were shown to be involved in TIDM pathogenesis through facilitation of pancreatic mononuclear cells infiltration (Tian *et al.*, 1997) and producing intense and generalized pancreatitits and insulitis associated with islet cell necrosis in NOD mice (Pakala *et al.*, 1997). Another report by Schloot *et al.* (2002) conducted on NOD mice, found that serum levels of IFN-γ were initially low but increasingly reaching the highest levels at diabetes onset. In contrast an early peak of serum IL-10 level was observed initially, but continued loss of IL-10 until progression toward diabetes was observed, confirming the fact that IL-10 was essential for an early phase of diabetes. This promoted the conclusion that TIDM is a Th_1 and Th_2 mediated autoimmune disease.

Functionally, Th_2 cytokines exert their effects through direct or indirect mechanisms:

1. Th_2 cytokines, in particular IL-10, may promote necrosis through occlusion of the microvasculature, thereby resulting in hypoxia and reducing the viability of the larger islets (Almawi *et al.*, 1999).
2. IL-10 is a potent B-cell activator, enhances MHC class II expression on B-cells, thus promoting peri-insulitis and insulitis (Gianani and Sarvetnick, 1996) or by altering the expression of endothelium-bound addressin, thereby stimulating accumulation of macrophages and B-cells (Wogensen *et al.*, 1994).
3. Due to its role as cytotoxic T-cell stimulatory factor, IL-10 may stimulate activated T-cells (Balasa *et al.*, 1998) and its essential for an early phase of diabetes.

In any event, Th_2 cytokines can no longer be viewed as "protective" of TIDM.

5.5.3 IL-6

The inflammatory cytokine IL-6, originally secreted from T-cells, B-cells and several non-lymphoid cells including macrophages, fibroblast endothelial cells and bone-marrow stromal cells (Goldsby *et al*., 2000).

The present results indicated an elevated levels of serum IL-6 in TIDM patients as compared to controls that added to the evidence that the disease is an immunoinflammatory disorder. This result is incommon with other reports (Myrup *et al*., 1996; Targher *et al*., 2001).

IL-6 is a powerful inducer of hepatic acute phase protein (C-reactive protein) which is known to increase inflammation and the development of vascular disease and atherosclerosis (Mendall *et al*., 1997). Elevated C-reactive protein level detected in infants and young children before the onset of TIDM (Chase *et al*., 2004) may provide an additional marker for risk of progression to TIDM. IL-6 is a "co-stimulatory signal" for T-cell activation made by certain APCs, IL-6 previously known as B-cell differentiation factor. It acts on most cells, but is particularly important in inducing B-cells to differentiate from antibody-forming cells (Roitt *et al*., 1998). A positive significant correlation is found between serum IL-6 level and CD_{19}^{+} cell subsets (r = 0.391).

IL-6 production is stimulated by pro-inflammatory cytokines IL-1 and TNF-α in human fibroblast (Zhang *et al*., 1988). The same observation was found in murine islet culture producing IL-6 in responses to a combination of IFN-γ and TNF-α (Campbell *et al*., 1989).

Mechanically, IL-6 may exert its effect by inducing a condition with increased energy expenditure in the islet, through elevated glucose oxidation and oxygen uptake accompanied by a partial inhibition of the glucose stimulated insulin release and lowering the islet cellular ATP contents (Sandler *et al*., 1990).

Viruses can also interact with and modulate the cytokine responses to IL-6. A possible link between virus infection and elevated IL-6 was postulated by

Kishimoto (1992). A nuclear factor controlling IL-6 gene expression (NF-IL-6) was also involved in the transcriptional regulation of various acute-phase protein genes. The NF-IL-6 was shown to recognize the enhancer core sequence of several viruses, suggesting a possible relationship of virus infection and IL-6 expression and possible autoimmune clinical outcome.

For all that mentioned before, soluble cytokines measured in the periphery are therefore promising surrogate markers of diabetes development and monitoring disease activity.

