

**IMMUNOLOGY IN DIABETES:**

**Humoral and cellular immunological studies on  
the pathogenesis of type 1 diabetes,  
insulin treatment,  
diabetic vascular complications  
and diabetic pregnancy.**

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To my father

who was introducing me to life  
when he died too soon

## DECLARATION

I hereby declare that the preparation and writing of this thesis has been carried out by myself. The research described in this thesis was conceived and conducted by myself, unless otherwise stated. In the case of studies involving collaboration with other workers, the contribution of these workers is acknowledged individually in the relevant sections of the thesis and in the published papers arising therefrom. In these collaborative studies, the immunological investigations, and in particular the assays of immune complexes, insulin antibodies, insulin-anti-insulin complexes, islet autoantibodies and circulating islet cell antigens, were done by myself.

Umberto DI MARIO

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## A B S T R A C T

In the last decade the immunological studies in diabetes have substantially modified some of the basic aspects of diabetology and specifically the classification of the disease itself.

The aim of the thesis is to investigate further some of the immune phenomena present in diabetes focussing attention on the immunological factors in the pathogenesis of type I diabetes, the immunological consequences of insulin treatment, the immunological phenomena in diabetic vascular complications and the physiopathological immune correlations between diabetic women and their neonates.

The immune phenomena investigated include organ specific auto-antibodies (among others islet cell antibodies), insulin antibodies, insulin-anti-insulin complexes, T cells subsets and activated T cells. Furthermore, circulating specific islet cell antigens were sought by an immunological approach. A wide range of monoclonal antibodies against islet cell antigens and T cell surface antigens have been used. In most of the techniques used, there have been personal technical modifications or developments.

The studies on the pathogenesis of type I diabetes were performed on a large group of insulin dependent diabetics at diagnosis and at regular intervals thereafter and in an uncommon and interesting group of diabetics with islet cell antibodies but not requiring insulin at diagnosis. Islet cell antibodies, and complement-fixing islet cell

antibodies, appear to be important markers in monitoring the islet cell damage, in detecting patients with other subclinical autoimmune disorders and in finding those diabetics, initially not requiring insulin, rapidly developing an insulin failure. Immune complexes have been found in circulation in newly diagnosed type I diabetic patients. In addition to the antibodies to islet cell and the immune complexes, some islet cell antigens also seem to be in circulation in type I diabetics, as determined by a new technical approach involving anti-islet monoclonal antibodies.

Studies on the consequences of insulin treatment were performed in type I diabetic patients within the first year of the insulin treatment and in long standing diabetics. They have shown that conventional heterologous insulin treatment has immunological consequences as determined by insulin antibodies, insulin-anti-insulin complexes and antigen non specific immune complexes and that human insulin is the least immunogenic of the currently available insulins.

The investigation on diabetic vascular complications has shown the presence of circulating immune complexes in patients with severe microangiopathy. The correlation has been demonstrated between this increase and an impairment of macroaggregate clearance by macrophages. A potentially important finding was the correlation between the increased levels of immune complexes and/or insulin-anti-insulin complexes and abnormalities of platelet specific proteins in diabetics with proliferative retinopathy. An inverse correlation was found between the presence of complexes and the levels of the T lymphocytes with the T8 phenotype.

The findings of the studies on the immunology of diabetic pregnancy have suggested a role for insulin antibodies and immune complexes in some maternal and neonatal clinical complications. Insulin-anti-insulin complexes were first described in the cord blood of neonates with characteristics different from those found in the diabetic mothers at the end of pregnancy. An interesting finding in pregnant diabetics was a higher increase in mononuclear cells expressing the 4F2 antigen than in those expressing MHC class II antigens, suggesting that also alerted but not fully activated T cells are present.

## ABBREVIATIONS

|           |   |
|-----------|---|
| AgAb      | Circulating immune complexes            |
| AID       | Autoimmune disorders                    |
| AHG       | Aggregated human globulins              |
| Clq-SP    | Solid phase Clq binding test            |
| CF-ICA    | Complement fixing islet cell antibodies |
| ICA       | Islet cell antibodies                   |
| ICSA      | Islet cell surface antibodies           |
| IDD       | Insulin dependent diabetes              |
| InsAb     | Insulin antibodies                      |
| Ins/iAb   | Insulin-anti-insulin complexes          |
| KgBt      | Conglutinin binding test                |
| MHC       | Major histocompatibility complex        |
| NIDD      | Non insulin dependent diabetes          |
| 4F2       | Monoclonal antibodies (see text)        |
| 5E9       | "                                       |
| TAC       | "                                       |
| OKT3      | "                                       |
| OKT4      | "                                       |
| OKT8      | "                                       |
| UCHT4     | "                                       |
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| HISL 1-19 | "                                       |
| BB-TECS   | "                                       |
| Leu3a     | "                                       |

SECTION

A

CHAPTER A 1

GENERAL INTRODUCTION :  
an overview of the subject



Chapter A1      GENERAL INTRODUCTION: an overview of the subject

- Al.1    Early studies on immunity in diabetes
- Al.2    Recent classification of diabetes
- Al.3    Factors involved in the pathogenesis of type 1 diabetes
  - Al.3a    The genetic factor
  - Al.3b    The environmental factor
  - Al.3c    The immune factor
- Al.4    Immunological consequences of insulin treatment
  - Al.4a    Insulin antigenicity
  - Al.4b    Insulin antibody formation
  - Al.4c    Clinical consequences of insulin immunogenicity
- Al.5    Immunological aspects of diabetic microangiopathy
  - Al.5a    Pathogenetic mechanisms of diabetic microvascular disease
  - Al.5b    Immunological aspects of microangiopathy
  - Al.5c    Insulin antibodies and immune complexes
- Al.6    Immunology in diabetic pregnancy
  - Al.6a    Immunology in normal pregnancy
  - Al.6b    Immunology in diabetic pregnant women
  - Al.6c    Immunology in the infants of diabetic mothers

## A1.1 EARLY STUDIES ON IMMUNITY IN DIABETES

Immunology developed from the study of resistance factors to infections. It has recently been proved that these defence mechanisms, instead of serving as a protection against disease, may give rise to serious disorders. From this fact, wide overlaps between immunology and other disciplines have become apparent. The influence of hormones or metabolic conditions on the immune system and of immune factors on the endocrine system in a healthy or diseased condition has received a great deal of attention.

Much of this attention has centered on diabetes.

Immunopathological phenomena have been described in various situations connected with diabetes. Treatment with heterologous insulin induces immune responses with potentially dangerous consequences (Page Faulk et al 1971; Jayarao et al 1974). Immunological changes are believed to play some role in late complications (Berns et al, 1962; Ortved-Andersen 1976). In the pregnant diabetic, insulin antibodies crossing the placenta may have untoward effects on the fetus (Fallucca et al; 1980). On the other hand, metabolic disturbances can seriously impair the immune defence mechanisms, facilitating aggression by exogenous agents (Bagdade et al, 1974).

Over the past ten years attention has been focused on the immunological events involved in the early stages of type I diabetes. Epidemiological studies have shown that this type of diabetes is associated with organ-specific autoimmunity (Irvine 1974; Irvine et

al,1970). A round-cell infiltration of pancreatic islets has been described in some juvenile diabetics who died shortly after the onset of diabetes (Gepts 1965). A modification of cell-mediated immunity has been found in several juvenile onset diabetics using different methodological approaches (Nerup et al 1971;Nerup et al 1974;MacCuish et al 1975).Circulating antibodies directed towards islet antigen determinants have been revealed both in diabetics with other autoimmune endocrine disorders (Bottazzo et al 1974;MacCuish et al 1974) and in most insulin dependent diabetics in the early stages of the disease (Lendrum et al 1975;Irvine et al 1977).

While evidence has been accumulating on the presence of several concomitant immunological changes close to the clinical onset of insulin dependent diabetes,other clinical and experimental work indicates both that some genetically determined disturbances of immune responses exist in this type of diabetes and that environmental agents are often involved in the immunological alterations observed.The study of the mutual influence of genetic,environmental and immunological factors has become the key to an understanding of the mechanisms leading to this disease.

## A1.2 CLASSIFICATION OF DIABETES: A REAPPRAISAL

In order to group patients for clinical and research purposes, it is of paramount importance to try to subdivide this syndrome according to some features which, with the present state of our knowledge, seem highly relevant. In the past, differences were between forms of diabetes secondary to other well-known disorders, mainly endocrinopathies, and forms that have no clear correlation with other diseases and, for this reason, are called primary or idiopathic diabetes. Within the latter forms of diabetes there are clinical differences in the onset and evolution of the disease that have been more clearly assessed. More recently, studies on the families of diabetic patients, studies on identical twins, the evaluation of autoimmune phenomena, particularly of antibodies against islet cell cytoplasm, the study of cell-mediated immunity, and genetic findings have clearly separated at least two substantially different forms within primary diabetes. In the past, these two forms were divided, principally, according to the age at onset of disease, the limit of division being arbitrarily around 25-30 years. The criterion of the age at onset, however, is not now considered satisfactory. In the last few years different classifications of diabetes have been reported (Bottazzo & Doniach, 1976; Cudworth & Woodrow, 1976; Irvine, 1977; Cudworth, 1978). A major problem at present is that no classification is satisfactory to both the clinician and the researcher. In 1979, the National Diabetes Data Group, together with the main associations for the study of

diabetes, developed a new classification of the disease (National Diabetes Data Group, 1979). Their aim was to give diabetologists a uniform framework in which to conduct clinical and epidemiological research or follow therapeutic guidelines. This classification divides primary diabetes according to insulin dependence. Nevertheless, the sole criterion of insulin dependence may be misleading because there are many non-insulin-dependent subjects that are treated with insulin for various reasons. Furthermore, among the non-insulin-dependent diabetic patients, there is a small group which, within a few months or years of diagnosis, develops a clear insulin dependence. To overcome these and other limits the National Diabetes Data Group proposed a parallel classification for research purposes. The main criteria of this classification are the detection of islet cell antibodies (ICA), an association with organ-specific autoimmune disorders, HLA typing and the mode of inheritance (Table A1.2/1). But this classification also has many theoretical and practical limits: the most important of which being that only a few diabetic clinics are able to perform genetic and immunological studies. In addition, these classifications reflect the state of knowledge on diabetes in Europe and North America. The possibility exists that among other populations the characteristics of these types of diabetes may be somehow different, or that certain ethnic groups may have peculiar forms of diabetes. In any event, irrespective of the criteria chosen to subdivide the diabetic syndrome, there is a proportion of patients that do not fit neatly into any group.

TABLE A1.2/1 Provisional research subclassification of Type 1 diabetes  
(National Diabetes Data Group 1979, modified)

| Diabetes mellitus   |   |
|---|---|
| Association with organ specific autoimmune disease or autoimmune phenomena      | Presence of islet cell antibodies (in relation to onset of insulin dependency)  |
| Other characteristics that offer promise of being used to sub-classify diabetes |   |
| Insulin-dependent type (IDDM)   |   |
| Subclass a  | Positive  |
| Subclass b  | Negative  |
| Subclass c  | Negative  |
|   | Usually persistent  |
|   | Usually transient   |
|   | Undetected  |
|   | Possible viral aetiology prevalence of HLA types (elevated or reduced relative to the general population), such as Subclass c DR2, DR3, DR4, B8, B15 etc.; antibody response to exogenous insulin |

## A1.3 FACTORS INVOLVED IN THE PATHOGENESIS OF TYPE I DIABETES

Considering the complexity of the problem, the discussion on the aetiology and the pathogenesis of diabetes remains quite speculative and attention should be especially focused on those aspects in which convincing findings have been reported.

Indirect and direct evidence of the presence of immunopathological phenomena in diabetes has been accumulated. An association between diabetes (primarily of the juvenile-onset type) and other diseases of autoimmune etiology has been described (Perlman, 1961; Solomon et al, 1965; Irvine, 1974; Nerup, 1974). Moreover, the presence of some organ-specific humoral antibodies has been observed in insulin-dependent diabetes mellitus (IDDM) (Landing et al, 1963; Blizzard et al, 1967; Irvine et al, 1970; Goldstein et al, 1970) as have alterations of cell-mediated immunity (MacCuish and Irvine, 1975; MacCuish et al, 1974; Nerup et al, 1974), and antibodies to pancreatic islet cells (Bottazzo et al, 1974; Irvine et al, 1977). The latter are also found in diabetics with other autoimmune disorders (Lendrum et al, 1975; MacCuish et al, 1974). More recently a series of abnormalities has been noted in the early stages of IDDM, including an increase in circulating immune complexes (Irvine et al, 1978), the presence of antibodies to surface antigens of pancreatic islets (Lernmark et al, 1978), an increased number of killer cells (Pozzilli et al, 1979) and circulating Ia-positive T cells (Rowley and Eisenbarth, 1982). On the other hand, suppressor T lymphocytes have been found to be

depressed (Buschard et al,1980;Galluzzo et al,1980). Type I diabetes, defined according to recently stated criteria (National Diabetes Data Group,1979),is heterogeneous, and may include adult females with a general trait towards autoimmunity, with characteristics very different from those found in young subjects with an acute severe onset of the disease (Table A1.3/1).

Three factors are at present known to be involved in the pathogenesis of type I diabetes:genetic susceptibility,environmental agents and immunological factors.



TABLE A1.3/1 Heterogeneity within Type 1 diabetes according to clinical and immunological factors

|                      | ←                              | ↔ | →                    |
|----------------------|--------------------------------|---|----------------------|
|                      | Organ specific<br>autoimmunity |   | Exogenous<br>insults |
| Age at onset         | any age                        |   | <30                  |
| Sex                  | F>M                            |   | M>F                  |
| Onset                | slow                           |   | acute                |
| HLA association      | DR3                            |   | DR3/4                |
| ICA                  | persistent                     |   | transitory           |
| AgAb                 | persistent                     |   | transitory           |
| K cells              | persistent                     |   | transitory           |
| AutoAb               | present                        |   | absent               |
| Autoimmune disorders | common                         |   | rare                 |

### Al.3a The genetic factor

The main data on genetic factors involved in type 1 diabetes derive from studies on the Human Leucocyte Antigens (HLA) of the major histo-compatibility system and from epidemiological research conducted using different approaches: population, family and twin studies (Nerup et al, 1974; Wolf et al, 1983; Srikanta et al, 1983).

The histocompatibility system genes located in the short arm of the sixth chromosome have been shown to be of paramount importance in a few disorders, including diabetes. In the same chromosomal area are the genes that control many aspects of the immune response, including the cell-mediated immune response and part of the antibody production. The HLA system is the most polymorphic system described in man with the genes at each locus encoding a different cell surface antigen. However, between HLA-A-B-C and -D there is a marked linkage disequilibrium, i.e., certain antigens tend to occur together more frequently than expected from their individual frequencies. The HLA genes present on one chromosome constitute the HLA haplotype and each individual inherits two alleles for each locus, one from the father and one from the mother.

#### \* Population studies

Population studies have demonstrated that a number of histo-compatibility antigens are more frequently found in insulin-dependent diabetics than in the normal population. Others, however, are rarer

(Nerup et al,1974;Morris et al,1976). DR3 and DR4, both in linkage disequilibrium with other antigens on A,B and C loci, are more frequently present.DR2,however, seems to have no association with diabetes.The presence of DR3 or DR4, therefore means an increased risk of developing type 1 diabetes and the simultaneous presence of both of these confers an additive risk.Conversely,DR2 seems somehow to afford protection against diabetes.It is now clear,however,that the strongest associations with the disease lie with the DR antigens, HLA DR3 (Sachs et al,1980;Deschamps et al,1980) and HLA DR4 (Deschamps et al,1980). Current evidence suggests that the findings reported originally regarding the HLA-A and HLA-B series antigens are secondary to those findings regarding the HLA DR series. In other words, the association of HLA-A,-B and -C antigens with diabetes comes about because of linkage disequilibrium with DR3 and DR4. Thus, HLA B8,B18,and A1 are in linkage disequilibrium with DR3 while HLA A2 and B15 are in linkage disequilibrium with DR4.Similarly, the apparent decrease in frequency of HLA B7 is due to the decrease of DR2 (Singal & Blajchman, 1973; Nerup et al,1974; Thomsen et al,1975; Morris et al,1976;Cudworth & Woodrow,1976). Direct evidence of heterogeneity within Type 1 diabetic patients comes from genetic studies showing that DR3 and DR4 seem to confer additive risk of type I diabetes. In Caucasians,the relative risk to heterozygote DR3/DR4 patients is considerably increased when compared with DR3/X and DR4/X patients even when they are homozygotes (Nerup et al,1980;Sachs et al,1980;Deschamps et al,1980).

Indirect evidence of differences between DR3 and DR4 diabetic patients derives from other studies (Table A1.3/2).In some cases,DR3 diabetic patients tend to have a persistent presence of ICA and

TABLE A1.3/2 Heterogeneity within type 1 diabetes according to clinical and genetic factors

| Evidence                            | D/DR3                                   | -A d d i t i v e- | D/DR4  | B8(DR3)/B15(DR4)<br>combined form              |
|-------------------------------------|---|-------------------|--|--|
| Relative risk for diabetes          |   |                   |  | ↑ relative risk, excess of patients            |
| Linkage disequilibrium              | B8,A1                                   |                   | B15,Cw3  | ↑ occurrence in concordant MZ twins            |
| Insulin antibodies                  | Non/Low responder (low antibody levels) |                   | High responder (produce high anti-body levels) | ↑ risk to sibling occurrence in familial cases |
| Islet cell                          | Persistent                              |                   | Transient                                      |  |
| Antipancreatic cell                 | Increased                               |                   | Not increased                                  |  |
| Age of onset                        | Any age                                 |                   | Young age                                      | Youngest                                       |
| Seasonal variation                  | No                                      |                   | Yes  |  |
| Levels of C-peptide Residual B-cell | High                                    |                   | Low  | Lowest   |
| Can present as Type II diabetes     | Yes                                     |                   | No   |  |

occurrences of other subclinical or overt organ-specific autoimmune diseases. They also tend to form low titres of insulin antibodies following insulin treatment. The risk of diabetes is increased in all age groups and in DR3 diabetic patients autoimmune mechanisms appear to be relevant in the pathogenesis. On the other hand, DR4 patients tend to have an increased production of antibodies to viruses and to heterologous insulin but there is no tendency towards autoimmunity. DR4- positive subjects seem to have an increased risk of developing the disease before the age of 16 (Ludwig et al, 1976; Irvine et al, 1978a; Irvine et al, 1978b; Bottazzo et al, 1978; Nerup et al, 1980; Cudworth, 1981). The decrease in frequency of DR2 antigens in diabetics may be secondary to the increases in DR3 and DR4 (Sachs et al, 1980) or subjects may be protected against type 1 diabetes possibly because their immune system can cope with adverse factors in the environment.

These data were obtained from population studies of Caucasian people. Different human races show substantial differences in the HLA system. Of relevance is also the fact that data presently available demonstrate different characteristics and prevalences of ketosis-prone diabetes in many African and Asian populations (Wakisaka et al, 1976). Properdin (complement factor B; Bf) polymorphism has received considerable attention in recent years. Raum et al (1979) suggested that the BfF1 allele was in strong linkage disequilibrium with a specific susceptibility gene associated with a disease of younger age at onset. BfF1 is a good example of racial differences in distribution of alleles occurring relatively commonly in Southern Europeans, as a component of the DR3-B18-Cw5-Aw30 or A25 haplotype (Deschamps et al, 1979; De Mouzon et al, 1979).

Combining the findings in two large studies in the British Isles, Wolf et al(1982) found that 21 out of 22 patients studied with the B18 haplotype possessed BfF1 in association with Cw5. All were DR3 positive. They were unable to confirm a younger age of onset. DR3-negative subjects were not associated with BfF1 but were associated with BfF or BfS. They concluded that the increase in frequency of the rare BfF1 allele occurred almost exclusively in haplotypes possessing B18 and Cw5 in linkage disequilibrium with DR3. They concluded that the rare BfF1 allele was increased in frequency and this occurred almost exclusively in haplotypes possessing B18 and Cw5 in linkage disequilibrium with DR3.

\* F a m i l y s t u d i e s

Family studies have shown that in siblings of type I diabetics there is a statistically significant association between the sharing of an identical haplotype and the development of type I diabetes (Nerup et al, 1977; Cudworth, 1980). Moreover, siblings of type I and type II diabetics tend to show the same type of diabetes as the probandi. This would support the existence of HLA-linked predisposing genes (Cudworth & Woodrow, 1976; Nerup et al, 1977; Barbosa et al, 1977; Cudworth, 1981; Deschamps et al, 1980). On the other hand, studies on type II diabetics have shown no association with HLA. Thus, the genetic predisposition in type II diabetics seems to act through different mechanisms.

## \* T w i n   s t u d i e s

Twin studies have confirmed a genetic susceptibility in type I diabetes but have shown that the importance given to the genetic factor alone in the development of the disease has perhaps been overstressed (Barnett et al,1981). In fact,in identical twins the concordance is near to 100% when the age at onset is above 45 years (Pyke,Nelson,1978) while the concordance is around 50% when the age at onset is less than 45 years.

HLA studies in twins have shown that HLA B8 was increased only in the concordant pairs while HLA B15 was equally increased in the concordant and in the discordant pairs.This finding reinforces the possibility of the presence of two different diabetogenic genes in linkage disequilibrium with HLA B8 and B15 respectively (Nelson et al, 1975; Cudworth,1981).

## \* D i s c u s s i o n

The risk of developing diabetes conferred by DR3 and DR4 seems to be related to the peculiar immunoresponsiveness found in these individuals.HLA-DR genes seem to be in linkage disequilibrium with other genes which regulate some immune responses (more generally termed Ir genes) and are present on the same chromosome.DR3 patients tend to show a pathological immune response towards autoantigens, while DR4 seems associated with an abnormal immune response to some exogenous agents (Bertrmas et al,1976;Irvine et al,1978).Thus,it seems that at least two HLA-linked diabetogenic genes,acting through

different mechanisms, may be inherited. The presence of these genes does not seem sufficient for the development of diabetes in individual patients. Other superimposed acquired factors must also be present.