5.6 Immunoglobulins and Complement Profile

5.6.1 Total Immunoglobulins (Igs)

Igs are circulating antibodies that protect their host by binding to and neutralizing some protein toxins, blocking the attachment of some viruses to cells, opsonizing bacteria, activating complement and activating NK cells (Ganong, 1997). They are soluble forms of B-lymphocyte antigen receptor (Goldsby *et al*., 2000). Our data demonstrated elevated level of Igs in TIDM patients which act as simple indicator to the frequency of infection in these patients. The increased serum levels of total IgM, IgG, and IgA in patients have also been demonstrated by Rayfield *et al*. (1982). On the other hand, the results of the present study are in disagreement with other workers who reported hypoimmunoglobin-G in TIDM patients accompanied with ketonuria (Shariff, 1982), while others found a non-significant elevation of serum Igs levls between diabetic patients and healthy controls (Hulnisky *et al*., 1990; Al-Hajaj, 2005).

5.6.2 Complement C_3 and C_4 Components

The complement system is a part of innate immune system composed of about 30 glycoproteins synthesized in the liver (Chapel *et al*., 1999). The complement test may be ordered to help diagnose the cause of recurrent microbial infection, and it may be used to help diagnosis and monitor the

activity of acute or chronic autoimmune disease (Fischbach, 1999). This study reported significant decreased levels of serum C_3 and C_4 in patients with TIDM than in healthy controls.

The decreased levels of serum C_3 and C_4 have also been reported by several investigators (Hulinsky *et al.*, 1990; Al-Hajaj, 2005). It appears likely that, the explanation of finding of low serum complement is the hyperglycemia, in which the blood sugar binds with the several sites on both α and β chains of C_3 and C_4 (non-enzymatic glycosylation), and elevate the glycosylated part of complement which can not be detected by conventional methods (Peak *et al.*, 1989). Binding of glucose to the active sites of C_3 may prevent the adhesion of C_3 on the microbial surface and hence impaired opsonization which in turn increased the infection in those patients (Hosteller *et al.*, 1990). It was known that many autoimmune antibodies present in the serum of diabetic patients, which binds to the complement and may in turn reduce its level in the serum (Roitt *et al.*, 1998).

5.6.3 Anti-GAD Autoantibodies

GAD autoantigen is neither beta-cell nor islet specific and is expressed predominantly in the nervous system and other tissues, including the testes, ovary, adrenal, pituitary, thyroid and kidney (Winter *et al.*, 2002).

The present results indicated that older children were more often tested positive than younger ones in females. This difference seems to be not significant between males in both age groups (table 4-19).

This result is in disagreement with Sabbah's (2000), but in agreement with Graham *et al.*, (2002) which indicated that GADA was less affected by age at clinical onset in patients than other autoantibodies marker. Islet cells reactivity as judged by the presence of antibodies to the GADA-65 were observed in 50% of the patients studied in both age groups (table 4-20), (55.6%) in >10 years old group were females. Our observation is in consistent with other studies and

supports the notion that autoimmunity is more common among females more than 10 years old (Raise *et al*., 1996; Sabbah, 2000; Graham *et al*., 2002).

The functional role of GADA in the pathogenesis of TIDM comes from their relationship to T-cell reactivity to GAD-65 autoantigen. Presentation of an immunodominant T-cell epitope from the human GAD-65 autoantigen is enhanced by GAD-65 autoantibodies through increasing the efficiency of antigen capture by APCs including Fc receptor (FCR)-Positive monocytes/macrophages (Reijonen *et al*., 2000). Since the human GAD-65 sequence contain a region duplicated in the P_2C protein of Coxsackie virus (Varela-Calvino *et al*., 2000), it has been speculated that a cellular response to the homologus viral sequence could induce T-cell reactivity to GAD-65 and as a consequence produce diabetes.

5.6.4 Anti-Viral IgG

Viral involvement has long been suggested in the etiology of TIDM.

The present results described finding of IgG antibodies against CVB_5 to be more frequent (20%) in TIDM patients than in controls (8%). A low prevalence of specific CVB-IgG may be due to use only one CVB serotype (CVB_5) and there may be another CVB serotype in the sera of TIDM patients which is not detected. The frequency of IgG antibodies against poliovirus (Oral sabin) was more (31.67%) in diabetic patients than in controls (26%). Also IgG antibodies against adenovirus were detected in only four diabetic children (6.67%).