### Al.3b The environmental factor

Clinical observations have shown that diabetes may appear shortly after a viral infection or after the ingestion of chemical products (Gundersen,1927;Craighead,1978;Cahill and McDevitt,1981). A variety of environmental events are thought to play a role in triggering the onset of type I diabetes.At present, the harmful agent has been found and described only in a few situations.

#### \* Viruses

Viruses appear to be the most likely agents to be involved in the development of many cases of diabetes.There is mounting evidence of their role in the aetiology of the disease but definite proof is lacking.Circumstantial evidence for the role of viruses has come from two distinct investigative processes. Interest in viral infections was aroused many years ago by the clinical observations in Scandinavian communities that a number of patients had suffered from a mumps infection some weeks or months before the clinical onset of diabetes (Gundersen,1927).

Epidemiological studies have shown that the onset of diabetes is more common in the cold months (Cudworth et al,1977;MacMillan et al,1977;Gamble 1977),coinciding with the peak incidence of some viral infections. Interestingly,this seasonal phenomenon is remarkably constant from year to year and it might also be concluded that in view of the epidemic nature of viral infections a single virus is

unlikely to be the sole pathogen. Studies have also shown an increased frequency and has elevated titres of antibodies to Coxsackie B4 virus in some type 1 diabetic patients at diagnosis (Gamble et al, 1969; Cudworth et al, 1977; El Hagrassy et al, 1980). Of interest is the incidence of diabetes in patients with congenital rubella infection, which Menser et al (1978) observed to be 20%.

Studies on animals have shown that a diabetic-like disease can be produced in some mice by the M variant of the encephalomyocarditis (EMC) virus (Craighead, 1981), while reovirus (Onodera et al, 1978), Venezuelan equine encephelitis virus (Rayfield et al, 1979) and Group B coxsackie viruses (Yoon et al, 1978) all produce a diabetic-like disease in rodents. It is relevant that only a few strains of mice develop hyperglycaemia after viral infections whereas other strains seem to be resistant. This finding stresses the role of a genetically determined abnormal immunoresponsiveness. The two lines of evidence come together when considering the histology of the islet of experimental diabetes in animals and of autopsy specimens in man. In mice infected with the encephalomyocarditis virus, insulitis occurs with a macrophage and lymphocyte infiltration (Craighead, 1977), and in animals infected with reovirus, virus particles can be identified in the B cells (Onodera et al, 1978). These findings are similar to those found in autopsy material from patients with type I diabetes (Gepts & De May, 1978; Gepts & LeCompte, 1981).

Following their observation of an association between diabetes mellitus and congenital rubella infection, Menser et al, 1978 reported that experimental congenital rubella infection in rabbits produced histological changes in the B cells similar to those produced by the M variant of the encephalomyocarditis virus. Yoon et

al(1979) linked the clinical and experimental findings in a patient who died from a viraemia associated with acute diabetes mellitus. They observed that homogenates of the patient's pancreas added to mouse, monkey, or human cell culture allowed recovery of the virus. Evidence that this was not a laboratory contaminant lay in the finding of an increase in titre of neutralising antibody in the patient before death. Furthermore, inoculation of mice resulted in hyperglycaemia and histology of the mouse islets showed B cell necrosis with an inflammatory cell infiltrate. The virus was identified as being similar to Coxsackie B4.

#### \* C h e m i c a l s

Chemicals may be responsible for diabetes (Craighead, 1978). A satisfactory assessment of their role is very difficult because of the extremely high number of substances potentially involved. In a few cases, a clear cause/effect relationship of chemical and disease has made identification of the substance possible (Notkins, 1979).

The mechanism through which chemicals may lead to islet cell damage is obscure. Of interest is the observation that repeated sub-diabetogenic injections of streptozotocin in some strains of mice produced hyperglycaemia and diabetes, and, surprisingly, an insulinitis with a round-cell infiltration of the pancreatic islets (Like & Rossini, 1976; Appel et al, 1978).

\* D i s c u s s i o n

More intriguing are the mechanisms via which environmental agents may lead to islet destruction. It is likely that a wide range of combinations exist. Severe viral infections or a high dose of a chemical agent may result in direct damage of the islets, but this seems unusual. On the other hand, viruses may interact with the host defence mechanisms in several ways. A prolonged virus infection may induce the production of antibodies to the islets through a mechanism of cross reactivity or by a direct modification of cytoplasmic or surface antigens; a modification of T cell subsets with a failure in the regulation of the host immune response may also be a consequence. Alternatively, the persistent antigenic stimulation may induce an abnormal proliferation of B lymphocytes and production of a wide range of organ-specific autoantibodies directed not only towards islets but also towards other cells (Onodera et al, 1983). For chemicals the mechanisms were thought to be different but it is noteworthy that, after repeated subdiabetogenic injections of streptozotocin in mice, there was a mononuclear cell infiltration of pancreatic islets simultaneous with insulin deficiency (Appel et al, 1978).

In any case environmental agents seem to produce diabetes only in individuals with a genetically abnormal immune response; furthermore, in most cases these agents seem only to trigger the immune mechanisms ultimately responsible for islet damage.

### Al. 3c The immune factor

From basic immunology it is known that cells can be damaged in different ways by immune mechanisms (Eisen, 1982; Roitt, 1980). In other words, cytotoxic antibodies, immune complexes and cell mediated immunity are the main tissue damaging factors. The possible involvement of these mechanisms in the early stages of type I diabetes is here discussed.

#### \* Cell damaging antibodies

Circulating antibodies may lead to a tissue lesion in a number of different ways. They may lead to phagocytosis by opsonization or by immune adherence, they may activate the complement system and lyse the cell or they may arm K cells and so predispose the target to be killed. There is no evidence that these mechanisms are actually operating in diabetes but, in any case, different kinds of antibodies are present in diabetes (Charles et al, 1983).

Islet cell antibodies (ICA) have been found so far in several conditions (Irvine, 1980; Bottazzo et al, 1981; Brogren & Lernmark, 1982). They tend to decline soon after diagnosis in type Ia patients. ICA have also been found in a few non-insulin-dependent diabetics, in patients with other autoimmune disorders but without diabetes, in subjects with an impaired glucose tolerance test, in gestational diabetics, in first degree relatives of type I diabetics and in a few normals (Irvine, 1980). There is a significant association between ICA

and thyrogastric antibodies. This association is much stronger in ICA persistently positive subjects (Irvine, 1980). The role of ICA in the pathogenesis of type I diabetes is still not clear; nor is the significance of their presence in non diabetic subjects. There is no clear evidence, so far, of a cell destructive effect of the antibodies in diabetes mellitus (Madsbad et al, 1980). ICA, crossing the placenta from a diabetic mother to the fetus (Gamlen et al, 1977), do not cause diabetes in the newborn. The fact that these antibodies react with all cell types present in the islet does not support a direct role for ICA on Beta cells. No significant difference was found between ICA positive and negative diabetics in plasma glucagon and insulin responses to an intravenous arginine infusion (Rubenstein et al, 1980; Tiengo et al, 1977). So far, the presence of ICA in diabetics is considered a valuable marker in separating type I from type II diabetics, selecting diabetics with subclinical autoimmune disorders when the antibodies persist for years and in selecting from NIDD those with the tendency to progress to insulin dependency. In fact, in these patients the presence of ICA has been shown to be a good indicator of the probability of becoming insulin dependent (Irvine et al, 1977; Irvine et al, 1979). In subjects without diabetes mellitus, ICA are important in the selection of those apparently normal subjects at risk of developing diabetes. In these cases, the presence of ICA precedes the impairment of glucose tolerance (IGT) (Gorsuch et al, 1981; Gorsuch et al, 1982). In gestational diabetes (GDM), ICA increase the probability of developing overt diabetes in the following years (Bottazzo et al, 1980; Steel et al, 1980).

It is not clear whether complement fixing islet cell antibodies (CF ICA) are a separate group of ICA or simply one immunoglobulin G

(IgG) subclass that avidly fixes complement (Bottazzo et al,1980; Bottazzo et al,1981). CF ICA seem to be present shortly before the clinical onset of the disease and rapidly disappear thereafter. CF ICA positivity is lower than that of ICA and almost all the CF ICA positive diabetics are also ICA positive (Chapter B1.3). CF ICA seem to be a good marker for an early clinical onset of diabetes in subjects at risk of developing the disease (Sai et al,1981;Bottazzo et al,1981).

A more interesting pathogenic significance seems to be held by antibodies reacting with the surface of islet cells (ICSA)(Lernmark et al,1978;Baekkeskov et al,1982). They are present in about two thirds of newly diagnosed type I diabetics and their presence declines thereafter (Lernmark et al,1981; Pujol-Borell et al,1982).There is a significant,but not very strong, correlation between the presence of ICA and that of ICSA in the same patients.It is still not clear whether ICSA react with all the cells in the islet or only with beta cells.ICSA have been found in some normals and,interestingly enough, also in subjects who developed overt diabetes after the ingestion of chemicals (Karam et al,1980). ICSA were found to be able to impair the function of islets in a perfusion system. It is of relevance that ICSA positive sera showed a complement dependent cytotoxic effect in cultures of dispersed rat islet cells; this effect was not found with ICA positive ICSA negative diabetic sera (Dobersen et al,1980).

\* I m m u n e c o m p l e x e s

Other possible tissue damaging mechanisms acting in diabetes involve circulating immune complexes (AgAb). Immune complexes may produce a tissue lesion through different mechanisms. They may interact with platelets, inducing a platelet aggregation, they may activate the complement system, leading to inflammation and they can interact with cells bearing complement (C) or Fc receptors. This phenomenon is very interesting because of the numerous effects of this interaction: the activity of the C-or-Fc bearing cells may be modulated in different ways and, in extreme conditions, may be either blocked or armed with a potential cytotoxicity. The formation and the properties of AgAb also differ substantially for a given antigen and antibody. The Ag/Ab ratio, the size of complexes, the lattice formation and the rate of formation/disappearance deeply influence the pathogenic potential of complexes. Increased levels of immune complexes have been found in newly diagnosed diabetics (using the solid phase Clq binding test, the Conglutinin radioimmunoassay and the Raji cell-binding assay) (Chapters B1.2, B1.3). The levels of immune complexes tend to decline soon after diagnosis, and within a few months of the onset of disease to reach levels not significantly different from those of normals. Whether AgAb are really involved in islet cell damage in type I diabetes is not clear. The most interesting aspect is the study of all the possible interactions between AgAb and cells bearing receptors for the Fc of immunoglobulins or for C3 and, in particular, the possible modulation of T cell and K cell responsiveness (Cordier et al, 1977; Pozzilli et al, 1982).



\* C e l l - m e d i a t e d   i m m u n i t y

A major role for this type of immune response in the early stages of IDDM has been suggested by pancreatic histopathological findings. A round-cell infiltration of the hyperactive islet is well in accordance with the possibility of cellular immune defence against a viral infection (Gepts, 1965; Gepts, 1980; LeCompte & Legge, 1972). When it was confirmed that these cells were small lymphocytes mostly directed towards beta cells and that plasma cells were not present, these concepts were reinforced. Cell-mediated immunity comprises immune mechanisms in which the specific recognition of the antigen is due to receptors on T lymphocytes. At present T cells are divided into regulators and effectors and there are two substantially different forms of effector T cells: those mediating delayed type hypersensitivity (DHT) and those that lyse target cells bearing the stimulating alloantigen (CTL). Cytotoxic properties are also retained by cells with Fc surface receptors other than B and T lymphocytes. The activity of part of these cells is dependent on the presence of specific antibodies and immune complexes, the so called antibody dependent cellular cytotoxicity (ADCC).

Cell-mediated immunity has been investigated in different ways (Buschard et al, 1980; Selam et al, 1979; Sensi & Pozzilli, 1979; Orita et al 1980; Buschard et al, 1980; Boitard et al, 1980).

The measurement of total T cells and the study of their functional capacity have received attention in many research works. When metabolic control is taken into account, there seems to be no substantial difference between the T cell number in diabetics and controls nor was the PHA stimulation of diabetic lymphocytes

significantly abnormal when the patients were studied in good metabolic control. A positive lymphocyte migration inhibition was found in several newly diagnosed insulin dependent diabetics when a bovine, porcine and human pancreatic antigen was used (Irvine et al, 1976). Lymphocytes from type I diabetics were found to be toxic to cultured human insulinoma cells (Huang & MacLaren, 1976).

More recently, new sophisticated techniques of identification, isolation and functional characterization of T-cell subsets have become available. T cells spontaneously form rosettes with erythrocytes and the measurement of this phenomenon under high affinity or low affinity experimental conditions has been developed. It is accepted that the low affinity E-rosette-forming cells have receptors for the Fc portion of immunoglobulin G and are capable of mediating the antibody dependent cellular cytotoxicity (West et al, 1977). In a recent study, about half of the IDD studied at diagnosis had raised levels of these cells. This increase was no longer found at a distance from diagnosis of more than 1 year (Pozzilli et al, 1980; Sensi et al, 1981). At present, the availability of monoclonal antibodies against antigens present in the lymphocytes has given further impulse to understanding the modification of the cell-mediated immune response. It is also rather interesting that an increased number of activated T cells were found, using monoclonal antibodies against antigens expressed by cells only in a particular functional stage (Rowley & Eisenbarth, 1982; Pozzilli et al, 1983).

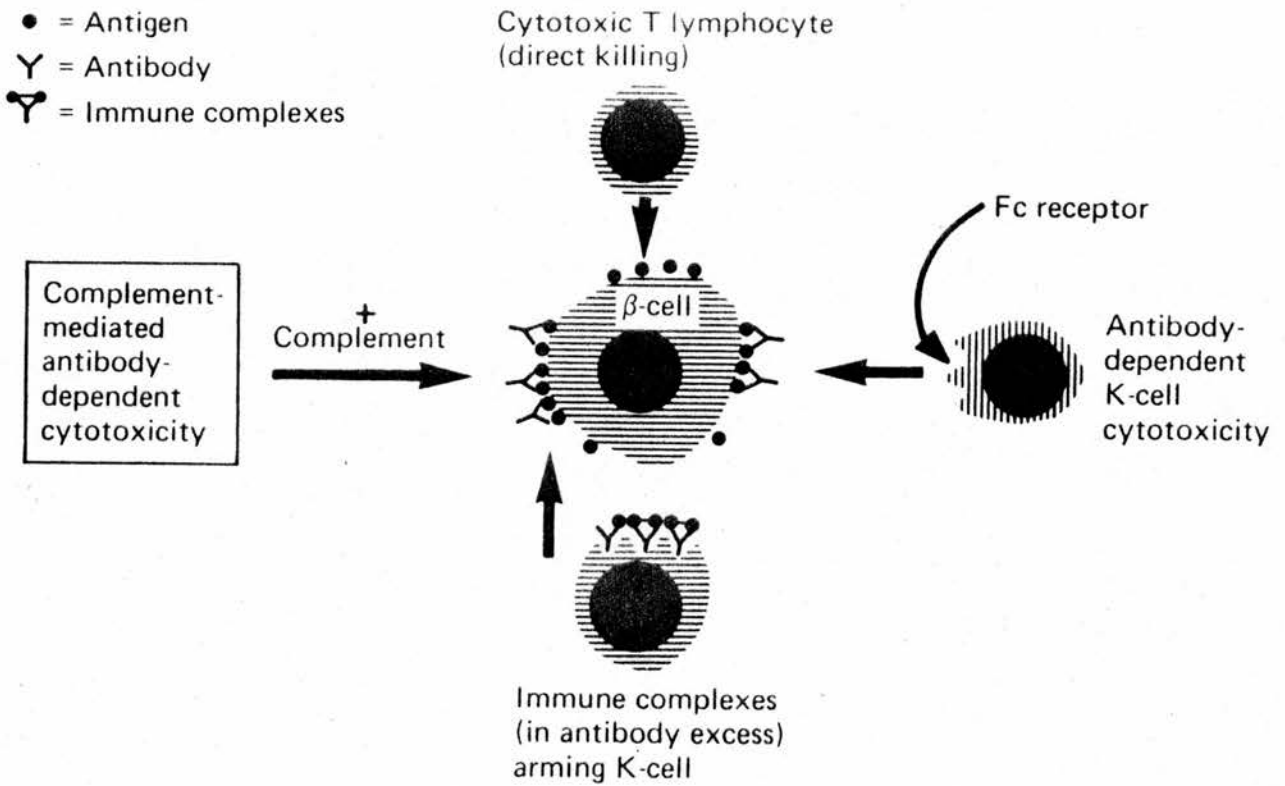
\* C o n c l u d i n g   r e m a r k s

The most obvious question is still unresolved: are these immunopathological mechanisms the cause of type 1 diabetes?

It is possible to say that a genetic, an environmental and an immunological factor are interacting in a wide spectrum of ways to produce the islet cell damage. In one extreme, the genetic susceptibility to organ specific autoimmunity leading to islet cell damage through immune mechanisms is probably different from those operating when a genetic predisposition modifies the immune defence mechanisms towards an aggression by exogenous agents, such as viruses or chemicals. In another extreme, an overwhelming viral infection or a chemical toxic to the pancreas may lead directly to islet destruction. The possible immune mechanisms operating in the beta cell destruction may involve cytotoxic T lymphocyte, killer cells armed by either specific antibodies or immune complexes in antibody excess and cytotoxic antibodies (Figure A1. 3/1). The intervention of these effector immune cells and the increase of these cytotoxic antibodies are probably secondary to a primary modification of the T regulator cells. In genetically susceptible individuals the environmental agent may act in a wide variety of ways, slowly modifying the host self antigens and interfering with helper/suppressor regulatory mechanisms or derepressing B cell clones towards self antigens. Certainly we are far from full elucidation of the pathogenesis of type I diabetes; the mounting evidence from recent research at least suggests we are on the right lines.

FIGURE A1.3/1

Possible immune mechanisms involved in the beta cell destruction in type 1 diabetes



## A1.4 IMMUNOLOGICAL CONSEQUENCES OF INSULIN TREATMENT

Although insulin has been known to have antigenic properties for 60 years (Joslin et al,1922;Tuft,1928,Lewis,1937), only in the sixties did the immunological aspects of insulin treatment receive the attention that it deserved (Federlin,1971).

It is known at present that the spatial configuration and the presence of contaminants are mostly responsible for the immunogenicity of commercial insulins. Insulin antibody formation is genetically influenced and the presence of insulin antibodies and of insulin-anti-insulin complexes are responsible for several clinical manifestations of immunological reactions, such as localized and diffuse allergy, insulin resistance, lipodystrophy, anaphylactic shock,thrombocytopenia, arthritis and vascular damage.

Al.4a      I n s u l i n   a n t i g e n i c i t y

The main cause of insulin antigenicity is the species heterogeneity (Maloney & Coval,1955;Berson and Yalow,1959;Lockwood & Prout,1965; Renold et al,1966; Page Faulk et al,1971). Both bovine and porcine insulins, the most commonly used in therapy,are immunogenic but bovine insulin seems to produce more antibodies than porcine insulin. When a patient is switched from treatment with bovine,or a mixture,of bovine-porcine insulin to porcine insulin (Andreani et al, 1972) a reduction in insulin antibody levels is usually observed. Bovine insulin,purified by sequential recrystallization,is more immunogenic than the equivalent form of porcine insulin (Kurtz and Nabarro, 1980;Reeves,1980;Reeves and Kelly,1982).This difference is likely due to the fact that bovine insulin presents three aminoacids different from the human molecule,namely,those in position 8 and 10 in the A chain and in position 30 in the B chain, whereas porcine insulin has only the amminoacid in position 30 in the B chain different from human insulin. Thus,generally speaking,insulin antibody levels seem related to the number of different aminoacids in the therapeutic insulin molecule.

The spatial configuration of the molecule also accounts for the antigenicity of heterologous insulin.The disulphuric bridges, the disposition of C-terminal aminoacids and the spatial configuration of insulin are important in establishing immunogenic determinants (Lockwood and Prout,1965;Levin,1969;Arquilla et al,1976; Keck,1975;-Berson and Yalow,1961). But there are, in fact,differences in insulin

antibody binding between porcine and whale insulins with identical aminoacid sequences (Levin,1969).

Pancreatic contaminants, such as a-component,b-component, glucagon, somatostatin,pancreatic and proinsulin may be important, in addition to insulin itself,in evoking an immune response against therapeutic insulin preparations (Schlichtkrull,1974;Schlichtkrull et al,1972;Bruni et al,1978;Kawazu et al,1979;Fizpatrick & Patel, 1981; Bloom et al,1979; Kurtz et al,1980; Kurtz et al,1983).

Physico-chemical factors are another cause of insulin antigenicity.The pH of insulin, the polymerization of the molecule, molecular changes such as arginine insulin,ethylester of insulin, monodesamino-insulin the presence of Zn,etc. may be responsible for the immunogenicity even when human insulins are used (Neubauer & Scone,1978;Deckert et al,1972;Hangen et al,1981;Wilson et al, 1962). Some of these factors induce changes in structure when insulin is already injected in tissues so that,even if the original preparations of insulin are almost pure, these physico-chemical changes may enhance an immune response (Hangen et al,1981;Wilson et al,1962; Deckert et al,1972;Renold et al,1964). The two aminoacidic chains have different immunogenic properties. The A chain appears to be more immunogenic than the B chain. (Wilson et al,1962;Berson and Yalow, 1963;Kumar,1979; Lockwood and Prout,1965). A few experimental studies have shown that, provided the A chain is not modified, the B-chain modifications of insulin do not substantially change the antigenicity but may modify its linkage with antibodies of different affinity (Federlin,1971;Wilson,1969;Faulk et al,1974; Kerp and Kasemir,1976).

Al.4b      I n s u l i n   a n t i b o d y   f o r m a t i o n

In the last two decades particular attention has been focused on insulin antigenicity mainly to obtain less antigenic insulin preparations and thus to avoid some inconveniences which are related to antibody formation in insulin treated diabetics.

Though a weak immunogen, insulin is able to elicit antibody formation both in the experimental animal and in humans treated with heterologous insulin (Berson et al,1956; Berson,1958; Root et al, 1972; Kumar et al,1975; Dixon et al,1975; Arquilla et al,1976; Baxter et al,1976; Davidson & Debra,1976; Gerbitz & Kenner,1978; Kurtz et al,1978; Kurtz et al,1979; Kurtz & Nabarro,1980; Ramila et al,1981; Arquilla et al,1981; Karlsson et al,1982).

Insulin antibody levels, easily detectable with common methods, are reached within a few weeks. In diabetics submitted to insulin treatment the level of antibodies increases progressively up to a year,thereafter it remains substantially the same at least as long as antigen is administered.Environmental conditions,i.e. infectious diseases, however, may interfere with antibody production and cause a sharp increase in circulating antibody. All the classes of antibodies, with known different properties, have been detected during insulin treatment and the affinity to antigen varies within the same class (Kerp and Kasimir,1976;Kumar, 1977) but the most clinically relevant are IgE and IgG antibodies (Berson and Yalow,1959).

In a few conditions insulin antibodies have been found in circulation irrespective of the injection of heterologous or exogenous insulin. Insulin antibodies have been found in newly diagnosed



insulin dependent diabetics before insulin treatment (Palmer et al,1982).If this finding is confirmed the presence of these antibodies may be explained as an autoimmune reaction towards partially modified insulin released by the damaged beta cells.Insulin antibodies were also found spontaneously present in a few diabetics with the so-called "insulin-auto-immune syndrome" (Kembo and Hirata,1974; Kawazu et al,1975).

There is evidence that insulin antibody formation is under genetic control in animals (Arquilla et al,1969;Barcinski & Rosenthal,1977;Keck,1977;Rosenwasser et al,1979; Scoer et al,1979; Spaeth et al,1981). Several research contributions have shown that in humans there is also a correlation between certain HLA types and the levels of insulin antibodies (Chapter B2.2b)(Bertrams et al,1976; Keck,1977; Schernthaner et al,1979; Kapp & Bucy,1981; Zeidler et al,1982; Schernthaner et al,1983). The genetic data indicate an association between low antibody responses and HLA-B8-DR3 as well as an association between B15- DR4,DR7 and high antibody responses.It seems that the Gm genes regulating the IgG-allotype determinants also have a remarkable influence on the immune response to insulin (Robbins and Kapp,1980; Nakao et al,1981).

Insulin antibodies usually found in circulation are an heterogeneous family of antibodies,some with a high affinity to insulin but the majority with a low affinity to the antigen.

The presence of insulin-anti-insulin complexes is expected in insulin treated diabetics with circulating insulin antibodies. In effect, these complexes comprised of insulin have been described in many diabetics (Jayarao et al,1974).

The insulin-anti-insulin complexes are usually of small size and they do not precipitate. It is doubtful whether insulin-anti-insulin complexes near the equivalence point may fix complement. These complexes are usually of small size. In antigen excess they show the structure  $Ag_2Ab$  and their molecular weight is roughly about 165,000 dalton (Kerp et al, 1976; Folling, 1976). At present there is no truly reliable and highly sensitive method to detect them.

Highly purified or human insulins have greatly reduced the production of insulin antibodies, as discussed in other parts of the work (Andreani et al, 1972; Andreani, 1973; Andreani 1974; Andreani et al, 1974; Yue & Turtle, 1977; Andreani et al, 1977; Weber et al, 1978; Wright et al, 1979).

Al.4c      C l i n i c a l   c o n s e q u e n c e s   o f  
                 i n s u l i n   i m m u n o g e n i c i t y

Therapeutic insulin and insulin antibodies may be responsible for many clinical and pathological effects (Karr et al,1931; Leslie, 1977). The main ones are allergic reactions, lipodystrophy, insulin resistance, reduction of bioavailability of insulin, prolonged insulin action and apparently there are effects on the clinical complications of infants of diabetic mothers and on the development of microangiopathy.

Insulin allergy is mainly caused by multiple factors, among which IgE, IgG antibodies and the activity of  $T_{DTH}$  lymphocytes are involved. It seems also to be influenced by genetic factors (Bertrams & Grunekle, 1977). Furthermore, many studies have reported that diabetic subjects affected by insulin allergy present elevated levels of circulating IgG-IAb and that after desensitization and treatment with highly purified insulin there is a decrease both in the allergy and the IgG-IAb levels (Andreani et al, 1974; Teutscher, 1975; Bruni et al, 1975; Kawanishi et al, 1977; Lunetta, 1982). Even highly purified insulins may occasionally induce insulin allergy (Goldman et al, 1976; Andreani et al, 1977; Bachmann et al, 1979; Carini et al, 1982). In these cases a previous transitory insulin therapy with conventional insulin is usually reported.

A possible role for IgG-IAb in lipodystrophy (Teutscher, 1974) and in lipohypertrophy (Andreani et al, 1974) was suggested in 1974 and subsequent research has confirmed that lipodystrophy may be reduced or eliminated by purified insulins (Teutscher, 1975; Hulst, 1976; Poulsen

and Deckert,1977).However, it should be pointed out that in a few subjects purified insulins may also induce lipoatrophy. Recently, Reeves et al.(1980) confirmed elevated levels of IgG-IAb in insulin-treated diabetic patients with lipoatrophy and made relevant findings to support the immune pathogenesis of lipoatrophy.

The presence of antibodies is responsible for some specific metabolic conditions (Gonen et al,1979; Gray et al,1981; Vaughan et al,1983). Since the insulin activity is dependent upon the free form of the hormone, one of the effects of the antibodies binding circulating insulin is the delay in its action and in some circumstances antibodies may even hinder the activity of insulin so that a resistance to the hormonal action derives.This resistance very rarely is absolute but may reach a high degree.From time to time occasional cases occur in which enormous amounts of insulin are needed to overcome resistance.

Another condition which has been studied more recently and in which insulin antibodies may play an important role,is pregnancy in insulin treated diabetic women. In effect, maternal antibodies cross the placenta, while insulin does not.Insulin antibodies in fetal cord blood are of the same level as in the mother's blood (Spellancy & Goetz,1963; Exon et al,1974; Kalhan et al,1975). Elevated insulin antibody levels have been correlated with newborn hypoglycaemia (Nagawa et al,1973). Indeed increased insulin and/or C peptide levels have been reported in the circulation of infants of diabetic mothers having high levels of circulating insulin antibodies (Sosenko et al,1979; Fallucca et al,1980).

Since insulin antibodies were first described there have been numerous investigations on the possible influence of prolonged

heterologous insulin treatment on the course of diabetic microangiopathy. This question is still unresolved. So far there have been conflicting indications from studies that have correlated the amount of insulin antibodies with the severity of microangiopathy (Page Faulk et al, 1971; Andersen, 1976). These mainly negative results are somehow expected. If insulin treatment has any influence on the course of complications through immune mechanisms, the study of free antibodies to insulin is not the best way to investigate this problem. These aspects will be discussed in details in chapter A1.5.

In the early seventies highly purified insulins were introduced in therapy (Andreani et al, 1973; Peacock et al, 1983) and in the early eighties human insulins were made available. Whereas highly purified and monospecies insulins have been proven to be of great utility in the treatment of allergic reactions, of lipodystrophy and other side effects, there is no evidence so far that purified insulins can substantially modify the course of microangiopathy. It must be said however that there are single cases which do not respond favourably to the purified insulin. In these cases it is presumed that the newly introduced human insulin may be useful. The newly prepared human insulins, both those developed by DNA recombinant technique and by semisynthesis, seem to have an immunogenicity similar or may be lower than that of purified porcine insulins. No significant difference has been observed in the insulin dose and in the biological effects on glucose metabolism at least after 6 months of follow up when patients previously treated with porcine monocomponent insulins were shifted to human insulins (Andreani et al, 1984; Iavicoli et al, 1983).

## A1.5 IMMUNOLOGICAL ASPECTS OF DIABETIC MICROANGIOPATHY

A1.5a Pathogenetic Mechanisms of  
Diabetic Microvascular Disease

Most aspects of the pathogenesis of diabetic microvascular disease remain unknown despite the great deal of research work in this area. Nevertheless, some factors or mechanisms responsible for the development of vascular damage have already been described. They can be divided into functional, biochemical, morphological and immunological changes.

\* Functional changes of small  
blood vessels

Modifications of haemodynamics

Intermittent vasodilatation of venules and capillaries, both in the retina and in the conjunctiva, are among the earliest changes in the microcirculation of diabetic patients (Ditzel, 1980; Ditzel et al, 1960). This is probably due to hypoxia, increased blood flow and increased blood viscosity.

Increased blood flow has been demonstrated by many studies, mainly in insulin-dependent diabetic patients. It is present in the

retinal circulation (Kohner,1976), in the kidney even at the onset of diabetes (Mogensen,1971) and in other tissues (Gundersen,1974).This increased blood flow is present in patients with a poor metabolic control and may be a consequence of a decrease in vascular resistance. In fact,several studies indicate an altered reactivity of vessels to vasoactive substances which can lead to decreased vascular resistance,which in turn may lead to an increased blood flow (Sieker et al,1955; Rhie et al,1982; Szentivanyi et al,1973; Cseuz et al, 1973; Sullivan et al,1979; Brody et al,1964; Ditzel et al,1972; Hostetter et al, 1981; Kreisberg 1982).Furthermore, the production of vasoactive hormones, and in particular of prostacyclin, is altered in diabetes (Christlieb,1976; Schernthaner et al,1981; Harrison et al,1981) .

The increased blood viscosity may be a consequence of abnormalities of red blood cells (Ditzel 1980; Mc Millan et al,1981), plasma proteins (Mc Millan 1975),platelets (Colwell et al,1981) etc.

#### Modification of permeability

The permeability changes are rather complicated. In diabetics an increased permeation of small blood vessels by albumin has been described (Williamson et al,1981),apparently as a consequence of an increased blood flow. On the other hand, inflammatory agents do not induce a substantially higher increase of albumin permeation in experimental diabetic animals in comparison with normal animals (Garcia-Leme et al,1974;Llorach et al,1976),perhaps because of the impaired vascular response to vasoactive substances found in diabetes. Furthermore,a change in electrical charge is found in diabetics and this can contribute to the loss of albumin in the first

stages of nephropathy while structural changes with the appearance of large pores may explain the massive proteinuria in the advanced stages (Myers et al,1982; Cohen et al,1977).

#### \* B i o c h e m i c a l   c h a n g e s

Some biochemical modifications are relevant in the deployment of immunological phenomena (Spiro,1976; Kijlstra et al,1978).

Biochemical studies of the permeability of the glomerular capillary wall have pointed out the importance of the positively or negatively fixed charged sites in the GBM in binding or trapping other penetrating macromolecules (Bohrer et al,1978; Brownlee & Spiro,1979; Gallo et al,1981). This permselectivity of the glomerular barrier is impaired in diabetic microangiopathy with a loss of glomerular electrostatic charge, a loss of ability to discriminate between macromolecules and with an increase in their transglomerular passage. The loss of glomerular fixed negative charges leads to an enhanced mesangial accumulation of circulating aggregates, a reduced affinity for cationic stains, a reduced restriction to filtration of circulating polyanions, a fusion of foot processes and eventually to an enhanced mesangial matrix production and albuminuria (Carrie and Myers 1980). Thus, among macromolecules, a few types of immune complexes, some of them potentially tissue destructive, may be more easily trapped or bound in the diabetic capillary walls (Barcelli et al,1981;Meade et al, 1981; Melato et al,1982). The fact that most of the glomerular electrostatic charges that are lost are negative seems to be due to a decrease in the basement membrane of sialic acid





residues and of glycosaminoglycans, that are negatively charged. In fact, a decrease in both sialic acid residues (Westberg et al, 1973; Kefalides, 1974; Canivet et al, 1979) and in glycosaminoglycans in diabetic kidney tissues (Farquhar, 1981; Williamson et al, 1983; Parthasarathy et al, 1982) has been reported.

Apart from the changes in electrostatic charges other important biochemical modifications have been observed in diabetes. The main finding has been an increase in type IV collagen in the basement membranes in the glomerulus, as a result of both an increased synthesis and a decreased degradation (Cohen et al, 1982; Brownlee et al, 1981). What is still not known is the reason for these abnormalities. In order to explain the increased synthesis of type IV collagen, it has been suggested that insulin deficiency, by causing an increased utilization of the alternate metabolic pathways of glucose, such as the polyol pathway, may mainly damage those cells which do not require insulin for the uptake of glucose and so are directly influenced by glucose levels (Williamson et al, 1981; Gabbay, 1975). Another possible explanation links together biochemical and functional changes of diabetic microangiopathy. The increases in blood flow and glomerular filtration rate may lead to an intravascular hyperpressure, thus accelerating the blood vessel damage (Hostetter et al, 1981; Williamson et al, 1977). In order to explain the reduced degradation of type IV collagen, the theory of increased glycosylation of proteins has been invoked. The increased glycosylation of collagen may indeed decrease its proteolysis, leading to an increased accumulation in the basement membrane (Uitto et al, 1982). This increase in circulation is mainly due to non-enzymatic mechanisms.

## \* Morphological changes

The main morphological modifications of blood vessels in diabetes are the retinal microaneurysms, the degeneration of pericytes, the segmental loss of endothelial lining cells, the capillary basement membrane thickening and the accumulation of proteins in the vascular basement membranes. Even if none of these changes is pathognomonic of diabetic microangiopathy, their contemporary presence is found only in diabetes. Very little is known about the causes of retinal microaneurysms, of pericyte degeneration, and of seemingly associated segmental loss of endothelial lining cells (Cogan et al, 1961; Kuwabara et al, 1963; Tilton et al, 1981). However, numerous researchers have investigated the capillary basement membrane thickening. Research on diabetic twins has shown an increase in width of the basement membrane in the gastrocnemius but not in the quadriceps muscle (Ganda et al 1983). This is obscure and differences in membrane width in the two muscles may be explained by differences in intravascular pressure. The blood pressure in the small vessels seems to be an important element (Gay et al, 1966; Berkman et al, 1973; Williamson et al, 1977; Mauer et al, 1978).

The accumulation of proteins, including immune factors, is of great relevance in diabetic microangiopathy (Mauer et al, 1976; Mauer et al, 1978; Cohn et al, 1978; Steffes et al, 1978; Jeraj et al, 1983). The presence of albumin among these proteins and their increased accumulation after unilateral nephrectomy or in the presence of hypertension, suggest that their presence is a result of an increased filtration of proteins with a consequent higher binding or trapping in the basement membrane. Nevertheless, the presence of some of these

proteins and, in particular, of the immunological proteins and macromolecules in the basement membrane may be harmful and contribute to the small blood vessel wall damage.

\* I m m u n o l o g i c a l   c h a n g e s

These aspects are extensively discussed in A1.5b.

Al.5b      I m m u n o l o g i c a l   a s p e c t s   o f  
                 m i c r o a n g i o p a t h y

\*    E v i d e n c e   f o r   n o n - m e t a b o l i c  
     f a c t o r s

The causes and the sequence of events of the lesions in small blood vessels in diabetics are still a matter of controversy. At present, it is widely accepted that metabolic abnormalities play a major role in inducing and accelerating the microangiopathic changes (Cudworth et al,1982). Nevertheless, there are many aspects of the pathogenesis of microangiopathy that cannot be explained only by metabolic derangements.

The occurrence and the progression of diabetic microangiopathy presents a wide individual variability.

- Microangiopathy may occur rather soon after the clinical onset of diabetes in patients with fairly good metabolic control and with a mild form of the disease. On the other hand, in some patients with an average unsatisfactory control, the late complications may not be recognizable even a few decades after diagnosis.
- Metabolic factors have been proved relevant in the initiation of nephropathy but they do not seem to have the same value when a fall in the glomerular filtration rate has taken place (Keen and Viberti,1981).
- From studies on diabetic twins, it has become apparent that there is a surprising concordance in the progression and

- severity of retinopathy in type 2 (non-insulin dependent) diabetic twins (Pyke & Tattersall,1973; Leslie and Pyke,1982).
- A heterogeneity within type 1 diabetes,with respect to susceptibility to microvascular disease and related to particular HLA antigens,has been suggested by several authors, though not confirmed by others. In any case, the assumption that the development of, or the protection from, microangiopathy may be influenced by inherited constitutional factors, either linked to HLA genes or not, is widely accepted (Marks et al,1981; Bodansky et al,1982; Gray et al,1982).
  - To some extent, there is a difference between types I and II diabetes in the severity and rate of progression of microangiopathic lesions,which is apparently related to factors other than metabolic events.
  - Other factors, including growth hormone and other hormonal factors,seem to play a role in controlling the insurgence and development of microangiopathy.

These and other considerations suggest that the pathogenesis of diabetic microangiopathy is multifactorial and an individual variability on a genetic basis may be expected. Furthermore, it is likely that different factors act at different stages of the pathogenetic sequence.

Rather than trying to postulate a single theory capable of explaining all the aspects of the small vessel damage, it may be more useful to orientate the research towards the individual aspects so far proved to be present in diabetic microangiopathy and try to understand their significance in the general multifactorial context. Of these aspects, only the immune phenomena will be discussed here.

\* I m m u n o l o g i c a l   p h e n o m e n a

Evidence is accumulating that a few potentially harmful immune factors are both in the circulation and in the blood vessel walls in diabetic patients with microangiopathy (Blumenthal,1962; Blumenthal et al,1962; Mohos et al,1963; Blumenthal et al,1964; Bloodworth,1965; Zampa & Mancini,1969; Andreani et al,1971).

In the sixties, interest in immunological mechanisms in late diabetic complications mounted after several researchers had reported immunohistopathological findings in the diabetic vascular lesions (Bloodworth,1968;Coleman et al,1962), as well as the possibility of inducing diabetic-like microangiopathy in normal animals through immune mechanisms and even with insulin treatment (Andreev et al,1970;McVerry et al,1980).

In the early seventies, this interest declined. Data available at that time suggested that the immunological abnormalities found could be merely secondary to other events and without a specific and destructive potentiality (Westberg and Michael,1972). Moreover, it was pointed out that exogenous heterologous insulin was not essential to the development of small blood vessel disease in diabetes.

In the late seventies, several different studies on circulating immune factors (Ortved Andersen,1976;Irvine et al,1977), on the interaction between soluble immune factors and other cellular systems (Iavicoli et al,1982), on the phagocytic function (Bagdade et al,1972) and on the physiopathology of the vascular basement membrane (Cruz & Moreau Lalande,1978; Cohen et al,1980) contributed new evidence on the immunological events in diabetic microangiopathy, at the same time revitalising interest in the subject.

### Histopathological findings in man

The results of histological studies may be summarized as follows:

- immunoglobulins, albumin, fibrin, complement, insulin and other proteins are deposited in the small blood vessel walls;
- deposits of immunoglobulins have been found in the ocular and dermal as well as glomerular vessels;
- in the diabetic kidney, serum proteins are present in the glomerular and tubular basement membrane and in mesangial nodules;
- serum proteins are deposited in most cases in a continuous linear pattern and sometimes also in a granular aspect along the capillary walls;
- immunoglobulins have no antibody activity to the glomerular basement membrane and added heterologous complement is not fixed;
- there are morphological similarities between the diabetic vascular lesions and those seen in other disorders of known immunological origin.

### Animal models

Studies on diabetic animals and experimental models capable of inducing microangiopathy in normal animals led to these main results:

- in diabetic animals, there is an increase in the glomerular basement membrane thickness, in the mesangial area and in immunological deposits;
- in normal animals, the injection of insulin together with Freund's adjuvant induces a diabetic-like vasculopathy;

- insulin injections produce microangiopathy only when insulin antibodies are formed;
- microangiopathy is experimentally induced in animals either sensitized with insulin/albumin or after injections of glucosylated plasma proteins.



Al.5c      I n s u l i n   a n t i b o d i e s   a n d  
                 i m m u n e   c o m p l e x e s

I n s u l i n   a n t i b o d i e s

Because of the species heterogeneity of therapeutic insulin, insulin antibodies, following exogenous insulin administration, were among the first immunological factors thought to play a role in vascular damage. As reported above, studies on animals supported this hypothesis. A few clinical studies have added some evidence of a role for insulin antibodies in subjects with severe diabetic complications (Ortved Andersen,1976;Jayarao et al,1974). Other research works have not been able to confirm an association between increased levels of insulin antibodies and the occurrence of microangiopathy (Bodansky et al,1982; chapter B3.2c).

This lack of a significant correlation between insulin antibody levels and clinical signs of microangiopathy was somehow to be expected. Most of the available methods of detecting insulin antibodies give a value related mainly to the free part of the antibody. If insulin has any role in the vessel wall damage, it is more likely brought about by the insulin anti-insulin complexes. Whereas this kind of complex in antibody or antigen excess is small in size because of the bivalency of insulin, insulin anti-insulin complexes near the equivalence point are of larger size and might activate complement components or induce the release of vasoactive substances when trapped in the vessel walls. Studies in this direction have recently been performed but no clear evidence for an

influence of insulin anti-insulin complexes on the genesis of the microangiopathic lesion has so far been established (Kilkpatrick & Virella,1980)(Chapter B3.4).