The presence of CVB5, poliovirus and adenovirus specific IgG antibodies are evidence of previous infection in TIDM children. This fact was confirmed by measuring the PBL proliferative percentage in sero-positive IgG diabetic children *in vitro* in response to CVB5, poliovirus and adenovirus, and the results indicated a high significant mean proliferative percentage for all tested viruses in those patients as compared to the sero-negative IgG diabetic children, Table

(4-24), (4-25), (4-26). This means that PBL of sero-positive IgG patients were boosted earlier either by natural infection or vaccination.

The low prevalence of anti-polio-IgG determined in healthy children may indicate a failure of poliovaccine to enhance the immune system, although these children presumely had taken many boosted doses of oral poliovaccine.

Several studies have found CVB-specific IgM antibodies to be more common in newly diagnosed children compared to healthy individuals (Frisk *et al*., 1985; Juhela *et al*., 2000; Yin *et al*., 2002). Others detected an increase of anti-enterovirus antibody levels (both IgM and IgG) preceding the appearance of signs of autoimmunity reflected either by synthesis of several autoantibodies or the development of clinical disease (Lonnrot *et al*., 1998). However, not all studies seeking association between the enterovirus infections (determined by increase in anti-EV antibodies) have reported positive results. Tuvemo *et al*. (1989) and Emekdas *et al*. (1992) found no evidence of increased antibody frequencies against CVB1-6 serotypes at the onset of childhood diabetes. Buschard and Madsbad (1984) demonstrated a lower antibody titer against CVB3-5 serotypes and adenovirus -7 in newly diagnosed TIDM children than in healthy controls.

These discrepancies could be due to the fact that in all these studies, the determination of viral infection was carried out indirectly through the determination of anti-viral antibodies and it is noteworthy that studies used multiple approaches to identify these viruses (serology, PCR, Faeces analysis) appear more likely to report an association with TIDM or islet autoimmunity, suggesting that the sensitivity of viral detection is an important factor.

Enteroviurses could be involved in the pathogenesis of TIDM by several different mechanisms:

1. During infection, viruses may reach the pancreatic islet and destroy insulin-producing beta-cells by virus-induced cytolysis (Roivainen *et al*., 2000).

2. Alternatively, beta-cell damage might result from virus-induced inflammatory reactions through producing inflammatory cytokines (IL-1β, IFN-α… etc.) (Chehadeh *et al*., 2000).
3. In addition beta-cell destruction might be based on molecular mimicry, because immunological cross-reactions between enteroviruses and beta cell autoantigens (GAD-65, Tyrosin phosphatease IAR/IA3 can take place at least *in vitro* (Harkonen *et al*., 2003).

5.7 Relation of Lymphocyte Proliferation with HLA

The present results indicated that stronger T-cell proliferation in response to CVB5, poliovaccine and adenovirus were related to HLA-DR_4 allele and HLA-DQ3 allele; whereas the HLA-DR3 and HLA-DQ2 were associated with weak responsiveness to the same antigens, table (4-27) (4-28). These results are in agreement with a report by Bruserud and Colleagues, (1985) who found that DR4, which is in linkage disequilibrium with the HLA-DQB1*0302 allele, associates with strong T-cell responses; whereas HLA-DR3 associated with HLA-DQB_1*02 allele associates with weak T-cell responses to enterovirus antigens. Juhela *et al*. (2000) reported the same observation in T-cell responses to enterovirus antigens in TIDM patients.

5.8 Relation of GADA with HLA

The results in table (4-29) indicated that GADA were found at the highest levels in index cases carrying DR3/DR4 heterozygous. This indicates that GADA expression is regulated genetically. It is known that there is an over-representation of DR3/DR4 heterozygous subjects among young children with newly diagnosed TIDM as compared with adolescents and adults with recent-onset disease (Karjalainen *et al*., 1989). These observations support the concept that a strong genetic susceptibility is associated with aggressive rapidly

progressing beta-cell destruction as reflected by marked GADA responses and clinical manifestation of TIDM at young age, while a weaker genetic predisposition results in a slower destructive process and disease presentation in adults. In this study a low frequency of GADA is observed in the patients who were homozygous for DR3. In contrast, Hagopian *et al*. (1995) and Sabbah (2000) reported that increased GADA concentration was the charactistic of DR3/DQ2 haplotypes. Another study conducted by Endi *et al*. (1997) reported that only T-cell reactive with GAD-derived peptides in the context of DR-heterodimers could be isolated form the periphery of TIDM patients, indicating that HLA-DR rather than DQ seems to be the principle restriction element used by T-cells present at the onset of the disease.