### I m m u n e c o m p l e x e s

The discovery of diabetic microangiopathy before the insulin era and its occurrence in insulin-treated as well as non-insulin-treated, diabetics suggest that immunological abnormalities, other than those simply related to therapeutic insulin, are involved in diabetic microangiopathy. More recently, attention has been focused on circulating and deposited immune complexes. In several different research works, an increase in immune complexes in circulation has been reported in a significant proportion of diabetics (Jayarao et al, 1974; Kumar and Quismorio, 1978; Bodansky et al,1982; Abrass and Lieberman,1983; Virella et al,1983). A few of these studies have reported an increase in complexes in diabetic patients with severe microangiopathy but not in those free of vessel wall lesions (Bodansky et al,1982)(Chapter B3.2c). This increase was found both in patients with proliferative retinopathy and advanced nephropathy but no substantial difference was found between insulin-treated and non-insulin-treated patients. The increase in immune complexes was even higher in diabetic patients with early retinopathy, also referred to as malignant microangiopathy (Andreani, 1980); the clinical manifestations in these cases have been referred to as an immune complex disease by some authors. From these studies, the presence of immune complexes seems to be closely related to the occurrence of

microangiopathic changes rather than the duration of the disease or the type of treatment.

## A1.6 IMMUNOLOGY IN DIABETIC PREGNANCY

A1.6a Immunology in normal pregnancy

Diabetic pregnancy is a very interesting model to study in order to further understand the significance of several immune phenomena.

In the present state of knowledge the immune system in normal pregnancy is considered to be in a state of "activation" rather than "depression" (Gleicher and Siegel,1983) and in many immunological aspects there is a strict interrelationship between mother and fetus, both in physiological and in pathological events.

In normal pregnancy, interesting modifications of the immune response are faced. An elevated production of counterregulatory hormones, which has been reported throughout the 9-months period, may influence the immune system. A decrease in both humoral and cellular immunoresponsiveness has been described (Gusdon,1976; Kaye,1973; Froelich et al,1980). The interrelations between mother and fetus are highly significant. The placental barrier selectively allows the passage of several molecules, including immunoglobulin G, whereas it blocks the passage of others (Vahquist,1958; Brambell,1974). Furthermore in pregnancy, the maternal immune system faces an antigenically non-self organism through the placental barrier. On the other hand, the fast growing immune system of the fetus is somehow protected and stimulated by the immune system of the host.

Several explanations have been proposed for this maternal immune protection.

The first hypothesis is based upon an immunological difference of the uterus. Thus allogenic spermatozoa are not recognized as foreign and promptly rejected by the immunocompetent system. On the other hand, since intrauterine graft of tissues other than fetal tissues are normally rejected, it is conceivable that human seminal plasma contains an immunosuppressive activity (Beer & Billingham, 1974; Lord et al, 1977; Stites et al, 1979; Denman, 1982).

The second theory invokes a decrease in the expression of HLA antigens on the trophoblast (Page Faulk, 1981). Whether the expression of transplantation antigens is really decreased is still a matter of controversy. It seems that HLA antigens, and both the maternal and paternal components of fetal transplantation antigens, are expressed on the placenta but in low density at the beginning of pregnancy and then they progressively increase and become detectable near the end of pregnancy.

A third possibility is based on the separation of maternal and fetal circulation. In clear contrast with this theory is the finding that fetal red cells, fetal lymphocytes and syncytiotrophoblast cells may cross the placenta during gestation.

At present the active immunosuppression found in pregnancy seems due both to humoral and to cellular immune factors.

Circulating factors such as hormones, alpha-fetoprotein, alpha<sub>2</sub> pregnancy associated globulin, immune complexes, anti-HLA antibodies, etc. are thought to have an immunosuppressive effect (Lord et al, 1977; Masson et al, 1977; Stites et al, 1979; Whyte and Loke, 1979; Stimson, 1980; Denman, 1982).

More recently the intervention of soluble suppressor factors, released by fetal suppressor T lymphocytes that then reach the maternal circulation, has been hypothesized (Olding and Olstone, 1975; Zuccarini et al, 1983).

Al.6b      I m m u n o l o g y   i n   d i a b e t i c  
p r e g n a n t   w o m e n

In the diabetic syndrome, several immune abnormalities have been found to be involved in the pathogenesis of type 1 diabetes (Bottazzo et al,1974; MacCuish et al,1974; Lernmark et al,1978; Pozzilli et al,1979)(Chapters B1.2a, B1.2b,B1.3), in the development of diabetic microangiopathy (Freedman et al,1960; Westberg & Michael,1972) (Chapters B3.2a, B3.2b, B3.2c, B3.2d), and as a consequence of heterologous insulin administration (Jayarao et al,1974; Andreani, 1974; Andersen,1976)(Chapters B2.3, B2.4, B2.5, B2.6).

In diabetic pregnancy, all these immunological phenomena are further complicated by the variable metabolic situation which influences several aspects of the immune response both in the mother and probably in the fetus (Barns and Morgans,1949; Macourt,1974; Pedersen,1975; Essex & Pyke,1979). Moreover, all the immunological factors or the defects antedating pregnancy have to be considered.

It is now well established that insulin antibodies, following heterologous insulin administration, cross the placenta and may be found for months in the newborn blood (Spellacy & Goetz,1963; Thorell,1966; Exon et al,1974; Mylvaganam et al,1983). On the other hand, maternal insulin does not reach the fetal circulation in significant amounts (Kalhan et al,1975).

Soluble immune complexes, found to be present in a good number of long-standing diabetics, have been reported to be present in the diabetic pregnant, but not, so far, in the neonatal circulation (Jorgensen et al,1966;Tamas et al,1975). Deposits of immune complexes

have been reported in placental vessels and into the basement membrane of the trophoblast. The presence of circulating immune complexes binding to placental Fc receptors seems responsible for the pre-eclamptic toxæmia. The hypothesis that immune complexes may interact with immunocompetent cells bearing Fc receptors on their surface, thus modulating or favouring an immunosuppressive effect, is stimulating .

Islet cell antibodies are present in some type 1 diabetic pregnant women. These antibodies may cross the placenta, as discussed in A1.6c. More important is the presence of islet cell antibodies in gestational diabetics. Here the presence of ICA predicts a probable insulin failure in the near future (Tingle et al, 1979; Mylvaganam et al, 1980; Steel et al, 1980; Ginsberg-Felner et al, 1980). Gestational diabetes with ICA shows a high association with HLA DR3 and/or DR4. The contemporary presence of these histocompatibility antigens and of islet cell antibodies suggests that a subgroup of gestational diabetics has many similarities with type 1 diabetes mellitus.

It is very likely that both the metabolic and the immunological factors present in diabetic pregnancy may have an influence on the course of pregnancy, on the function of the placenta and on the normal development of the fetus.



Al.6c Immunology in the infants of  
diabetic mothers

\* Humoral immunity

Insulin antibodies and islet cell antibodies easily cross the placenta and are found in the circulation in neonates of antibody positive, diabetic mothers.

The pathological significance of the presence of insulin antibodies in the neonatal circulation has not yet been elucidated but theoretically, they may be responsible for fetal hyperinsulinism and, thus, for the macrosomic and/or hypoglycaemic reactions in the neonate. In particular, the maternal insulin antibodies, on reaching the fetal circulation, face an immunological situation different from that of the mother. In diabetic pregnant women, the antigen is a heterologous therapeutic insulin, partially purified or of low immunogenicity, administered as a bolus, usually twice a day. Since maternal insulin does not cross the placenta in significant amounts, in the fetal circulation maternal insulin antibodies encounter the antigenically different endogenous foetal insulin continuously secreted throughout the day. Furthermore, in the fetal circulation the antigen/antibody molar ratio varies with the progression of pregnancy, so, from a condition of antibody excess in early pregnancy, a more balanced antigen antibody ratio is reached in late pregnancy.

Islet cell antibodies which have been passively transferred from the mother, may be found in the neonatal circulation but they do not

seem to have any specific deleterious effect on the fetus. They usually remain in the circulation for some months after birth but no correlation between their presence and glucose metabolism abnormalities or other neonatal clinical complications has so far been found. Circulating immune complexes have not yet been described so far in the circulation of infants of diabetic mothers. It is likely that the immune complexes described in some diabetic pregnancies may be passively trapped, or actively bound to Fc receptors of the placenta and this produces local damage with indirect pathological consequences for the mother and the fetus.

\* C e l l - m e d i a t e d i m m u n i t y

Eosinophilic infiltration of the pancreas of infants of diabetic mothers has been described (Silverman, 1963). It is likely that this infiltration is due to alterations in the immune response. The thymus of infants of diabetic mothers shows signs of involution in contrast to the generalized macrosomia (Horger et al, 1975).

Using monoclonal antibodies it has been shown that neonatal T lymphocytes do not express class II antigens on their surface after prolonged pokweed mitogenic stimulation (Miyawaki et al, 1982). The percentage of T suppressor lymphocytes in the cord blood appears to be surprisingly decreased thus suggesting that the immunosuppressive effect described in the neonatal circulation is not correlated to the number of cells but probably to other factors, possibly secreted by the immunocompetent cells themselves (Hayward and Kurnick, 1981).

C H A P T E R    A 2

PLAN OF THE STUDIES  
INCLUDED IN THE THESIS

Chapter A2      PLAN OF THE STUDIES INCLUDED IN THE THESIS

- A2.1    Background of the studies
- A2.2    Aims of the studies
- A2.3    General design of the studies

## A2.1 BACKGROUND OF THE STUDIES

The present study is concerned with the immunological aspects of diabetes mellitus.

The personal scientific interest of the author has been, and continues to be, on the overlap between immunology and diabetes, that area that is in more general terms referred to as immunoendocrinology.

The interest in the immunology of diabetes has developed only two decades ago. The detection of insulin antibodies after heterologous insulin treatment, the severe problem of microangiopathy, and the suspicion of aggravating immunological factors, and epidemiological and histopathological studies on the juvenile form of diabetes slowly raised interest in the role of immunological events in diabetes. At the end of 1974, however, the discovery of antibodies directed to the pancreatic islet in newly diagnosed insulin dependent diabetics by Bottazzo and Doniach in London and by Irvine's group in Edinburgh convinced all those in the field of the importance of immunity in diabetes.

This work started from the author's original observation that circulating immune complexes are present in diabetic patients. Their presence was described in different diabetic conditions: in some newly diagnosed insulin dependent diabetics, in long standing insulin-treated diabetics and in some patients with microangiopathy (B1.2a, B2.2a, B3.2a).

The finding of increased circulating amounts of antigen-antibody complexes, not only confirmed the occurrence of immunological phenomena in diabetes, but suggested their possible role in still obscure pathological events.

Since complexes were found to be increased in such different diabetic conditions, they were thought to be heterogeneous. The preliminary observation of a possible correlation of immune complexes with islet cell antibodies, insulin antibodies and vascular complications respectively in newly diagnosed diabetics, in long standing insulin treated diabetics and in diabetics with microangiopathy stimulated the author to pursue research in a number of directions.

Thus three main lines of research were begun.

The first was on the pathogenesis of type 1 diabetes (subchapter B1.2). The presence of circulating immune complexes needed to be confirmed in a substantial number of newly diagnosed insulin dependent diabetics (B1.2c). The possible correlation between the presence of immune complexes and the occurrence of circulating antibodies reacting with pancreatic antigen was analyzed both at diagnosis (B1.2c) and in an interesting minority of diabetics, the so-called islet cell antibody persisters (B1.2d), who were also genetically and immunologically better defined (B1.2b). These studies confirmed the increased presence of some types of complexes in newly diagnosed diabetics, and in islet cell antibody persisters, and their correlation with the occurrence of islet cell antibodies.

The second was on the consequences of insulin treatment (subchapter B2.2). The possible correlation between insulin antibodies and the presence of islet cell antibodies was investigated in type 1

diabetics at the same time after diagnosis and in islet cell antibody persister diabetics (B2.2b). The genetic influence on the immune response, as evaluated by HLA phenotypes, was also studied (B2.2b). The production of antibodies to exogenous insulin was found to be linked to particular HLA types whereas no correlation was found between insulin and islet cell antibodies.

The third was on the immunological phenomena in diabetic vascular complications (subchapter B3.2). In order to assay the possible pathogenetic role of circulating immune complexes in patients with microangiopathy, their presence needed firstly to be confirmed in a larger group of patients with different degrees of microangiopathy (B3.2b). Following this, selected patients with severe microangiopathy and others with no apparent sign of microangiopathy were studied (B3.2c). Since an increase in circulating complexes can be the result of an increased formation or of a decreased clearance, the function of fixed phagocytes was analyzed in vivo in patients with microangiopathy and correlated with the presence of complexes (B3.2d). This showed that some types of immune complexes are increased in both type 1 and 2 diabetics with severe microangiopathy and that such an increase is likely to be due to a reduced phagocytic clearance.

Following on from this earlier work - done mainly in Edinburgh in the years 1976-1981 - a more general plan of study has been designed which follows the three lines detailed above. A fourth line has been added, the immunology of diabetic pregnancy, to better understand the role of immunity in diabetes.

The cultural and theoretical background of the study have already been discussed in chapter A1.

## A2.2 AIMS OF THE STUDIES

The aims of the studies included in this thesis are:

1. To investigate the role of humoral immune factors in the pathogenesis of type 1 diabetes. Circulating islet cell antibodies, pancreatic specific antigens and immune complexes are evaluated and correlated with other immunological and genetic parameters in an attempt to monitor the islet cell damage in the early stages of type 1 diabetes and to better understand the pathogenetic phenomena.
2. To evaluate the immunogenicity of insulin preparations differing in purity and in their sources, i.e. semisynthetic human insulins or insulins extracted from animal pancreases. The host immune response to exogenous insulin is evaluated through the humoral immunological consequences of the treatment.
3. To study the possible role of humoral immune factors in the development of diabetic microangiopathy and their correlation with other abnormalities likely to be present in the same condition, i.e. platelet, coagulation and lymphocyte changes.
4. To better understand the immunological aspects of the fetomaternal relationship in diabetic pregnancy. In this particular condition the sum of the abnormalities related to diabetes and of the



modifications of the immune response characteristic in pregnancy may have physiopathological consequences on the outcome of pregnancy and on the neonate, that are largely unexplored.

## A2.3 GENERAL DESIGN OF THE STUDIES

This study follows four different lines in the field of immunity in diabetes.

This division into four lines is somewhat artificial, there being many areas of overlap. Such a division, however, best describes how the research has been planned and certainly simplifies its discussion.

### i) Pathogenesis of type 1 diabetes (Chapter B1)

Attention is focussed on humoral immunological phenomena in prospective studies on type 1 insulin dependent diabetics and on that interesting minority of diabetics who show immune abnormalities but initially do not require insulin.

Immune complexes are studied prospectively in a large number of type 1 insulin dependent diabetics and correlated with the presence of islet cell antibodies, complement fixing islet cell antibodies, viral antibodies, insulin antibodies and HLA types (B1.3).

Immune complexes, islet cell antibodies, complement fixing islet cell antibodies and autoantibodies to thyroid and gastric-parietal cells are studied prospectively in newly diagnosed diabetics, initially treated with diet or oral agents but showing islet cell antibodies in their serum (B1.4).

Since circulating antibodies to islet cells and soluble antigen-antibody complexes were found to be present and in correlation in the early stages of type 1 diabetes, it has been decided to search for circulating specific pancreatic antigens as possible early markers of islet cell damage. Thus monoclonal antibodies against pancreatic antigens are used, in specifically designed techniques, to try to reveal circulating pancreatic antigens in the early stages of type 1 diabetes (B1.5).

ii) Immunological consequences of insulin treatment (chapter B2)

The main objectives ~~are~~ to correlate the presence of soluble immune complexes with the type of treatment and with the presence of insulin antibodies and also to compare the immunogenicity of different insulin preparations.

The presence of immune complexes is evaluated in a large number of insulin and non-insulin treated diabetics and correlated with the type of treatment and other variables, such as age and sex, and duration of the disease (B2.3).

The correlation between immune complexes and insulin antibodies in randomly selected insulin treated diabetics is analyzed (B2.4).

The immunogenicity of conventional and highly purified heterologous insulins, as far as insulin antibodies and immune complexes are concerned, is investigated in a one year prospective study on newly diagnosed type 1 diabetics (B2.5).

Finally the immunogenicity of conventional, monocomponent and semisynthetic human insulins is studied in type 1 and 2 diabetics at

the beginning of their insulin treatment and in the following six months (B2.6).

iii) Immunology of diabetic vascular complications (chapter B3)

After having established the increase in the circulation of some types of immune complexes and the impairment of phagocytic function with a decreased clearance of macromolecules in diabetics with severe microangiopathy, the relationship between insulin antibodies and different kinds of immune complexes in patients with and without microangiopathy is investigated (B3.3).

The presence of humoral immune factors, such as immune complexes, insulin antibodies and insulin complexes, is investigated and the possible correlation with other abnormalities reported to be present in diabetics with severe retinopathy, i.e. the modification of platelet and coagulation factors is evaluated (B3.4).

The possible modification of T lymphocyte subsets and their correlation with immune complexes are investigated in long standing diabetics with various degrees of microangiopathy (B3.5).

iv) Immunology of diabetic pregnancy (chapter B4)

Attention is focussed on both the humoral and cellular phenomena in insulin treated diabetic pregnant women and on the possible physiopathological consequences of these factors on the outcome of pregnancy and on the neonate.

Insulin antibodies, immune complexes and islet cell antibodies are studied in a large group of insulin treated diabetic pregnant women. The possible correlation between these immune factors and the type of insulin treatment and the maternal metabolic control are considered. The main objective is to investigate the possible role of immune factors in clinical complications in the mother and/or neonate (B4.2).

Insulin antibodies and insulin-anti-insulin complexes is evaluated in both the maternal circulation during pregnancy and in neonatal cord blood. The placental transfers of insulin, of insulin antibody and of insulin-anti-insulin complexes and their physiological consequences have received particular attention (B4.3).

Since pregnancy is a unique condition as far as cellular immunity is concerned, and diabetic pregnancy is likely to be further complicated by cell-mediated phenomena related to diabetes, it is considered to be of some importance to study T cell subsets in diabetic pregnant women (B4.4) and to search for activated T cells by the use of an appropriate number of monoclonal antibodies defining T cell surface antigens (B4.5).

CHAPTER A 3

METHODS: GENERAL LINES

Chapter A3      METHODS : GENERAL LINES

- A3.1    Circulating immune complexes
  - A3.1a    Methods of detection: general lines
  - A3.1b    Solid-phase Clq binding test
  - A3.1c    Conglutinin binding radioimmune assay
  - A3.1d    Raji cell radioimmune assay
  - A3.1e    Technical notes
  - A3.1f    Immune complexes in normal subjects
- A3.2    T cell subpopulations
  - A3.2a    Monoclonal antibodies to T cell surface antigens
  - A3.2b    An original method to facilitate the evaluation of T-cell subsets
  - A3.2c    An original method to evaluate the MHC class II antigen positive T cells
- A3.3    Insulin antibodies
  - A3.3a    Andersen's method
  - A3.3b    Christiansen's method
- A3.4    Insulin-anti-insulin complexes
- A3.5    Islet-specific autoantibodies
  - A3.5a    Islet cell antibodies
  - A3.5b    Complement fixing islet cell antibodies
- A3.6    Circulating islet cell antigens
  - A3.6a    Monoclonal antibodies reacting with

pancreatic islet cell antigens

A3.6b Techniques to detect circulating  
islet antigens



## A3.1 CIRCULATING IMMUNE COMPLEXES

A great deal of useful information has accumulated over the past ten years on the composition, biological activities and detection of immune complexes in human disease. This period has also seen an increase in the number of variable techniques for the detection of complexes (WHO Scientific Group Report, 1977; Thompson RA 1980). Almost all of the tests for immune complexes are antigen non-specific since they depend upon the various biological properties of complexed IgG, which are in turn expressions of activity of altered Fc domains. An inevitable consequence of this non-specific expression of activity is the failure of available techniques to distinguish between complexes arising from specific disease processes and those which are the result of a natural encounter with environmental agents or perhaps expressions of altered autoimmunity with increasing age. This is of more than academic importance and requires that the possible physiological roles of immune complexes be more fully explored. A better understanding of the mechanisms by which immune complexes are detected and a further refinement and development of immune complex technology will hopefully facilitate a greater comprehension of their potential physiological and undoubted pathological roles.

A3.1a      M e t h o d s   o f   d e t e c t i o n :  
                 g e n e r a l   l i n e s

Although the pathological importance of the formation of soluble immune complexes has been recognised for almost seventy years, only recently have apparently suitable techniques for their detection been developed and refined. At this time there are about thirty such techniques which depend upon one or another of the variety of physical or biological properties displayed by immune complexes. Of this large number of techniques, only a few appear sufficiently sensitive for clinical or experimental purposes in human disease (WHO Scientific Group Report, 1977; Fust et al, 1981). None of these methods distinguishes between non-specifically aggregated immunoglobulins and immune complexes. They are based on the properties of complexed immunoglobulins, which differ from those of monomeric immunoglobulins. Depending on the principle of the method, they may be broadly divided into those revealing complexes through the physical properties of complexes and those through the biological properties.

a) Methods based on the physical properties of the complexes:

Among this group are those methods based on size changes of aggregated immunoglobulins and those based on solubility changes. The increase in size may be analyzed by sucrose density gradients, by gel filtration or ultrafiltration and by analytical ultracentrifugation. These methods are more important in the purification of complexes than in routine evaluations of serum immune complex levels in patients. The solubility changes of aggregated immunoglobulins may be

revealed by low temperature or by polyethylene glycol, molecular weight 6000. This latter method has been extensively used for routine assays but its very low specificity recommends its use only as an initial step in the further purification of complexes.

b) Methods based on the biological properties of complexes:

The reactivity of immune complexes with receptors, either on free molecules or on the surface of cells, is the basis of numerous techniques.

Among the methods utilizing receptors on free molecules, there is a large group based on the properties of the first component of complement (Clq). Clq avidly binds the Fc portion of complexed immunoglobulins and different techniques have been devised to reveal this binding. Among these, the most commonly used are the fluid phase Clq radioimmune assay and the solid phase Clq binding test (see below). The direct measurement of anticomplementary activity or the interaction of complexes with complement is also used as the basis of several other tests. Other free molecules used to bind complexes are the rheumatoid factor, low affinity IgM antibodies and a bovine protein, conglutinin, which binds complex-bound C3. The conglutinin binding test is now widely used (see below).

Among the methods based on the reactivity of complexes with cellular receptors, the Raji cell radioimmune assay must be mentioned. This human lymphoblastoid cell line, derived from Burkitt's lymphoma patients, presents both Fc and complement receptors on the cell surface (see later). Other cellular tests include those using platelets or macrophages.

None of the methods described above is sufficiently specific, reproducible or sensitive enough to detect circulating immune complexes with precision.

\* C o m p a r i s o n   b e t w e e n   d i f f e r e n t  
t e c h n i q u e s

At present it is widely accepted that different methods usually detect different types of immune complexes. Of course, the types of complex detected depend on the antigen(s) or antibody(s) involved. But, for a given antigen and antibody, the valency of the antigen, the class of the antibody and the antigen/antibody ratio also influence the size of complexes, the degree of lattice formation, the possibility of activating the complement cascade, of interacting with platelets and of reacting with cells bearing Fc and C receptors. Furthermore, differences in the specificity and sensitivity of the various methods account for the discrepancies often found in the results obtained with them.

A few collaborative studies were organized to compare different methods. A WHO collaborative study for the evaluation of 18 methods of detecting immune complexes in serum showed a considerable difference in the pattern of reactivity of sera from diseases under study in the various tests (Lambert et al, 1978). The study suggests that, for each disease, it is possible to select one or more tests most likely to detect abnormal reactivity and to screen patients for immune complexes. A parallel application of different methods, based on different principles, is advisable. In another recent collaborative

study, in which the author was involved, the conclusions were similar (Celada and Migliorini, 1982; Migliorini et al, 1983).

Since immune complexes are polymorphic and since there is now definitive evidence of variability in the quality of complexes from disease to disease and in the same individual from stage to stage of the disease, the attention of researchers is now focussed on the possibility of purifying complexes and identifying the antigens involved in the immune complex formation.

At present antigen non-specific methods, possibly different techniques based on different principles, are confined to screening patients' sera or to following the same patient over a period of time.

A3.1b The Solid-phase Clq binding  
test (Clq - SP)

a Preparation of immunological  
reagents for Clq - SP

a.1 Purification of Clq

The methodology described is that devised by Yonemasu and Stroud (1971) with minor personal modifications.

\* Reagents and equipment:

EGTA; Na<sub>2</sub>EDTA; NaOH; NaCl; Na acetate; acetic acid; KH<sub>2</sub>PO<sub>4</sub>;

Na<sub>2</sub>HPO<sub>4</sub>;

magnetic stirrer; ultracentrifuge (with a potential load of at least 130ml); dialysis tubes

\* Preparation of solutions:

bf1: 0.026 M EGTA in 2.2 litres of distilled water (d.w.), reach pH 7.5 with concentrated NaOH

bf2: 0.75M NaCl; 0.02M Na acetate; 0.02M acetic acid; 0.01M Na<sub>2</sub>EDTA in 500ml of distilled water; adjust to pH 5 with NaOH

bf3: 0.06M EDTA in 4 litres of d.w. adjust to pH 5

bf4: 0.005M KH<sub>2</sub>PO<sub>4</sub>; 0.005M Na<sub>2</sub>HPO<sub>4</sub>; 0.75M NaCl, 500ml pH 7.5; 0.01M EDTA, (8ml 1N NaOH; in 500ml of d.w.

adjust to pH 7.5 with NaOH

bf5: 0.035M Na<sub>2</sub>EDTA

in 4 litres of d.w.

adjust to pH 7.5

bf6: 0.75M NaCl;0.02M Na acetate;0.02M acetic acid;

0.01M EDTA

in 500ml of distilled water

adjust to pH 7.5

\* Procedure for the purification of Clq:

The method is devised for 1 unit of serum ( 127ml).

For more than 1 unit, use adequate quantities of buffers.

(i) first precipitation

- dialyze 1 unit of fresh human serum against bfl, 1 litre, for 4 hr at 4°C
- continue dialysis against another litre of bfl for 1hr at 4°C
- spin at 10 000g for 15'
- wash precipitate with bfl, 50ml
- spin at 10 000g for 15'
- dissolve with bf2, 32ml
- spin at 5 000g for 5' to remove undissolved.

Keep the supernatant

(ii) second precipitation

- dialyze the mixture against bf3, 4 litres, for 4 hr at 4°C
  - spin at 10 000g for 15'
  - wash precipitate with bf3, 50ml
  - spin at 10 000g for 15'
  - dissolve with bf4, 32ml
  - spin at 5 000g for 5' to remove undissolved.
- Keep the supernatant

(iii) third precipitation

- dialyze the mixture against bf5, 4 litres, for 5 hr at 4°C
- spin at 10 000g for 15'
- wash the precipitate with bf5, 50ml
- spin at 10 000g for 15'
- dissolve with B6, 16ml
- calculate the concentration, with a spectrophotometer (OD 1=1.4mg)
- aliquot and store at -70°C

a.2 Radiolabelling of protein A (or anti human immunoglobulin antibody)

The procedure is according to Dorval et al.(1975) with minor personal modifications.

\* Reagents and equipment:

<sup>125</sup>I;Sephadex G.50 or G.25; protein A (or anti human



immunoglobulin antibody); chloramin T; Na meta-bisulfite; Bovine serum albumin; chromatographic column 200 x 10mm; fraction collector (if available)

\* Preparation of solutions:

bf1: phosphate buffered saline (see part B)

bf2: chloramin T 0.8mg/ml in bf1

bf3: Na metabisulfite 1mg/ml in bf1

bf4: BSA, 5% in bf1

\* Procedure:

- prepare the chromatographic column with Sephadex G25 (or G50) in bf1

- add 1mCi of  $^{125}\text{I}$  to 50ul of protein A, 1mg/ml

- add bf2, 5ul and mix 1 min

- add bf3, 5ul and mix

- count the radioactivity

- add bf4, 250ul and mix

- separate  $^{125}\text{I}$ -protein A from  $^{125}\text{I}$  on the Sephadex column collecting 1ml fractions

- pool the  $^{125}\text{I}$ -protein A peak (usually around the 3rd-5th fraction)

- divide into aliquots and store at  $-20^{\circ}\text{C}$  till used

### a.3 Preparation of immunoglobulin aggregates

\* Reagents and equipment:

human IgG;

thermostatic bath at 63°C

\* Heat aggregation:

- dilute human IgG to a concentration of 10mg/ml and  
divide into aliquots

- heat 20 min at 63°C

- store at -20°C

b. Preparation of other reagents  
for ClqSP

b.1 Buffers

bf1 Phosphate buffered saline, pH 7.2, 0.15 M (quantities  
for 10 litres):

Sodium chloride, 80g; Potassium chloride, 2g;

Di-sodium hydrogen phosphate (0.008M), 11.5g;

Potassium di-hydrogen phosphate, 2g;

Sodium azide, 0.2g/l

bf2 Gelatin solution:

Gelatin powder, 0.05% in bf1

bf3 Di sodium ethylene-diamino tetra-acetic acid, pH 7.5:

0.2M in d.w. adjust to the right pH with NaOH 1N

bf4 PBS + Tween 20:

Tween 20 0.05% in bf1

bf5 PBS + Bovine serum albumin:

bf1 + BSA 1%

### c. Procedure of Clq - S P

This procedure is a minor personal modification of those described by Svehag (1975) and Hay et al. (1976).

#### c.1 Coating tubes with Clq

- Clq is diluted to 10ug/ml in bf1
- Diluted Clq (100ul) is added to polysterene tubes (Immulon, Dynatech, 250ul capacity tubes)
- incubate 3 days at 4°C
- wash 3 times with bf4
- fill tubes with bf2
- incubate 2h at room temperature (r.t.)
- wash 3 times with bf4

#### c.2 Complement inactivation of serum (13).

- add serum to be tested, 10ul, to bf3, 20ul and mix
- incubate 30 minutes at 37°C
- transfer the mixture to an ice bath

#### c.3 Preparation of the standard curve

- dilute one aliquot of the immunoglobulin aggregates to 500, 250, 125, 62, 31, 16, 8, 4, ug/ml in normal serum
- include these dilutions in duplicate (or in triplicate) in each experiment

#### c.4 Assay

- add the complement inactivated human serum (10ul) to

Bf1.(90ul) and mix

- incubate 1 hour at 37° and 30 minutes at 4°C
- wash 3 times with Bf4
- add <sup>125</sup>I protein A, 25ng/ml in Bf5, 100ul
- incubate 1 hour at 37° and 30 minutes at 4°C
- wash 3 times with Bf4
- count in a gamma counter

#### c.5 Calculation of results

- use a semilogarithmic paper and put the absolute value of aggregates on the semilog axis and the radioactivity values (cpm) on the y axis
- read the absolute values of aggregates for each test serum
- taking into account the quantity and the dilution of serum, give results in ug AHG equivalents/ml of serum.

A3.lc    The   c o n g l u t i n i n   b i n d i n g   r a d i o -  
i m m u n o a s s a y   ( K g B t )

a.   P r e p a r a t i o n   o f   i m m u n o l o g i c a l  
r e a g e n t s   f o r   K g B t

a.1   P u r i f i c a t i o n   o f   c o n g l u t i n i n

The purification is performed according to the methods of Lachmann ,1967,(Weir,1978) and Casali et al,1977, with minor personal modifications.

\*   P r e p a r a t i o n   o f   t h e   y e a s t

i) Reagents and equipment:

baker's yeast;mercaptoethanol;iodoacetamide;NaCl;  
KCl;Na<sub>2</sub>HPO<sub>4</sub>;KH<sub>2</sub>PO<sub>4</sub>;sodium azide;  
autoclave;centrifuge 4 x 500ml;incubator 37° C;  
stirrer;pH meter

ii) Solutions:

bf1: phosphate buffered saline (see ClqSP)

bf2: mercaptoethanol 0.1M

bf3: iodoacetamide 0.02M;NaCl 8.5 g/l;

1/5 v/v of phosphate buffer 0.2M, pH 7.2

bf4: veronal buffered saline pH 7.2;NaCl 8.5 g/l

Barbitone 0.575 g/l;Soluble barbitone 0.185g/l;

NaCl 8.5 g/l; MgCl<sub>2</sub> 0.168 g/l;

CaCl<sub>2</sub> 0.028 g/l

iii) Preparation of the yeast:

- 1 lb of baker's yeast is added to 2l of bfl
- autoclave for 30' at 120°
- centrifuge at 1000g for 10'
- wash in bfl 3 times
- add 1.7ml of bf2 to 250ml of yeast in bfl
- incubate at 37° and stirring for 2 hrs
- centrifuge at 1000g for 10'
- wash with bfl
- resuspend in 500 ml of bf4
- stir 2h at r.t.
- adjust pH to 7.2
- centrifuge at 1000g and wash in bfl 3 times
- resuspend in 2l of bfl
- autoclave 30' at 120 °C
- centrifuge at 1000g and wash extensively in bfl
- resuspend in 1l of bf5
- divide into 50ml aliquots

\* Purification of bovine conglutinin:

i) Reagents and equipment:

CaCl<sub>2</sub>; KH<sub>2</sub>PO<sub>4</sub>; NaCl; Na azide; Na<sub>2</sub> EDTA; H<sub>3</sub>PO<sub>4</sub>;

Na OH;HCl; dialysis tubes;  
 thermostated bath 56°;stirrer; centrifuge rotor 100ml  
 x 4 or rotor 500ml x 4;ultracentrifuge;DEAE column

ii) solutions

- bf1:  $\text{CaCl}_2$  40g/dl  
 bf2: PBS (see above)  
 bf3:  $\text{Na}_2\text{EDTA}$  0.01M in PBS, adjust pH to 7.2  
 bf4:  $\text{H}_3\text{PO}_4$  0.1M  
 bf5: Phosphate buffer 0.01M, pH 5.4  
 bf6: mix 1/3 of phosphate b. 0.1M, pH8  
           1/3 of NaCl 0.2M  
           1/3 of  $\text{Na}_2\text{EDTA}$  0.02M, pH8  
 bf7: mix 1/3 of phosphate b. 0.1M, pH8  
           1/3 of NaCl 0.1M  
           1/3 of  $\text{Na}_2\text{EDTA}$  0.02M, pH8  
 bf8: mix 1/3 of phosphate b. 0.1M, pH8  
           1/3 of NaCl 0.3M  
           1/3 of  $\text{Na}_2\text{EDTA}$  0.02M, pH8  
 bf9: mix 1/3 of phosphate b. 0.1M, pH8  
           1/3 of NaCl 0.6M  
           1/3 of  $\text{Na}_2\text{EDTA}$  0.02M, pH8

iii) initial purification

- centrifuge 10l of bovine blood ,2000g for 10' and  
   keep the serum
- heat the bovine serum at 56°C for 45'
- add 1 ml of yeast and 5 ul of bf1 to 5 ml of bovine

serum

- stir at 4°C for 1h
- centrifuge at 1000g for 10'
- wash 3 times with bf2
- resuspend in bf3 (0.5 ml for each 1 ml of yeast previously added)
- stir 10' at r.t.
- centrifuge at 1000g for 10' and keep the supernatant
- resuspend the precipitated yeast in bf3 (1 ml for each 1 ml of yeast previously added)
- centrifuge at 1000g for 10' and the supernatant
- pool the supernatants
- adjust to pH 5.4 with bf4
- dialyse 24h against bf5
- centrifuge at 5000g for 15'
- resuspend in 10 ml of bf6

iv) further purification

- use a DEAE chromatographic column 20x2 cm and a fraction collector
- equilibrate the column with bf7
- carefully add the conglutinin to bf6
- use bf8 as the first gradient
- use bf9 as the second gradient
- collect 1ml fractions and measure the optical density with a spectrophotometer
- the peak eluted by the second gradient contains the



## conglutinin

- check purity with an antiserum to conglutinin

## a.2 Radiolabelling of protein A

(see Clq-SP)

## a.3 Preparation of immunoglobulin aggregates

(see Clq-SP)

b. Preparation of other reagents  
for K g B t

bf1 carbonate Ca buffer 0.05M pH 9.6:

$\text{NaHCO}_3$ , 0.035M;  $\text{Na}_2\text{CO}_3$ , 0.015M;  $\text{CaCl}_2$ , 0.2mM;

Na azide, 0.2g/l

bf2 VBS pH 7.2:

Barbital, 0.575g, quantities for 1 litre;

Soluble barbital, 0.185g;  $\text{NaCl}$ , 8.5g;  $\text{MgCl}_2$ , 0.168g;

$\text{CaCl}_2$ , 0.028g; Na azide, 0.2g

bf3 VBS + Tween 20:

bf2 + Tween 20 0.05%

bf4 VBS + Bovine serum albumin:

bf2 + BSA 0.1%

c. Procedure of K g B t

c.1 Coating tubes with conglutinin:

- Kg is diluted to 5ug/ml in bf1

- diluted Kg (100ul) is added to polypropylene tubes (Immulon, Dynatech, 250ul tubes)
- incubate 3h at 37°C
- leave 4°C until used
- wash 3 times with bf3

#### c.2 Preparation of the standard curve

- dilute one aliquot of the immunoglobulin aggregates to 250, 125, 62, 31, 15, 8, 4 ug/ml in fresh human serum
- incubate 30min at 37°C
- keep at 4°C until used

#### c.3 Assay

- prepare the standard curve as indicated in A.3
- fresh serum (10ul) + Bf2 (90ul) are added to tubes
- incubate 2h 4°C
- wash 3 times in bf3
- add <sup>125</sup>I-protein A, 5ng, in bf4 (around 30-50000 cpm)
- incubate 4h at room temperature
- wash 3 times in bf3
- count radioactivity in a gamma counter

#### c.4 Expression of results

(see Clq-SP)

### A3.1d The Raji cell radioimmune assay (RAJI)

The method is according to that of Theophilopoulos et al with several personal modifications (Theophilopoulos et al,1976).

#### a. Preparation of immunological reagents for RAJI

##### a.1 Preparation of cells

- For the culturing of cells see other general texts on this topic
- prepare about 2 million cells for the assay
- wash cells in medium (RPMI 1640, Modified Eagles medium, or others)
- count the cells and check the viability (for instance by trypan blue exclusion)
- wash the right amount of cells for the assay and resuspend in 50 ul of medium

##### a.2 Radiolabelling of protein A (or antihuman immunoglobulin antibody (see Clq-SP)

##### a.3 Preparation of immunoglobulin aggregates (see Clq-SP)

#### b. Preparation of other reagents for RAJI

b.1 Media

- m1 Modified Eagles medium (MEM)
- m2 MEM + 1% bovine serum albumin

b.2 Buffers

- bfl PBS (see Clq-SP)

c. Procedure for RAJI

c.1 Preparation of sera

- dilute cells 1/4 in bfl, final volume 100 ul

c.2 Preparation of the standard curve

c.3 Assay

- add 25 ul of diluted serum (or standard) to RAJI cells suspended in 50 ul of serum
- incubate at 37°C for 45 minutes
- add 1 ml of "m1" to wash (spin 800 g, 10 minutes, room temperature). Resuspend the pellet in 1 ml of "m1" and repeat twice
- add 5-10 ug/tube of <sup>125</sup>I-protein A in 50 ul of m2 resuspend the pellet
- incubate at 4°C for 30 minutes
- add 1 ml of m2 to wash (spin 800 g, 10 minutes, 4°C). Resuspend the pellet in 1 ml of m2 and repeat twice
- count the radioactivity

c.4 Calculation of results

see Clq-SP

A3.1c      T e c h n i c a l   n o t e s

\*      Standardization, performance and sensitivity of the techniques as assessed by aggregated Ig (AHG) binding. Fc receptor uptake of AHG by Raji cells.

Aggregated Ig curves were included on all occasions assays were performed. In terms of this standard the RAJI, Clq-SP and KGBt techniques appear equally sensitive and more sensitive than the Clq-FP. Each of the former assays detects less than 10 ug/ml of aggregated Ig whilst the lower limit of detection for the latter assay appears to be about 50-60 ug/ml. However, this comparison is fallacious; the Clq-FP, for example, is more sensitive than the Clq-SP technique in tests on rheumatoid arthritis sera (personal observation). Nevertheless, Clq-SP is a useful technique in other diseases such as diabetes mellitus. In general terms very little variation was seen in the binding of aggregated Ig in either Clq-SP or KGBt assays over several months of use. Some variation in the background non-specific precipitation of Clq in Clq-FP was evident from batch to batch of Clq and also from day to day. Although this variation was in the order of only 5-6% of Clq precipitated, it was sufficient to make the interpretation of results between tests difficult. In practice, because of the lack of binding of aggregated Ig in the Clq-FP technique below about 50 ug/ml, it is difficult to express results as equivalents of aggregated Ig in ug/ml and thus some form of internal standardization, by including the same positive and negative sera in every test, is a useful practice. This form of standardization is

advisable for all the immune complex techniques. The poor binding of Ig in the Clq-FP technique is probably related to the large size of aggregates produced by heating Ig at 63°C. About 60% of Ig aggregated by heating at 63°C has a molecular size of 1 million or greater and the remainder is apparently monomeric Ig. Clq-FP does not appear to detect such large complexes or aggregates as successfully as it detects smaller molecular weight material.

In our hands the binding of aggregated Ig to Raji cells was subject to quite marked variation over some months of use although consecutive tests usually gave reproducible results. This variation in apparent binding ability of Raji cells can usually be circumvented by deriving serum binding values from aggregated Ig curves; in this way sera tested several months apart gave reasonably correlative results. Nevertheless, RAJI is by far the most technically demanding of the four techniques we have used.

Although evidence exists that immune complexes are preferentially taken up by attachment to C3/C3d receptors on RAJI cells (Theophilopoulos et al, 1976), complement independent mechanisms may be important for their attachment. Aggregated Ig, in the absence of normal serum, is taken up equally well in the presence of complement. The binding of aggregated Ig in heat inactivated sera is difficult to assess because heat inactivation to destroy complement components also induces aggregation of Ig (Zubler et al, 1976) but some uptake of Ig is evident. It has been sometimes found an unsatisfactory correlation between RAJI and other complement dependent techniques for complexes; when RAJI has been used, evidence of complexes, which could not be confirmed with the Clq-SP, Clq-FP or Kg-B techniques, has been found. Although differences in assay sensitivities may

account for these findings, the uptake of complexes onto RAJI cells by complement independent mechanisms may well be a plausible explanation.

\* Correlation between results obtained with different assays.

As some studies, including those presented here, have shown (see below) the concordance rate among results of assays can be high. For example, in tests of diabetic sera it has been found a good correlation between RAJI and Clq-SP. However, unsatisfactory correlations are sometimes apparent. Such unsatisfactory correlations may be consequences of differences in the sensitivity of the assays or may be reflections of the heterogeneity of physical and biological characteristics of complexes found in disease. Even when the prevalence of complexes detected in the same population by different techniques is similar, the results for individual sera may not be in accord. This was illustrated in a study of long-standing diabetic patients (Chapter B3.2c) where the prevalence of detectable AgAb by Clq-SP and KgBt was respectively 51% and 39% but the results of the assays were in agreement in only 56% of sera tested. In this particular example, the lack of correlation may be explained by differences in the size of complexes which are detected by Clq and KgBt techniques. From such examples it may be inferred that no single technique is in itself sufficient to investigate the occurrence and characteristics of immune complexes in disease.