Conclusions and Recommendations

6.1 Conclusions

1. Significant increase of the $HbA1_C$ percentage in most newly diagnosed patients, and no impact of the age of children on the metabolic decomposition.
2. Significant decrease of serum levels of C-peptide in diabetic patients compared to healthy controls, but no significant differences appear between both age groups.
3. The HLA-class I (A9 and B8) and class II (-DR3, DR4, DQ2 and DQ3) antigens were significantly increased in TIDM patients and they played an important role in the etiology of the disease, while DR2 and DQ1 antigens were significantly decreased in the patients.
4. In siblings a significant increase was observed in HLA-DR4 antigen compared to control group.
5. Defining of PBL phenotypes by means of CD markers showed no significant differences in the percentage of CD_4^+ cell subsets in the patients in comparison to controls, while a significant decrease of CD_3^+, CD_8^+, $CD_{45}RA^+$ and CD_{56}^+ cells percentage with significant elevation of CD_4^+: CD_8^+ ratio were observed.
6. A significant elevation of activation markers $CD_{45}RO^+$, CD_{38}^+ and HLA-DR^+ cells percentage with the CD_{19}^+ cell subset were detected in the patients.
7. Although the PBL proliferation percentage showed no significant differences between patients and controls in response to CVB5, adenovirus except poliovaccine; proliferative percentage was significantly higher in patients who were sero-positive for CVB5; Poliovirus and

Adenovirus specific IgG compared to sero-negative IgG patients for the tested viruses.

8. Strong T-cell proliferation in response to CVB5, poliovaccine, and adenovirus were related to the HLA-DR4 and HLA-DQ3 risky alleles, whereas the HLA-DR3 and HLA-DQ2 alleles were associated with week responsiveness to the same antigens.
9. Cytokines are important to the outcome of autoimmune diabetes, which were indicated by a significant elevation of serum levels of IFN-γ, IL-10 and IL-6 in the patients.
10. Significant increase in mean serum levels of total Igs has been reported in TIDM patients compared to controls.
11. Significant decrease in mean serum levels of C_3 and C_4 complement components in TIDM patients.
12. GADA were present in 50% of diabetic children. Older children were tested positive for GADA more than younger ones, especially females.
13. High proportion of GADA was found in the TIDM patients carrying HLA-DR3/DR4 heterozygous.
14. A high proportion of anti-CVB5 IgG and anti-polio IgG were found in diabetic patients compared to controls, while anti-adeno IgG were detected in diabetic patients only.

6-2 Recommendations

1. It is potentially valuable to predict TIDM in siblings by screening for HLA risky alleles in correlation with autoantibodies such as GADA, ICA, IAA, and also C- reactive protein because early treatment of TIDM with tight glycemic control preserves beta-cell function, prevents the development of diabetic ketoacidosis and provides an opportunity for entrance into trails to prevent TIDM.
2. Further studies of the induction of Fas (CD_{95}) receptor on beta-cells and ligation with ($CD_{95}L$) on the surface of CD_4^+ or CD_8^+ cells as possible role of autoimmune beta-cell death are recommended.
3. Measuring other mediators like nitrous oxide (NO) which may reflect the disease severity.
4. Using molecular techniques (PCR) to identify the viral infection in addition to serological methods which appear more likely to report an association with TIDM.

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Appendix – I: Basic information sheet for children and adolescent patients

Name		**Date of birth**		**Sex**		
Diabetes since		**Associated diseases**	**Celiac**	**Thyriod**	**Obesity**	**Others**
Age of onset						
Family history	**TIDM**	**Father**	**Mother**	**Brother**	**Sister**	**Others**
	TIIDM					
Feeding history	**Breast / months**		**Cow milk**		**Mixed**	
Psychosocial history						
Infectious diseases						
Vacciation						
Occupation		**Social state**				
Previous hospital admissions						

Appendix II: HLA- antisera which are used in the present study.

HLA- A	HLA- B	HLA- C	HLA- DR	HLA- DQ
A1	B4	C1	DR1	DQ1
A2	B5	C2	DR2	DQ2
A3	B6	C3	DR3	DQ3
A9	B7	C4	DR4	
A10	B8	C5	DR5+8	
A11	B12	C6	DR6+7+12	
A23	B13	C7	DR7	
A24	B14		DR7+9	
A25	B15		DR7+12	
A26	B16		DR8	
A28	B17		DR8+12	
A29	B18		DR10	
A30	B27		DR11	
A31	B35+53		DR14	
A32	B37		DRw52	
A33	B38		DRw53	
A34	B39+67			
A2+28	B40			
A25+32	B41			
A28+33	B44			
A28+32+31+10+29	B45			
	B49			
	B49+50			
	B51+52+53+5			
	B55			
	B62			
	B63			
	B65			
	B73			
	B73+27			
	B73+7			
	B60+12+21+70			

CV

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العنوان الإنكليزي:

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Evaluations of Immunogenetics, Viral infections, Humoral and cellular immune Responses in Children with Type 1 Diabetes Mellitus

A Thesis
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Dedication

To my parents, brothers and sisters who backed me to be

To memory of my brother Salah

List of Abbreviations

ALP	Alkaline Phosphatase
APC	Antigen Presenting Cells
BFA	Buffered Formal Citrate
BSA	Bovine Serum Albumin
Con-A	Concavalin-A
CPM	Count per minute
CTLA-4	Cytotoxic T-Lymphocyte Antigen Gene
CVA	Coxsackie virus A
CVB	Coxsackie virus B
DAISY	Diabetes Autoimmune Study in the Young
DCs	Dendritic Cells
DDW	Deionized Distilled Water
DM	Diabetes Mellitus
EF	Etiological Fraction
Evs	Enteroviruses
FCS	Fetal Calf Serum
GAD_{65}	Glutamic Acid Decarboxylase
GADA	Glutamic Acid Decarboxylase Autoantibodies
hCMV	Human Cytomegalovirus
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
HRP	Horse Reddish Peroxidase
HSP_{70}	Heat Shock Protein
IA-2A	Insulinoma-Associated Protein 2 Antibodies
IAAs	Insulin autoantibodies
ICAs	Iset cell Autoantibodies
IDDM	Insulin Dependent Diabetic Mellitus
IF	Immunofluorescence
IGF_2	Insulin-Like Growth Factor 2 Gene
IFN-γ	Interferone Gamma
IL	Interleukin
iNos	Nitric Oxide Synthase
IP	Immunoperoxidase
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MODY	Maturity Onset Diabetes in the Young
MTB	Magnesium Tris Buffer
NAD	Nicotinamide Adenin Dinucleotide
NDDG	National Diabetes Data Group
NIDDK	National Institute of Diabetes and Diagestive and Kidney

	Disease
NIDDM	Non Insulin Dependent Diabetes Mellitus
NK	Natural Killer
OGTT	Oral Glucose Tolerance Test
PBLs	Peripheral Blood Lymphocytes
PBS	Phosphate Buffer Saline
PF	Preventive Fraction
PNPP	P-nitorphenyl phosphate
RIA	Radioimmunoassay
RPMI-1640	Roswell Park Memorial Institute-1640 Medium
RR	Relative Risk
SRID	Single Radial Immunodiffusion
TGF-β	Transforming Growth Factor-Beta
TIDM	Type 1 Diabetes Mellitus
TMB	Tetramethyl benzidine
TNF-α	Tumor Necrotic Factor-Alpha
VNTR	Variable Number of Tandem Repeats
WHO	World Health Organization

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Supervisor Certificate

We certify that this thesis was prepared under our Supervision at the College of Science, Al-Nahrain University, as Partial Fulfillments of the Requirements for the degree of Doctor of Philosophy in Biotechnology / Immunology.