- \* Expression of results and definition of positive immune complex values.

It is widely accepted that quantitation in the described methods may be achieved by reference of sample duplicates to the uptake of serially diluted heat aggregated immunoglobulins and the results expressed in micrograms of aggregate equivalents per millilitre of undiluted serum (ugAHG<sub>eq</sub>/ml). As an alternative method, quantitation may be referred to "in vitro" made complexes with a known antigen and antibody in a prefixed ratio. Results may also be expressed as a percent of the radioactivity added, calculating the ratio between the radioactivity found in the sample and that added. Others prefer to express results as a percent of the maximum bound, that is to say, the ratio between the radioactivity found in the sample and that of the highest fixed amount of aggregates in the standard curve.

The definition of the prevalence of immune complexes in disease is obviously related to the values obtained for apparently normal subjects in the assay used. Although some opinions suggest that immune complexes are undetectable in healthy normal subjects, the experience of the author indicates otherwise. About 10% of randomly selected blood donors give moderately raised binding values in Clq-SP, Clq-FP and Kg-Bt assays (Figures A3.1/1, A3.1/2, A3.1/3). The prevalence of complexes is related to age but not to the sex of the subjects and may be a reflection of serum autoantibodies (Delespesse et al, 1980). In addition, gel filtration studies of normal sera show "heavy" molecular weight IgG present and some variation in the levels of complement fixing immune complexes can be shown during the day (Di

Mario et al,1980). Some of this diurnal variation is perhaps due to the formation of complexes after food ingestion. In the RAJI assay,20-25% of blood donors may give raised binding values (Figure A3.1/4) - an observation confirmed in other laboratories. This may be related to the use of cultured cells as a matrix for the detection of complexes; that is to say, some of the values obtained in RAJI may be false positives due perhaps to the presence in the sera of antibodies to cell surface membrane antigens or to antigens adsorbed onto the cell during culture.

In conclusion, since the values of immune complexes in the normal population show a positive peaked asymmetric distribution, the 90th percentile of the values of the normal population was chosen as the limit of positivity for the Clq-SP and the KgBt techniques For Clq-FP,the 90th percentile, the 95th percentile or the mean of the controls plus 2 standard deviations of the mean may in practice make little difference. However, in RAJI, because of the high values and wide range of values obtained for normal subjects, the author, along with other researchers,has found it necessary to arbitrarily designate 20 mcg/ml of aggregated Ig equivalents as the upper limit of immune complex negative. This value is usually the minimum quantity of aggregated Ig which can be detected with confidence. As a function of this wide variation of binding of normal sera to RAJI cells,and the inter-test variability, necessitating expression of RAJI results in terms of units derived from standard curves, the test may be unduly influenced by the choice of serum used as a complement source for the dilution of aggregated Ig.In studies with RAJI,for the dilution of aggregated Ig sera derived from pools of normal sera or sera with a binding value approximately equal to the mean of a large

number of normal sera were used. Although this practice facilitates the sensitivity of the test, it also delineates a number of normal sera as apparently positive.

To reduce to a minimum the variability of the limit of positivity from test to test, it was decided to assay a very large group of normal sera to be used as controls in the same experiment. Out of these samples ten sera were chosen to statistically represent the entire normal population studied, i.e. their median and interquartile range were the same as the total group. Frozen aliquots of these ten sera were included in each experiment and only an inter-assay variability of less than 10% of their values was tolerated.

FIGURE A3.1/1

AgAb detected by Clq-SP in 80 normal subjects. Measurements falling at or above the 90th percentile of the group are considered positive. The limit of positivity is indicated by a dotted line.

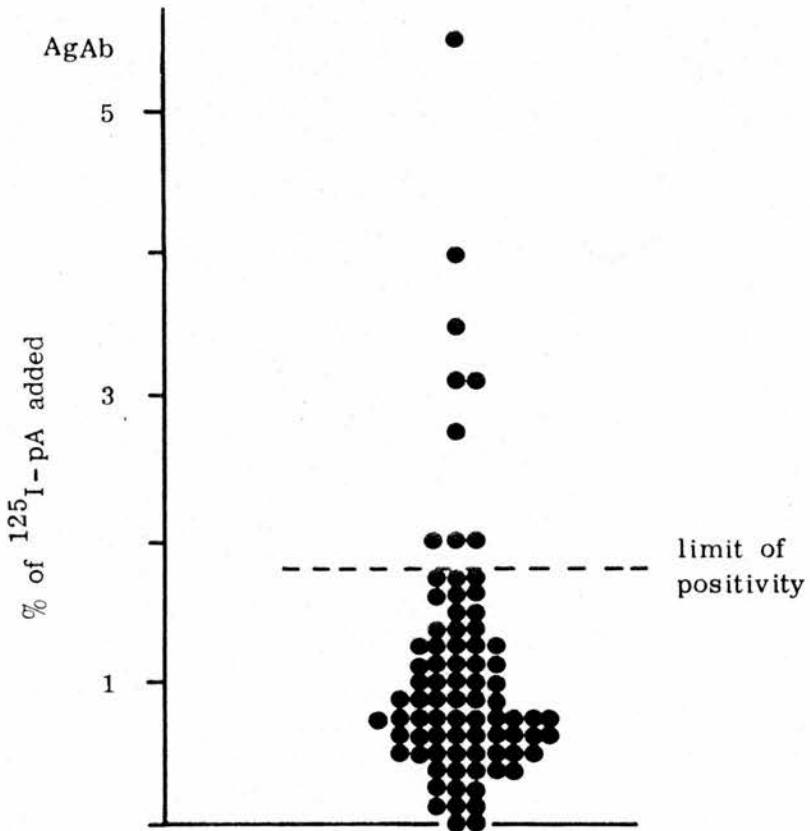


FIGURE A3.1/2

AgAb detected by Clq-FP in 83 normal subjects. Measurements falling at or above the 90th percentile of the group are considered positive. The limit of positivity is indicated by a dotted line.

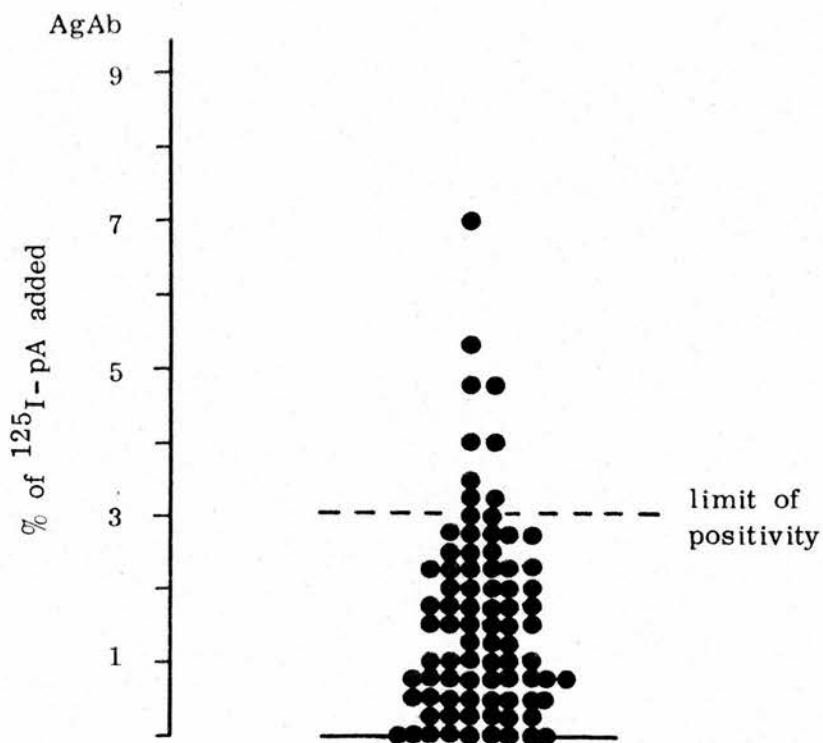


FIGURE A3.1/3

AgAb, as detected by KgBt, in 105 normal subjects. The dotted line represents the 90th percentile. Measurements falling at or above the 90th percentile of the group are considered positive. The limit of positivity is indicated by a dotted line.

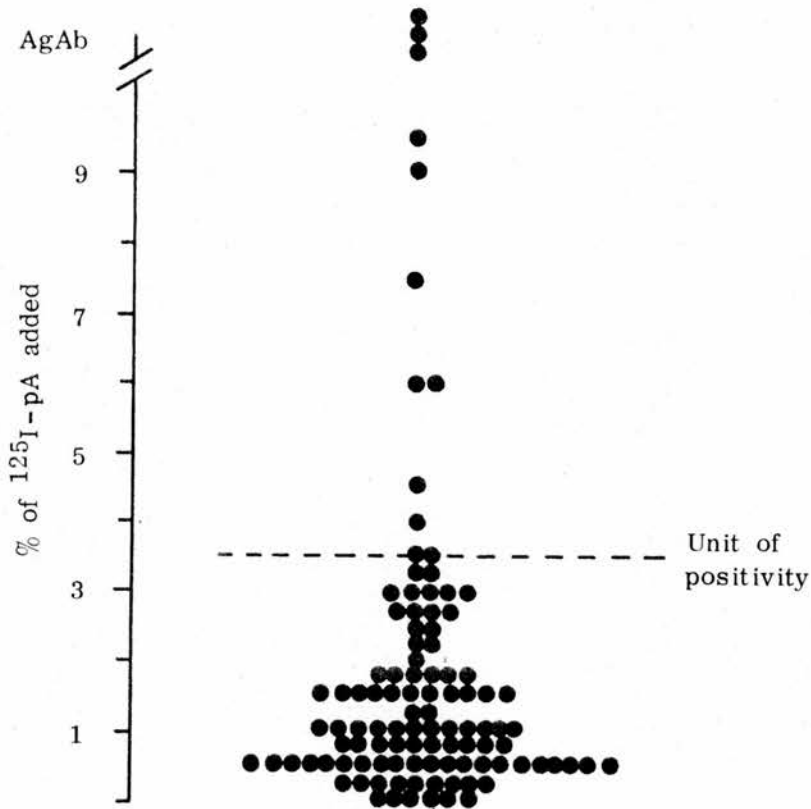
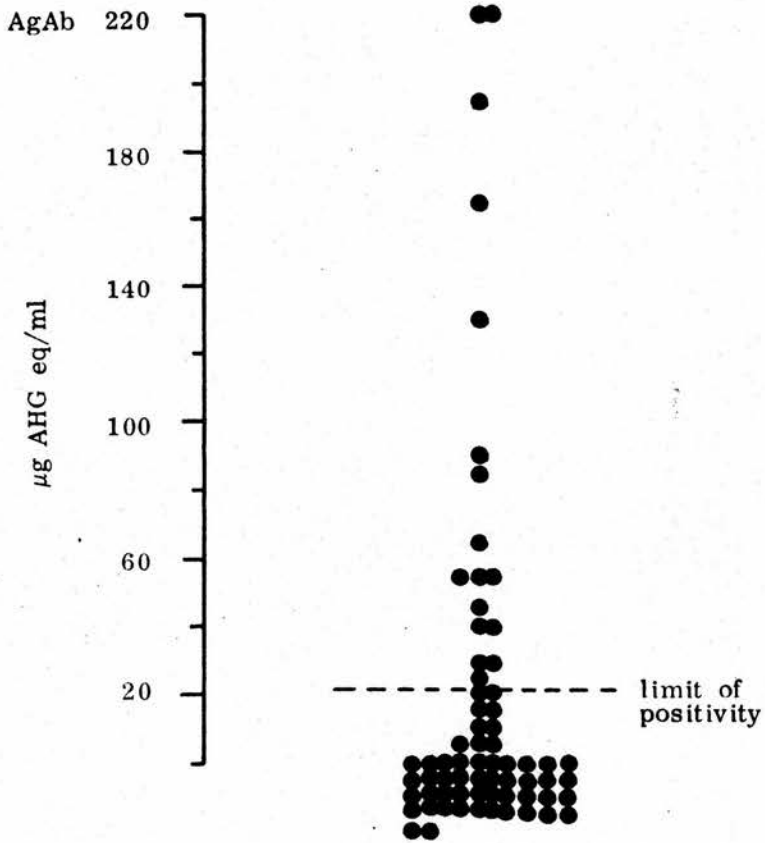


FIGURE A3.1/4

AgAb, as detected by RAJI, in 68 normal subjects. The limit of positivity was chosen as 20 ug AHG eq/ml.



A3.1f Immune complexes in normal subjects

Since the methods presently available do not distinguish between complexes related to specific disease processes and those found in other more common conditions, e.g., viral infections, it is not surprising that circulating AgAb are also found in some apparently healthy subjects. In evaluating the presence of AgAb in a particular disorder it is necessary to compare the results with those found in an apparently normal population. However, the limit dividing normal from pathological values is arbitrary and may vary according to the characteristics of the normal population, which have been chosen as reference.

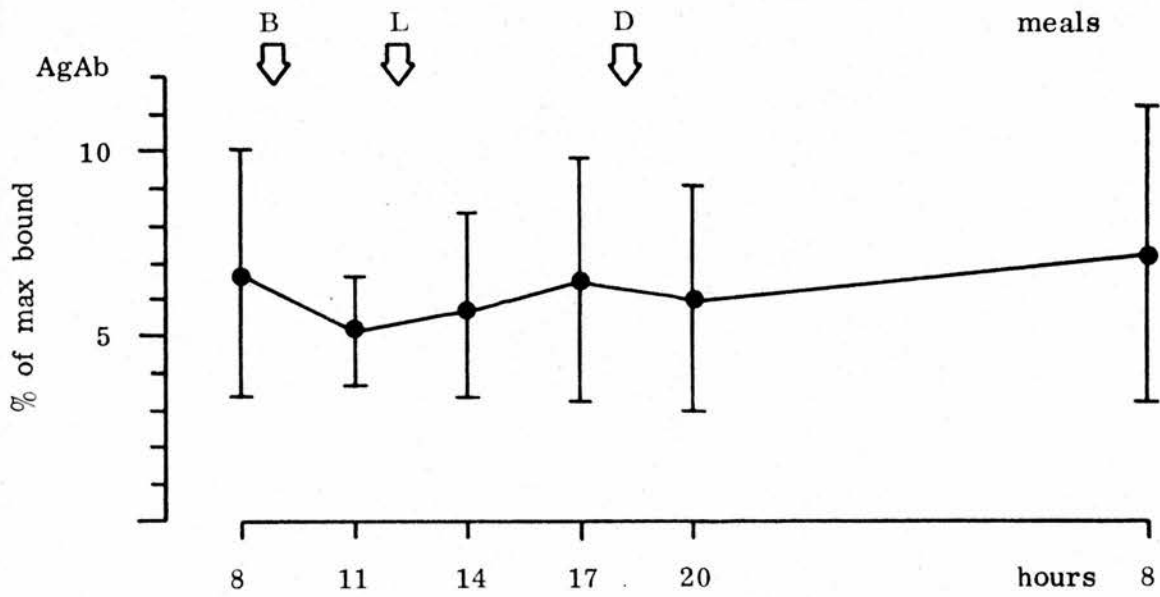
The presence of circulating AgAb in a clinically normal population has been investigated using four methods based on different principles: the solid phase Clq binding test (Clq-SP), the fluid phase Clq radioimmune assay (Clq-FP), the Raji cell radioimmune assay (RAJI) and the conglutinin binding test (KgBt) (Di Mario et al, 1980). With the first three methods, immune complex levels showed a peaked asymmetric distribution with a positive tail including about 10% of subjects (Figures A3.1/1, A3.1/2, A3.1/3) while with the fourth method immune complex values were more widely spread (Figure A3.1/4). No difference between immune complex values in males and females was found. There was a significant trend for the prevalence of immune complexes to increase with age when methods were used which detected complexes through C3 binding. In subjects tested at regular intervals during a period of 24 hours, using the fluid phase Clq



binding assay, significant variations in immune complex levels were found but no consistent pattern of variation at different hours of the day or after meals could be seen (Figure A3.1/5). These hour-to-hour variations are likely to pose a problem in the investigation of small quantities of AgAb in individual subjects or small groups of subjects. They may be compensated for by studying considerable numbers of patients of a particular disease group and comparing them with to age-matched controls, or by analysing multiple samples obtained at different times from small groups of patients and comparing them with to similar samples from control subjects.

FIGURE A3.1/5

AgAb in the serum during 24 hours in 13 normal subjects. The figures are the means of the immune complex values for each of the different time intervals. The standard deviations are shown. B=Breakfast, L=Lunch, D=Dinner.



## A3.2 T CELL SUBPOPULATIONS

T cells and activated T cells were detected and evaluated by the use of monoclonal antibodies (see below). Original technical developments to facilitate the evaluation of T cell subsets (A3.2b) and of class II MHC positive T cells (A3.2c) are described.

\* T c e l l s u b s e t e n u m e r a t i o n

T cell subsets have been enumerated by an indirect immunofluorescence technique.

- Purify lymphoid cells by density gradient centrifugation, check the viability and count the cells;
- Add monoclonal antibody against T cell surface antigen (0.05ml of the appropriate dilution to  $5 \times 10^5$  cells); incubate 30 minutes at 4°C and wash twice;
- add rabbit anti-mouse antibody conjugated with fluorescein; incubate 30 minutes at 4°C and 2 washings;
- read under a fluorescence microscope.

A3.2a      M o n o c l o n a l   a n t i b o d i e s   t o  
T   c e l l   s u r f a c e   a n t i g e n s

\*      Monoclonal antibodies defining regulator T lymphocytes

OKT3

This monoclonal antibody (Ortho Immune Diagnostics) is directed against a T cell surface molecule of 20000 dalton present in all mature T lymphocytes and a minority of thymocytes (Reinherz & Schlossman, 1980; Reinherz et al, 1983). This antigen participates in cell-mediated lysis by both of the major cytotoxic T lymphocyte (CTL) subsets: in fact antibodies to this structure block killing by T4 and T8 CTL. It seems that is closely linked to a recognition structure for antigen. It was used here to enumerate T lymphocytes and as a T cell cytotoxic antibody in the double staining technique (A3.2c).

OKT4

This monoclonal antibody (Ortho Immune Diagnostics) is directed against a T cell surface molecule of 62000 dalton present on the majority of thymocytes and on about 60% of peripheral T cells. This antigen participates in cell-mediated lysis: antibodies to this structure block killing by T4 CTL. T4 positive T cells are directed

at class II MHC antigens on target cells. The T4 were shown to provide inducer (helper) function in T-T and T-B cell interactions (Reinherz & Schlossman,1980; Ballieux & Heijnen,1983).

#### Leu3a

This monoclonal antibody (Beckton & Dickinson) phenotypes the helper/inducer T cell subsets (Ledbetter et al,1981). It is analogous to the monoclonal OKT4.

#### OKT8

OKT8 (Ortho Immune Diagnostics) is directed against a T cell surface molecule of 76000 dalton present in the majority of thymocytes and on about 30% of peripheral T cells. This antigen participates in cell mediated lysis: antibodies to this structure block killing by T8 CTL. T8 positive T cells are directed at class I MHC antigens on target cells. A number of studies have indicated that the T cell subset defined by the T8 antigen is heterogeneous with respect to function. It contains cytotoxic cells as well as cells involved in the suppression of the immune response (Reinherz & Schlossman,1980; Ballieux & Heijnen,1983).

## UCHT4

This antibody is analogous to OKT8 or Leu2a (Beckton & Dickinson) monoclonal antibodies which phenotype the suppressor/cytotoxic T cells (T8 positive T cells) (Beverley, 1982).

\* Monoclonal antibodies defining activated T lymphocytes

The monoclonal antibodies used were: 4F2, 5E9, anti-Tac, DA6.231, DA6.164, L243.

## 4F2

This monoclonal antibody is a IgG2a kappa immunoglobulin and binds rabbit complement and protein A (Haynes et al, 1981; Haynes, 1981). 4F2 antigen is a 120000 dalton protein present on haematopoietic and non-haematopoietic cells. B and T lymphocytes bind negligible amounts of 4F2 antibody, polymorphonuclear cells do not bind the antibody whereas monocytes bind the 4F2 antibody. 70% of concanavalin A activated T cells bind strongly the 4F2. The antigen is newly synthesized by activated lymphocytes. The antigen is one of the first antigens to be expressed on activated T cells. (The antibody was kindly donated by DR GS Eisenbarth, Joslin Clinic, Boston, USA).

## 5E9

This monoclonal antibody is a IgG1 kappa myeloma protein which binds neither complement nor protein A (Eisenbarth et al,1980; Haynes 1981). 5E9 antigen is a 87000 dalton protein but it may exist in dimeric form with a molecular weight of 190000. The antigen has the functional property of binding transferrin. It is present on all rapidly dividing lymphoid or non-lymphoid cell lines. It is very similar, or identical, to a monoclonal antibody termed OKT9. B and T lymphocytes, monocytes and polymorphonuclear cells do not bind this antibody. About 50% of PHA activated lymphocytes bind 5E9 antibody. 5E9 is a useful marker of human cell activation. (The antibody was a gift of Dr GS Eisenbarth, Joslin Clinic, Boston, USA).