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m

اللَّهُ نُـورُ السَّـمَوَات ِوَالأَرْضِ مَثَـلُ نُـورِهِ
كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ المِصْبَاحُ فِي زُجَاجَةٍ
الزُّجَاجَـةُ كَأَنَّهَـا كَوْكَـبٌ دُرِّيٌّ يُوقَـدُ مِـن
شَـجَرَةٍ مُّبَارَكَـةٍ زَيْتُونَـةٍ لاَّ شَـرْقِيَّةٍ وَلاَ
غَرْبِيَّةٍ يَكَادُ زَيْتُهَا يُضِـيءُ وَلَوْ لَمْ تَمْسَسْـهُ
نَـارٌ نُّورٌ عَلَـى نُـورٍ يَهْدِي اللَّهُ لِنُـورِهِ مَنْ
يَشَـاءُ وَيَضْـرِبُ اللَّهُ الأَمْثَـالَ لِلنَّـاسِ وَاللَّهُ
بِكُلِّ شَيْءٍ عَلِيمٌ (٣٥)

صدق الله العظيم
سورة النور / الآية ٣٥

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تقييم الوراثة المناعية والأخماج الفايروسية والاستجابة المناعية الخلطية والخلوية للأطفال المصابين بداء السكري النوع الأول

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الخلاصة

تم تصميم هذه الدراسة للتحري عن دور مستضدات التطابق النسيجي - للصنف الاول والصنف الثاني في احداث المرض ، التغييرات الخلوية للخلايا اللمفية في جهاز الدوران والتي تشمل فحص الواسمات السطحية (CD) وفحص التحول للخلايا اللمفية باساتخدام المشطر وباستخدام بعض الفايروسات والتي يفترض ان لها دور كأحد مسببات مرض السكري من النوع الاول مثل فايروس Coxsackie B وفايروس شلل الاطفال وفايروس الـ Adenoبالاضافة الى تحديد معدل المستويات المصلية للحركيات الخلوية Cytokines والكلوبيولينات المناعية IgM,IgG,IgA والمكونين الثالث C3 والرابع C4 للعامل المتمم والاجسام المضادة الذاتية لـ GAD بالاضافة الى قياس المستويات المصلية للكلوبيولين المناعي IgG والخاص بفايروس CVB وفايروس شلل الاطفال وفايروس الـ Adeno لغرض معرفة هل هناك تعرض مسبق لمرضى السكري لهذه الاصابات الفايروسية.

شملت الدراسة (٦٠) مريضا حديثي الاصابة بمرض السكري النوع الاول (مشخصين بالاصابة خلال فترة اقل من خمسة اشهر) تم اختيارهم من المركز الوطني للسكر / الجامعة المستنصرية. جميع المرضى هم تحت المعالجة اليومية بجرعات الانسولين . تراوحت اعمار المرضى من (٣-١٧) سنة وتم تقسيمهم الى مجموعتين اعتمادا على العمر : ٣٦ مريض مساوي او اقل من عشرة سنوات و ٢٤ مريض اعمارهم اكثر من عشرة سنوات . بالاضافة الى ذلك شملت الدراسة عينة سيطرة مكونة من ٨٠ شخص (يبدون اصحاء) متطابقون من حيث العمر ، الجنس ، العامل العرقي (عراقيين عرب) وذلك لغرض فحص مستضدات التطابق النسيجي . من ضمن عينة السيطرة تم الاختيار العشوائي لـخمسون شخص لغرض تكملة بقية الفحوصات كما هو الحال لمجموعة المرضى. تم تقسيم هؤلاء الاشخاص وحسب اعمارهم الى مجموعتين ، الاولى تضم ٢١ طفل اعمارهم مساوية او اقل من عشرة سنوات والثانية تضم ٢٩ طفل اكثر من عشرة سنوات . كذلك شملت الدراسة مجموعة ثالثة من اخوة المرضى مكونة من ٥٠ فرد لغرض اجراء فحص مستضدات التطابق النسيجي وتراوحت اعمارهم من (٣-١٦) سنة .

سجلت الدراسة الحالية النتائج المهمة التالية :

- زيادة ملحوظة في نسبة الهيموغلوبين السكري **HbA1c** مع انخفاض معنوي للمستويات المصلية للببتيد – **C –peptide** في مرضى السكر حديثي التشخيص . ولم يسجل اي تأثير للعمر على الفعاليات الايضية .
- بالنسبة لمستضدات التطابق النسيجي- الصنف الاول ، اظهر المرض زيادة معنوية في تكرار **A9** (٤٠,٠٠ ضد **18.75%**) و **B8** (**28.33** ضد**8.75%**) عند المقارنة مع عينة السيطرة. بينما اظهرت مستضدات التطابق النسيجي الصنف الثاني زيادة معنوية في المستضد **DR3** (**53.33** ضد ٢٦,٢٥%) وللمستضد**DR4**(٥٠.**00**ضد ١٢.**50**%) وانخفاض معنوي في المستضد **DR2** (**6.6**٦ ضد ٢٥.**00**%) عند المقارنة مع مجموعة السيطرة. بالاضافة الى هذا فان مرض السكري النوع الاول اظهر ارتباط معنوي مع المستضد **DQ2** (**33.33** ضد ١٥.**00**%) والمستضد **DQ3** (**40.00** ضد **20.00%**). كما تصاحبت هذه الزيادة المعنوية في اخوة المرضى مع المستضد **DR4** (**34.00** ضد **12.5%**) عند مقارنتهم مع مجموعة السيطرة .
- اظهرت الخلايا اللمفية الموجبة للواسمات **CD3,CD8,CD45RA,CD56** انخفاضا معنويا ملحوظا ولكن هذا الانخفاض لم يكن معنويا للخلايا الموجبة ل **CD4** عند المقارنة مع عينة السيطرة. بالمقابل اظهرت النتائج زيادة واضحة في الخلايا الموجبة للواسمات الفعالة والتي تتضمن **CD45RO,CD38,HLA-DR** في دم المرضى بالاضافة الى زيادة معنوية في نسبة الخلايا الموجبة للواسم **CD19** ونسبة **CD4:CD8** في دم المرضى .
- لم يظهر فحص التحول للخلايا اللمفاوية فروقا معنوية في استجابة الخلايا اللمفاوية اثناء التحفيز بالمشطر **Con-A** وكذلك باستخدام الفايروسات **CVB** و **Adenovirus** كمستضدات في مجموعة المرضى مقارنة بمجموعة الاصحاء ولكن نسبة التحول للخلايا اللمفية وبعد تحفيزها خارجيا بلقاح شلل الاطفال اظهرت انخفاضا معنويا عن مجموعة الاصحاء . لوحظ ان هناك استجابة قوية في نسبة التحول للخلايا اللمفية عند تحفيزها بالمستضدات الفايروسية قيد البحث عند الاشخاص الحاملين لمستضدات **HLA-DR4** و **DQ2**.

- لقد اظهرت المستويات المصلية لكل من IFN كاما ، IL-10 و IL-6 ارتفاعا معنويا في مجموعة المرضى بعد فحصها بطريقة ELISA.
- اما بالنسبة لنتائج بعض المؤشرات المناعية الدموية المستخدمة قيد الدراسة فقد سجلت مستويات الكلوبيولينات المناعية صنف IgM, IgG, IgA زيادة معنوية في مجموعة المرضى بينما سجلت الدراسة انخفاضا معنويا للمستويات المصلية للمكونين الثالث C3 والرابع C4 للمتمم .
- الاجسام المناعية الذاتية لGAD ظهرت في ٥٠% من الاطفال المرضى وخاصة الاعمار الكبيرة وفي الاناث (55.6%) اكثر من الذكور (47.8%) بالاضافة الى هذا فان النسبة العظمى من هؤلاء المرضى هم من حاملي المستضد الهجين HLA-DR3/DR4 .
- وجود نسبة كبيرة من المضادات الخاصة بالكلوبيولينات المناعية IgG ضد فايروس CVB وفايروس شلل الاطفال في الاطفال المرضى مقارنة بالاطفال الاصحاء بينما لوحظ وجود الكلوبيولينات المناعية نوع IgG والخاصة ضد فايروس ال Adeno في عينة المرضى فقط.

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