## anti-Tac (or TAC)

It is a monoclonal antibody that is reactive with human T cells activated by phytohaemoagglutinin, pokeweed mitogen, concanavalin-A, streptolysin O and allogeneic cells. It does not react with fresh peripheral blood T cells, B cells, monocytes, thymocytes and B cells activated by pokeweed mitogen or Epstein-Barr virus (Uchiyama et al, 1981a; Uchiyama et al, 1981b). The Tac antigen is expressed on T cells, activated by pokeweed mitogen, that have the functions of help and suppression. Two different kinds of helper T cells are present in pokeweed-stimulated T cell cultures: the Tac<sup>+</sup> helper T cells, that are radioresistant, and the Tac<sup>-</sup> helper T cells, that are more radiosensitive. Tac<sup>+</sup> T cells, separated from peripheral blood

mononuclear cells after in vitro allosensitization, showed specific cytotoxic killer cell activity against allogeneic cells, whereas Tac- cells did not have this function. Tac antigen seems different from class II MHC antigens expressed on activated T cells: Tac+ and class II- and Tac- and class II+ leukemic T lymphocytes have been reported. It seems that the Tac antigen is part of the T cell growth factor receptor. In conclusion the anti-Tac monoclonal antibody can define activated and functionally mature human T cells. The Tac antigen is expressed on T cells not only after in vitro activation but also after in vivo activation by specific antigens. In other words it can be used to analyze the activation and the terminal differentiation of T cells.

#### DA6.231

DA6.231 is an IgG1 non-complement-fixing monoclonal antibody directed to a framework determinant of human MHC class II molecules. DA6.231 reacts with all homozygous typing cell lines representing the common DR alleles (DR1-8). DA6.231 reacts with circulating peripheral blood B lymphocytes and many B cell neoplasias. The DA6.231 determinant is expressing on >80% of peripheral blood monocytes and on activated T lymphocytes. In in vitro assay systems of lymphoproliferation, DA6.231 blocks both DR and SB specific responses. DA6.231 does not block the binding to cells of authentic anti-HLA-DC monoclonal antibodies suggesting that it does not react with DC-encoded molecules. When tested against a panel of mutant cell lines de-



rived from DR1 and DR3 haploid parent cell lines, DA6.231 identifies a determinant common to SB and DR molecules. The DA6.231 determinant appears to be predominantly expressed on beta polypeptides (Van Heyningen et al,1982; Guy et al,1982; Cohen et al, 1983).

#### DA6.164

DA6.164 is an IgG1 non-complement-fixing monoclonal antibody directed to a framework determinant distinct from that recognized by DA6.231. DA6.164 reacts with all homozygous typing cell lines representing the common DR alleles, with the exception of DR7 cell lines. DA6.164 appears to have higher affinity for class II molecules derived from DR3, DR5 and a subset of DRw6 cell lines than for molecules from DR1, DR2, DR4 and some other DRw6 alleles. SB specific responses are not blocked by DA6.164. With panels of deletion mutants derived from DR1 and DR3 parent cell lines, DA6.164 identifies a determinant present only on DR encoded antigens. The DA6.164 determinant is labile and does not react with isolated chains of class II molecules. However the reaction of DA6.164 with cell lines is invariably blocked by prior incubation of the cells with DA6.231 suggesting that the DA6.164 determinant is also on beta polypeptides. DA6.164 is expressed on >80% of peripheral blood monocytes and is present on activated T cells and normal and neoplastic B lymphoid cells (Van Heynenegen et al,1982; Guy et al,1982; Cohen et al,1983).

(DA6.231 and DA6.164 monoclonal antibodies were a kind gift from

Dr K Guy, MRC Clinical and Population Cytogenetic Unit,  
Edinburgh,UK).

L243

This monoclonal antibody is directed against human class II MHC molecules. It binds to human B cell lines but not to T cell lines in normal conditions. The antigen is expressed on some activated T cells. It precipitates 28000 and 34000 dalton chains from extracts of B cell lines. It is cytotoxic for human cell lines (Lampson & Levy,1980). (The antibody was donated by Dr GS Eisenbarth, Joslin Clinic, Boston, USA).

A3.2b    A n o r i g i n a l m e t h o d t o f a c i l i -  
t a t e t h e e v a l u a t i o n o f  
T - c e l l s u b s e t s

An important development in the characterization of cell surface functional antigens has been the development of monoclonal antibodies. To date, more and more specificities are described in the scientific literature and no doubt even more will be found in the future. For example, accurate characterization of T and B lymphocyte subclasses, K/NK cells and activated T cells requires at least 10 monoclonal antibodies per blood sample. If a fluorescence activated cell sorter (FACS) is available, the number of samples poses no problem. At present, however, this is not the case and many researchers find themselves, therefore, in the unenviable position of spending a great deal of time on preparing a single slide for each monoclonal antibody.

In this report an adaptation of existing equipment in order to facilitate the workload is described.

M a t e r i a l s   a n d   M e t h o d s

The equipment consists of Medicell glass-bottomed plates in conjunction with an inverted fluorescence microscope. The plates, which are readily available (Medicell International LTD) since they are routinely used for HLA-DR tissue typing, are made of a thin

sheet of glass, subdivided into 60 flat circular areas by raised rings, attached to a plastic frame with an inner ridge. The plates can be covered with a transparent plastic cover which fits over the inner ridge of the frame thus effectively reducing the rate of evaporation of the cell samples. The latter are placed within the circular areas where diffusion is prevented by the rings; alternatively the plates can be moved along by hand.

The method described here is for blood mononuclear cells although it can be applied to any other cell preparation.

- Isolate mononuclear cells by density gradient centrifugation on Ficoll-Hypaque.
- Wash cells three times in Phosphate Buffered Saline (PBS), pH 7.3, and suspend them at a concentration of  $2.0 \times 10^6$  cell/ml in RPMI 1640 medium containing 10% heat inactivated foetal calf serum (complete medium). 1.0 ml of this suspension is enough for a panel of up to 10 monoclonal antibodies.
- Divide the cell suspension into 0.1-0.2 ml aliquots in small plastic centrifuge tubes (10x60mm).
- Spin tubes at 1,700 g for 1.5 min, preferably in a centrifuge equipped with a fixed-angle rotor. Remove supernatant by tipping tubes then add 50 ul of the appropriate monoclonal antibody. Quickly vortex tubes to suspend cells and incubate for 30 min in an ice-bath.
- Wash cells three times with PBS at 1,700 g for 1.5 min then add 50ul of the appropriate dilution of fluorescein conjugate antimouse IgG antibody. Vortex tubes and incubate for 30 min in an ice-bath.
- Wash cells three times in PBS and finally suspend them in

50ul of complete medium.

- Place 5ul of each cell sample in a circular area of the Medicell plate and leave for 3-5 min to allow cells to sink to the bottom.
- Count the proportion of rim fluorescence positive cells in the inverted fluorescence microscope.

#### C o m m e n t s

The length of time required to complete the characterization of mononuclear cells from a single subject is a very acute problem in all the laboratories. The method described has now been used for over one year and has been found extremely useful. Many of the problems connected with eye adaptation to dark room conditions are avoided since the microscope reader does not need to leave the machine to prepare single slides.

In addition to increasing the number of cell samples that can be read at any given time, there is the bonus that the number of cells required for each monoclonal antibody can be greatly reduced ( $0.2-0.5 \times 10^5$  cells). This can be of considerable importance when phenotyping mononuclear cells from small blood samples, in particular those from small children and difficult patients.

A3.2c    A n   o r i g i n a l   m e t h o d   t o  
e v a l u a t e   c l a s s   I I   a n t i g e n  
p o s i t i v e   T   c e l l s

Attention of investigators has recently been focused on that subset of lymphocytes which expresses the class II (or DR) MHC antigen on their surface. This class II antigen is a marker of T cell activation and is normally absent on non activated or otherwise uncommitted T cells. On the other hand, this antigen is normally present on the surface of B cells. The fact that this surface antigen is present in both B cells and in a few T cells creates some technical difficulties.

Since the percentage of activated T cells is usually very low, a method of easily and clearly identifying this T cell subset is needed. Most investigators identify and isolate T cells on the basis of their ability to form spontaneous rosettes when incubated with sheep red blood cells (WHO Scientific Group Report, 1977; Weir, 1978; Hudson & Hay, 1981). This widely used technique is time consuming and not accurate enough for the evaluation of activated T cells: some T cells are lost - and a particular subpopulation of T cells may be preferentially removed; on the other hand, some B cells still remain in the rosette enriched T population, making it difficult to discriminate the activated T cells from the contaminating B cells. Other investigators use a double immunofluorescence staining - with both fluoresceine and rhodamine - to label respectively the antibody defining the DR antigen and that defining another antigen of either T

or B cells. This technique is rather elaborate and tedious; the immunofluorescence pattern is usually faint, the different cells need to be read with different fluorescence microscope filters and the results obtained are usually poorer than expected.

In order to overcome these difficulties, a straightforward double staining technique using commercially available monoclonal antibodies has been devised.

#### M e t h o d s

a) immunological or other specific reagents needed: anti-class II antigen monoclonal antibody (this antibody should not be cytotoxic), anti-total T cell monoclonal antibody (for instance Leu la, Becton- Dickinson or OKT3, Ortho Diagn.), ethidium bromide, fresh rabbit complement, rabbit anti-mouse antibody conjugated with fluorescein. All the monoclonal antibodies are diluted in medium (RPMI 1640) plus 10% foetal calf serum.

#### b) procedure:

- purify lymphoid cells by density gradient centrifugation; check viability of cells and count them;
- add anti-class II antigen monoclonal antibody (0.05 ml appropriate dilution to  $5 \times 10^5$  cells); incubate 30 minutes at 4°C and wash twice in medium;
- add rabbit anti-mouse antibody conjugated with fluorescein; incubate 30 minutes at 4°C and wash twice in medium;
- add a cytotoxic anti-T cell monoclonal antibody (0.05 ml

appropriate dilution) and fresh complement (200 ul rabbit or guinea pig complement after checking for spontaneous cytotoxicity); incubate 30 minutes at 37°C;

- add ethidium bromide at a concentration of 50ug/ml and read with a fluorescence microscope, fitted with a filter for fluorescein.

The B cells will appear green on the surface, the class II negative T cells will appear red in the nuclei whereas the class II positive T cells will show a red nucleus and a green surface.

#### C o m m e n t s

With the technique described, the difficulties found with the other methodologies seem to be overcome. Under the fluorescence microscope it is possible to see clearly the bright red of the nuclei of the T cells, the green continuous linear fluorescence of the surface of the B cells and a few cells with a red nucleus and a fluorescent green surface, i.e. the class II MHC antigen positive T cells. Both colours are read with the same filter, i.e. that for fluorescein. The technique is fast: 3 or 4 hours are needed to complete it. No significant amount of cells are lost with this procedure which requires only washings but not separation of cells. Furthermore the total number of B and of T lymphocytes are easily obtained.



### A3.3 INSULIN ANTIBODIES

The methods to detect insulin antibodies are numerous and differ in principle (Christiansen,1970; Andersen et al,1972; Mustaffa et al, 1977; Gerbitz & Dixon,1978; Kurtz et al,1980). Two methods were chosen to measure insulin antibodies in this study. Serum insulin binding capacity was evaluated by the method described by Andersen et al,1972, as developed by Mustaffa et al,1977, with minor modifications and by the method described by Christiansen,1970.

#### A3.3a Andersen's method

##### a. Reagents

Guinea pig anti-insulin serum (Wellcome), diluted 1:8000; rabbit anti-guinea pig serum (Wellcome), dilute 1:60; normal guinea pig serum, diluted 1:50; bovine <sup>125</sup>I-insulin, at a working dilution of 20uU/ml; charcoal suspension 5g/100ml; Sodium-phosphate buffer 0.04mM/l; albumin solution 5g/l

## b. Procedure

1st day

- Add guinea pig anti insulin serum, 100 ul, to phosphate buffer, 100 ul,
- add rabbit anti guinea pig serum, 100 ul
- mix and incubate overnight at 4 °C

2nd day

- add to the mixture the serum to be tested, 100 ul
- add radiolabelled insulin, 100 ul of the working dilution
- mix and incubate at 4°C

3rd-5th day

- leave to incubate at 4°C

6th day.

- add normal guinea pig serum, 100 ul
- mix and incubate at 4 °C

7th day

- centrifuge at 2800 rpm at 4°C for 45 minutes
- keep the precipitate
- add the charcoal suspension to the supernatant
- mix for 5 minutes
- centrifuge at 2800 rpm at 4°C for 30 minutes
- keep the supernatant
- count the first precipitate and the second supernatant

c. Expression of results

Insulin antibody levels are calculated as follows:

supernatant / precipitate x [guinea pig antiserum titre] x 10

and are expressed in uU/ml.

A3.3b Christiansen's method

## a. Reagents

Agarose (Litex Agarose, Denmark); anti-human-immunoglobulin antibody (Dako, Denmark); bovine  $^{125}\text{I}$ -insulin; phosphate buffer, pH 8.6.

## b. Procedure

1st day

- add 50 ul of the serum to be tested to 50 ul of buffer
- add  $^{125}\text{I}$ -insulin to the mixture
- leave to incubate overnight

2nd day.

- heat 50 ml of buffer, diluted 1/2 in distilled water, plus 1g of agarose for a few minutes
- pour 13.5ml of the agarose solution in 20ml tubes inside a thermostatically controlled bath at 52 °C
- add 1.5 ml of anti-human-immunoglobulin antibody to each of the tubes containing agarose
- pour gently one tube of the agarose-antibody mixture in a 10cmx10cm glass slide, pre-heated with a Bunsen burner
- leave the agar mixture to cool
- make 12 wells into the agar gel
- put 5 ul of the test serum-radiolabelled insulin mixture in

each of the wells

- fill the electrophoretic baths with 1/3 of buffer and 2/3 of distilled water
- put dialysis paper on the anodic side of the glass
- switch on the electricity
- leave antibodies to migrate for 18 hours

3rd day

- cut the precipitation rings around the wells
- put the strips in tubes with 1 ml of distilled water
- count the radioactivity

#### c. Expression of results

Insulin antibody levels are calculated as follows:

$$\frac{\text{cpm of tested serum} - \text{cpm mean of normals}}{\text{total cpm added}} \times (\text{uU of insulin added})$$

Results are expressed in mU/ml

## A3.4 INSULIN-ANTI-INSULIN COMPLEXES

The method used is a development of the methods described by Jayarao et al,1973, and Virella et al,1980.

The method is based on the assumption that when bound and the free radiolabelled insulin are separated at neutral pH with one of the numerous of different ways, after having added radiolabelled insulin to a serum containing insulin antibody, only free insulin antibodies are measured. On the other hand, when the pH of the medium is below three, insulin antibodies are dissociated from the antigen and the measurement of insulin binding capacity at that point reflects the total amount of insulin antibody. The difference between "total" and "free" insulin antibody values indicates the presence of insulin-anti-insulin complexes.

## a. R e a g e n t s

## Glycine-HCl buffer

prepare glycine 0.2N 3l and HCl 1 l

add 684 ml of HCl 0.2N to 3l of glycine

reach pH 3

## Barbital buffer

Na barbital 14.7g and Na acetate 9.7 g in 500 ml of d.w.

add 1800ml of NaCl 0.85g/100ml and 100 ml of HCl 0.1N

reach pH 7.4

Dextran charcoal

Charcoal norit A 5g/100ml in barbital buffer

Dextran 80 0.5g/100 ml

Mix equal volume of charcoal suspension and dextran solution

Acid dextran charcoal

As above in glycine-HCl buffer

Albumin solution

Albumin 3% in barbital buffer

b. Procedure

- split the serum to be tested into two aliquots.
- dialyse one aliquot overnight in buffer glycine-HCl (pH3),
- add serum to dextran-charcoal in glycine-HCl,
- incubate for 30 minutes at room temperature,
- centrifuge at 2800rpm for 20 minutes
- dialyse the supernatant versus barbital buffer, pH 7.4.
- add radiolabeled insulin to this mixture
- add radiolabeled insulin also to the other aliquot, adequately diluted (1:10)
- incubate for two hours at 37°C,
- add dextran-charcoal
- centrifuge at 2800 rpm for 20 minutes
- count the supernatant and the precipitate.

c. E x p r e s s i o n o f r e s u l t s

The insulin-anti-insulin complexes are calculated as the difference between the "total" antibody and the "free" antibody. The "total" and the "free" antibodies are calculated as follows:  $100 - (\text{cpm in the precipitate} - \text{cpm in the supernatant} / \text{total cpm}) \times 100 - \text{background}$ .

Positive values are above the mean + 2 SD of normal values and are expressed in percentage (i.e. >10%). Positive values of Ins/iAb are above the mean + 2 SD of values found in normal controls (i.e. >7). The sensitivity of this technique in assaying the sole presence of insulin antibodies is slightly different from Andersen's method.



### A3.5 ORGAN SPECIFIC AUTOANTIBODIES

#### A3.5a I s l e t c e l l a n t i b o d i e s

##### a. R e a g e n t s

Fresh human sharply frozen pancreatic tissue

Anti-human-immunoglobulin serum conjugated with fluorescein

##### b. P r o c e d u r e

- prepare unfixed 5nm cryostat sections of group 0 frozen human pancreas
- add patient's undiluted serum to pancreas sections
- incubate 30 minutes at 37°C
- wash for two 15 minute periods in neutral buffer
- rinse in buffer
- incubate with anti-human-immunoglobulin antiserum conjugated with fluorescein at appropriate dilution for 30 minutes at 37°C
- wash as above
- read under a fluorescence microscope

A3.5b      C o m p l e m e n t   f i x i n g   i s l e t   c e l l  
a n t i b o d i e s

a.   R e a g e n t s

Fresh human sharply frozen pancreatic tissue

Fresh normal human serum as source of complement

Anti-human-C3 antiserum conjugated with fluorescein

b.   P r o c e d u r e

- add patient's undiluted serum to pancreas slices as above
- incubate for 30 minutes at 37°C
- add fresh normal human serum as a source of additional  
      complement
- wash for 2 15-minute periods in neutral buffer
- add anti-human-C3 conjugated with fluorescein
- incubate 30 minutes at 37°C
- wash as above
- read under a fluorescence microscope

## A3.6 CIRCULATING ISLET CELL ANTIGENS

The search for circulating specific antigens has been done by the use of monoclonal antibodies reacting with human pancreatic islet cell antigens.

A3.6a Monoclonal antibodies reacting  
with pancreatic islet cell  
antigens

\* Overview on anti-islet monoclonal antibodies

The monoclonal antibodies reacting with islet cells antigens so far produced (Eisenbarth et al,1984) can be grouped as follows:

1. Heterospecific monoclonal antibodies which initially were produced to neurons or malignant cells.
2. Murine heterospecific monoclonal antibodies produced following immunization of mice with rat islet cells (A4A11), a rat islet cell

tumor line (5D6,A1D2), human islets and human insulinoma tissue (HISL 1 to 19)

3. Rat monoclonal autoantibodies produced by fusing spleen cells from a diabetic prone BB rat (A1G12, BB-TECS).

4. Human monoclonal autoantibodies produced by fusing circulating lymphocytes from a child with type I diabetes with a human myeloma cell line (B6).

In addition to anti-islet antibodies two monoclonal antibodies which react with subsets of thymic epithelial cells have been recently described. These antibodies, BB-TECS (made by fusing spleen cells of a diabetes prone BB rat) and DM-TECS 1 (made by fusing circulating lymphocytes from children with type I diabetes with a human myeloma line) react with the endocrine portion of the thymus and their existence may indicate that the autoimmunity of type I diabetes extends to the thymus.

| mcAb          | Type  | Antigen                  | Cells                         |
|---------------|-------|--------------------------|-------------------------------|
| A2B5          | Mouse | CQ ganglios.             | Islet,neuron & neuroend. tiss |
| 3G5           | Mouse | Ganglioside              | Islet,neuron & neuroendocrine |
| Tet. Tox.+3D8 | Mouse | GD,G <sup>T</sup> gangl. | Islet,neuron & neuroendocrine |
| 4F2           | Mouse | 120K glycopr.            | Islet,monoctyte, act.T cell   |
| LC7/2         | Mouse | 120K glycopr.            | Islet,tumor line              |
| A4A11         | Mouse | Unknown                  | Islet                         |
| 5D6           | Mouse | Unknown                  | Islet,fibroblast              |
| A1D2          | Mouse | 24K glyco                | Islet                         |
| B6            | Human | Unknown                  | A cells                       |

|              |           |         |                              |
|--------------|-----------|---------|------------------------------|
| AlG12        | Rat-Mouse | Unknown | "Nuclear" islet              |
| BB-TECS      | Rat-Mouse | Unknown | Thymic endocrine, islet      |
| DMTECS 1     | Human     | Unknown | Thymic nonendocr. epithelium |
| HISL 1 to 17 | Mouse     | Unknown | Islet cells frozen pancreas  |
| HISL 19      | Mouse     | 64K     | Islet cells frozen pancreas  |

\* Anti-islet monoclonal antibodies used in this study

Among the monoclonals described above, four anti-islet monoclonal antibodies were chosen to be used in this study.

#### HISL-5 and HISL-19

These two monoclonal antibodies have been very recently raised in mice after immunization with human frozen pancreatic islet cells. A number of monoclonal antibodies were produced in this way in Dr GS Eisenbarth laboratory and sequentially numbered. The monoclonal antibody HISL-19 reacts with a 64000 dalton molecular weight protein present on the human islet. It is not known the molecular weight of the antigen relative to the monoclonal HISL-5. These two monoclonal antibodies give a bright staining of human pancreatic frozen sections with a pattern identical to that of spontaneously occurring antibodies.

## A2B5

This monoclonal antibody was initially produced following immunization of mice with chicken retina neurons (Eisenbarth et al,1979). Studies of the antigenic material extracted from brain revealed that this monoclonal antibody reacts with cQ gangliosides, complex gangliosides with four sialic acid residues. Such complex gangliosides are predominantly found in neuronal and in neuroendocrine tissues, including pancreatic islets. A2B5 reacts specifically with human pancreatic islets on frozen sections and gives an indirect immunofluorescence staining identical to that of islet cell autoantibodies.

## 3G5

3G5 is a monoclonal antibody that reacts with complex neuronal gangliosides present in neuronal and in neuroendocrine tissues, such as pancreatic islet cells. This monoclonal antibody does not react with a solid phase ganglioside preparation from rat brain, with which antibody A2B5 reacts. Thus its receptor is a ganglioside different from antibody A2B5 (Eisenbarth et al,1984).

A3.6b      T e c h n i q u e s   t o   d e t e c t  
c i r c u l a t i n g   i s l e t   c e l l  
a n t i g e n s

The identification of soluble pancreatic antigens was performed by the use of monoclonal antibodies in fluid phase. The monoclonal antibody is radiolabeled with  $^{125}\text{I}$ ; the serum to be tested and the radiolabeled monoclonal antibody are left to incubate; the free monoclonal antibody and the complexed form are separated by precipitation with polyethyleneglycol (PEG).

\*    R e a g e n t s

Monoclonal antibodies A2B5 (10 mg/ml), 3G5 (4mg/ml), HISL-5 (8 mg/ml), HISL-19 (10 mg/ml),  $^{125}\text{I}$ , PEG 6000 12%, phosphate buffer, bovine serum albumin

\*    I o d i n a t i o n   o f   m o n o c l o n a l s

The chloramine T method was used (see chapter A3.1)

\*    P r o c e d u r e

-    add 50 ul of serum to 200 ul of phosphate buffer with

- bovine serum albumin 3%
- add 50 ul of iodinated monoclonal antibody (approximately 2 ng/tube)
  - incubate 1 hour at 37 °C
  - add 300ul of the PEG solution
  - mix accurately at 4°C
  - spin at 3000 rpm for 20 minutes
  - count the supernatant and the precipitate

\* Expression of results

Results are expressed as in percent as follows:

$\text{cpm precipitate} / (\text{cpm precipitate} + \text{cpm supernatant}) \times 100$



SECTION

B

CHAPTER B 1

IMMUNOLOGICAL FACTORS IN THE PATHOGENESIS OF TYPE 1 DIABETES

Chapter B1      IMMUNOLOGICAL FACTORS IN THE PATHOGENESIS  
OF TYPE 1 DIABETES

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B1.5 Search for circulating specific antigens in the early stages of type 1 diabetes by monoclonal antibodies

B1.5a Aim of the study

B1.5b Materials and methods

B1.5c Results

B1.5d Discussion

B1.6 Conclusions

B1.1        EXPERIMENTAL DESIGN OF THE STUDIES ON THE PATHOGENESIS OF  
              TYPE 1 DIABETES

The prevailing concept of the aetiopathogenesis of most cases of type 1 diabetes is that in genetically susceptible individuals a combined process of an exogenous aggression and autoimmune reaction determines the destruction of insulin producing cells. However most of the pathogenetic mechanisms are still obscure (For details and references see chapter A1.3).

Four observations are highlighted here.

- Circulating immune complexes, activated T cells, killer cells and antibodies to islet antigens are present in the early stages of type 1 diabetes.
- Circulating immune complexes are presently detected with methods that do not give information on the antigen(s) involved in their formation. Some types of complexes seem to be increased at diagnosis in diabetes and then to decline in parallel with islet cell antibodies.
- The presence of humoral immune factors, such as islet cell antibodies, complement fixing islet cell antibodies, immune complexes and other factors seem to be important prognostic markers not only in insulin dependent diabetics but also in some other diabetics not requiring insulin.

- The selective destruction of the pancreatic islet beta cells may continue for months or years before and after the clinical onset of diabetes. It is possible that circulating islet antigens, either free or complexed with antibody are in the circulation both before and after the initial clinical manifestation of the disease.

Starting from the above observations the following studies have been undertaken:

- a) The following over a period of time of the main humoral immunological factors suspected of having a role in type 1 diabetes, from diagnosis up to a few years after, and to evaluate the possible correlations between them.
- b) The evaluation of the clinical prognostic value of different immunological markers in diabetic patients not requiring insulin at diagnosis.
- c) The search for circulating pancreatic antigens in newly diagnosed type 1 diabetics.

In the study described in chapter B1.3, the humoral immune response in type 1 diabetics has been investigated at diagnosis and thereafter. When the study was done, no other similar investigation on a large group of diabetics had been reported by others. Islet cell antibodies, complement fixing islet cell antibodies, viral antibodies, different types of immune complexes, insulin antibodies and HLA types were evaluated in a group of about 150 newly diagnosed type 1 diabetics. In the majority of them a regular follow up till 3 years after diagnosis has been carried out.

In chapter B1.4 a study of some immunological events in diabetics not requiring insulin at diagnosis is described. The presence of immune complexes and of islet cell antibodies have been evaluated in newly diagnosed diabetics treated with diet or oral agents. Those diabetics not requiring insulin at diagnosis and who showed islet cell antibodies in their sera have been followed over a period of time. The clinical prognostic value of islet cell antibodies, complement fixing islet cell antibodies and immune complexes has been evaluated.

An attempt to find possible specific circulating islet cell antigens in early stages of type 1 diabetes and to relate them to the pathogenetic events is described in chapter B1.5. Since the auto-antibodies to islet cells so far described do not seem to have a direct correlation with islet damage, the detection of islet cell antigens released in the circulation in parallel with islet cell damage might provide a invaluable marker of the pathogenetic events in type 1 diabetes. No investigation on circulating islet cell antigens has been reported so far.

Patients have been included in these studies as a result of collaboration with the Diabetic Department, Royal Infirmary, Edinburgh, UK, the Diabetic Unit, University of Rome, Italy, and the Joslin Clinic, Boston, USA.

Viral antibodies have been assayed in the department of Bacteriology, Edinburgh University, UK.

HLA studies have been done in part in the Nuffield Department of Surgery, University of Oxford and in part in the Endocrine Unit/-Immunology Laboratories, Royal Infirmary, UK.

Part of the the islet cell antibody evaluations in study Bl.3, and the autoantibodies to gastric parietal cells and to thyroid cell cytoplasm, have been done routinely in the Endocrine Unit/Immunology Laboratories, Royal Infirmary, Edinburgh, UK.



## B1.2 EARLIER STUDIES BY THE AUTHOR

B1.2a Soluble immune complexes in  
newly diagnosed diabetics:  
a preliminary observation

The presence of immune complexes in the sera of newly diagnosed insulin-dependent diabetics, compared to that in controls, and their association with the occurrence of antibodies to islet cells and viruses, are reported and discussed.

M a t e r i a l s   a n d   m e t h o d s

Sera were obtained between October and March from thirteen newly diagnosed diabetics. Control sera for these patients were obtained during the same period from age- and sex-matched healthy subjects, including hospital personnel. All sera were stored in 500 ul aliquots at  $-40^{\circ}\text{C}$ .

The Raji cell radioimmunoassay as described by Theofilopoulos et al (1976) was used. The amount of soluble immune complexes in each test serum was expressed as being equivalent to so many ug aggregated human gamma globulin (AHG) per ml serum (Chapter A3.1).

Islet cell antibodies (ICA) and other organ-specific auto-antibodies were detected by the indirect immunofluorescence test

(Chapter A3.5; Irvine et al, 1977). Sera that were positive for ICA when tested neat were titrated using doubling dilutions.

The sera of the thirteen newly diagnosed insulin-dependent diabetics and their age- and sex-matched controls were screened and titrated for the following viral antibodies: to Coxsackie virus B1-5 by a metabolic inhibition test; to Epstein-Barr virus by an indirect immunofluorescence test; and to rubella virus using a haemagglutination inhibition test. Sera were screened and titrated for complement fixing antibody to the following antigens: influenza A, B and C; parainfluenza type 1; measles; mumps S and V; respiratory syncytial virus; adenovirus; cytomegalovirus; varicella zoster; Herpes simplex; *Coxiella burnetii* phase 2; *Chlamydia* group B and *Mycoplasma pneumoniae*.

All analysis were done using Fisher's exact test, except where indicated.

## R e s u l t s

Seven out of the 13 patients were positive for immune complexes, 6 of these 7 were were islet cell antibody positive whereas none of the complex negative showed ICA in their serum. Three immune complex positive and three complex negative patients showed also circulating viral antibodies. The occurrence of soluble immune complexes in the sera of seven out of thirteen newly diagnosed insulin-dependent diabetics clearly correlated with the presence of ICA ( $P < 0.01$ ), but not with the presence or titres of antibodies to Coxsackie B4. The prevalence and titres of antibodies to the other viruses investigated were similar in the diabetics and in the age- and sex-matched controls. There was no correlation between the occurrence of soluble

immune complexes in the serum and the presence of ketosis or the level of the blood sugar.

#### D i s c u s s i o n

The close correlation between the presence of soluble immune complexes and ICA in the serum, and the absence of such a correlation with viral antibodies and other autoantibodies, suggests that the antigens involved in these immune complexes may be derived from islet cells. This would suggest that in such patients there has either been a recent release of islet cell antigens or that such antigens are being continually released after the onset of clinical diabetes of the insulin dependent type. The fact that the symptoms of diabetes before diagnosis were present for as long as 4 months in patients with immune complexes at diagnosis suggests the latter, and this would accord with the observation that some B cells of the islets persist after the clinical onset of this type of diabetes, as evidenced by their ability (albeit diminished) to produce C peptide (Heding & Rasmussen, 1975). Moreover, ICA reacts with all cells in the islets, although it is only the B cells that are markedly affected in Type I diabetes (Doniach, 1974; Egeberg et al, 1976) .

Soluble immune complexes comprising islet cell antigen and antibody could be important in relation to antibody-dependent cell-mediated cytotoxicity, whereby K cells may be specifically armed by such complexes in antibody excess. If so, the question remains why it is that the B cells are selectively destroyed, unless A and D cells have greater powers of regeneration. Alternatively, immune complexes, in relation to islet cell antigens, may simply be a consequence of

islet cell damage and not be involved in producing further islet cell damage.

B1.2b Autoimmunity and HLA antigens in type 1 diabetes

The present study was undertaken to analyse in greater depth which, if any, of the three HLA groups in linkage disequilibrium with B8, B15 and B7, respectively, could be shown to be correlated with the autoimmune aspects of type I diabetes.

M a t e r i a l s   a n d   m e t h o d s

Type I diabetics were selected from those attending the Out-Patient Diabetic Department of the Royal Infirmary, Edinburgh, and divided in three groups. Group I included 101 recently discovered insulin dependent (type 1) diabetics, with no other associated clinic autoimmune disease. 50 patients had ICA detectable in the serum within 1 month of diagnosis (group Ia) while 51 did not show islet cell antibodies (Group Ib). The mean age at diagnosis was respectively 22.3(+1.8) and 25.2(+ 1.8) years respectively in group Ia and Ib patients. Group II consisted of 39 insulin dependent (type 1) diabetics who had ICA detectable in their sera at least three years after diagnosis. Their mean age at diagnosis was 25.1 (+2.4) and they did not show any clinical sign of autoimmune disorders. The diabetics in group III showed clinical manifestations of organ specific autoimmune disorders and were subdivided into group IIIa if they were ICA positive at 3 or more years after diagnosis and group IIIb if they were ICA negative within 3 years of diagnosis. The mean age at diagnosis was 41.1 (+3.7) years respectively in group IIIa and IIIb patients. 300 healthy Caucasians from the Oxford area served as HLA-typing controls.

ICA was detected by the indirect immunofluorescence test as previously described (chapter A3.5). Antibodies to thyroid cytoplasm and gastric parietal cells were also detected by indirect immunofluorescence and antibodies to thyroglobulin by tanned cell haemagglutination (a titre of 1:25 or more being taken as positive) (Irvine et al, 1970).

HLA typing was performed to study the following 37 antigens. A series: 1,2,3,11,25,26,28,28 and W23,24,30,31,32,33. B series: 5,7,8, 12,13,14, 15,17,18,27,37,40 and 21,22,35,38,39,41. C series: W1,2,3,4, 5 (Morris et al, 1976).

Statistical analysis used the chi-squared test (with Yates' correction when indicated) (C) and Student's t-test (S) as shown.

## R e s u l t s

### Diabetics without clinical evidence of associated autoimmune disease

An increased prevalence of HLA-B8 was found in patients in groups Ib (47%), Ia (61%) and II (73%) when compared with the control population (28%). Apart from the increased prevalence of A1 in group II patients (67% vs 35% in the normal population,  $p < 0.001$ ), there was no significant difference in any of the other HLA types tested, particularly B7, B15, B18, B40 and CW3, between the groups and the controls. There was no tendency for B15 to be increased in group II patients (Figure B1.2/1).

There was a significantly higher prevalence of B8 in those in whom ICA persisted (group II) compared to those in whom ICA was negative (group Ib). Although there was a higher prevalence of B8 in

ICA positive compared to ICA negative patients within group I, this did not reach statistical significance.

The prevalence of thyroid and gastric autoantibodies was significantly increased in patients in group Ia and in group II when compared with those in group Ib. While patients who had thyroid and gastric antibodies in their sera tended to have a higher prevalence of B8, this did not reach statistical significance.

There was no difference in the sex distribution nor in the mean age at diagnosis in patients who were ICA positive (group Ia), ICA negative (group Ib) or in whom ICA persisted for more than 3 years (group II) .

#### Diabetics with clinical evidence of other autoimmune diseases

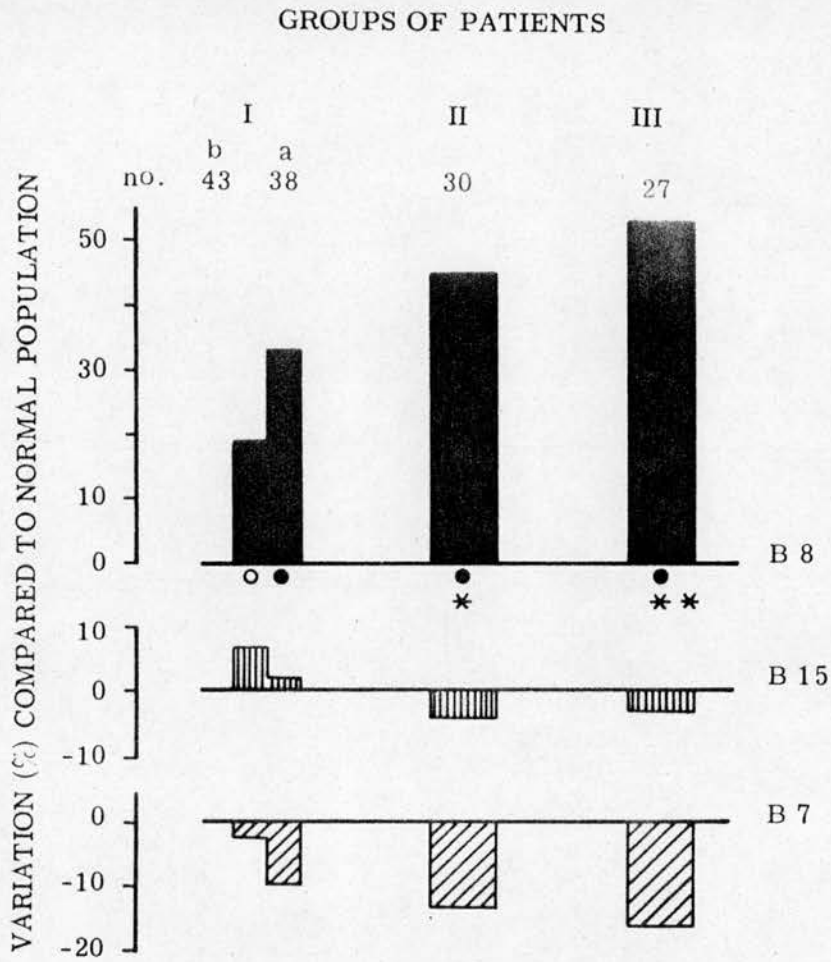
In patients in group III the prevalence of B8 (81%) and of A1 (59% vs 35% in the normal population,  $p < 0.025$ ) were significantly higher when compared with those found in the normal population while the prevalence of B15 did not show any tendency to be increased. Moreover the frequency of B8 was significantly higher in diabetics in group III, either ICA positive after 3 years or ICA negative within 3 years of diagnosis (78% and 89%, respectively), in comparison with ICA negative patients in group Ib (47%).

There was a significantly increased prevalence of thyroid and/or gastric autoantibodies in group III patients compared to those in group Ib. Again the occurrence of these autoantibodies was not significantly associated with B8.

In the 46 patients within group III there was a significantly higher prevalence of females and the mean age at diagnosis was greater than for patients in group I or II.

FIGURE B1.2/1

The prevalence (in %) of B8, B15 and B7 in diabetics in groups I, II and III. The level 0 represents that found in the normal population.



- $p < 0.0005$
  - $p < 0.025$
  - \*  $p < 0.05$
  - \*\*  $p < 0.01$
- } (C) compared to control population
- } (C) compared to group Ib



## D i s c u s s i o n

This piece of research has demonstrated that in type I diabetics without other clinical autoimmune disease, HLA-B8 is significantly correlated with the persistence of ICA in the serum. This, together with the observation that B8 is also significantly increased in diabetics with other overt autoimmune diseases irrespective of their ICA status, suggests that in type I diabetes B8 is associated with autoimmunity either in terms of persistence of ICA or of clinical autoimmune disease. Moreover, the presence, and particularly the persistence, of ICA in the serum is significantly associated with the occurrence of thyroid and/or gastric autoantibodies. These findings contradict the reports by Lendrum et al(1975;1976) but substantiate the trends reported previously by the Edinburgh Group (Irvine et al,1977;Morris et al,1976).While an increased prevalence of thyroid and/or gastric antibodies was to be expected in diabetics with poly-endocrinopathy, its occurrence in group Ia and especially in group II patients indicates that the presence of ICA is associated with a diathesis towards organ-specific autoimmunity in general. However, no significant correlation could be shown between B8 and the occurrence of thyroid and/or gastric antibodies in the serum, which is in agreement with other studies on patients with clinical autoimmune thyroid disease (Irvine et al,1978) and with pernicious anaemia or subclinical autoimmune atrophic gastritis (Mawhinney et al,1975).

The younger age and lower female prevalence of ICA positive diabetics without other clinical autoimmune disease (groups Ia and II) compared to diabetics with other immunological disease (group III) suggests that the subclinical organ-specific autoimmunity in the

former may progress in some with time to overt clinical disease especially in females.

The fact that, apart from B8, the only HLA antigen of the A, B and C series to have a significantly increased prevalence in groups II and III was A1, suggests that only the HLA group A1, B8 and DR3 is associated with the autoimmune aspects of type I diabetes. Interestingly, B15 showed a higher prevalence in group I patients but tended to show a lower prevalence in groups II and III. Although these trends did not reach statistical significance, they indicate a lack of involvement of the second linked group of HLA antigens in autoimmunity in diabetes. On the other hand, this second HLA group as indicated by B15 and CW3 is associated with the tendency to mount a higher humoral immune response to heterologous insulin, but the opposite tends to be the case in the presence of A1/B8 (Bertrams et al, 1976; chapter B2.2b).

The third HLA group, A3, B7 and DR2, is protective against the development of type I diabetes (Ludwig et al, 1976), although it is not known whether this is achieved through a resistance to autoimmunity or to pancreatotropic viral infection (or other exogenous reagent). B7 has been shown to be associated with a high response to flagellin (Morris et al, 1977). In diabetics with B7 a significantly increased incidence of cutaneous allergic reactions to heterologous insulin has been described (Bertrams & Gruneklee, 1977). However, neither the production of antibodies nor the occurrence of cutaneous allergic reactions to heterologous insulin is concerned with autoimmunity in the pathogenesis of type I diabetes. Thus, while all three of the HLA groups associated with type I diabetes may be linked

with different immune response genes, only the HLA group which includes B8 is related to organ specific autoimmunity.

Bl.2c      I m m u n e   c o m p l e x e s   i n   n e w l y  
d i a g n o s e d   t y p e   1   d i a b e t i c s :  
a   p i l o t   s t u d y

Evidence is accumulating that in type I diabetes, immunopathological mechanisms may contribute to islet cell damage. In chapter Bl.2a the finding of soluble immune complexes (AgAb), detected by the Raji cell assay, in a small group of newly diagnosed insulin dependent diabetics has been reported. A correlation between the presence of AgAb and islet cell antibodies (ICA) in the serum was found. To confirm this preliminary report AgAb and ICA have been here studied in a larger group of IDD patients at diagnosis and at intervals up to one year following diagnosis. Immune complexes were detected by two sensitive techniques differing in principle because of possible heterogeneity of AgAb in diabetes and the known discriminating ability of available techniques for different types of AgAb (Lambert et al, 1978).

M a t e r i a l s   a n d   m e t h o d s

110 newly diagnosed insulin dependent diabetics attending the Out-Patient Diabetic Department of the Royal Infirmary, Edinburgh were studied. Serum from each patient was obtained within one week of diagnosis and from 37, 33, 33 and 37 patients of the same group at 1, 3, 6, and 12 months respectively after diagnosis. One hundred and thirty blood donors of comparable age and sex distribution were used as a control group.

Solid phase Clq radioimmunoassay (Clq-SP): Clq was isolated from fresh human serum by the method of Yonemasu and Stroud (Yonemasu &

Stroud,1971). The assay was carried out as a minor modification of the method described by Hay et al,(Hay et al,1976)which is based on the method of Svehag(1975).The sera were pretreated with EDTA according to Zubler et al(1976).  $^{125}\text{I}$ -labelled Staphylococcal protein A was used to detect AgAb bound to Clq coated tubes. Protein A was labelled according to Dorval et al. (1975) (see chapter A3.1).

Raji cell radioimmunoassay (RAJI): was used to detect AgAb by a minor modification of the technique of Theofilopoulos et al(1976). Raji cell bound AgAb were detected by the addition of  $^{125}\text{I}$ -labelled Staphylococcal protein A.

For the expression of results and definition of positivity see chapter A3.1

ICA was detected as previously described (chapter A3.5).

The Chi squared test (C), with Yates' correction where indicated, and Fisher's exact test (F) were used as shown.

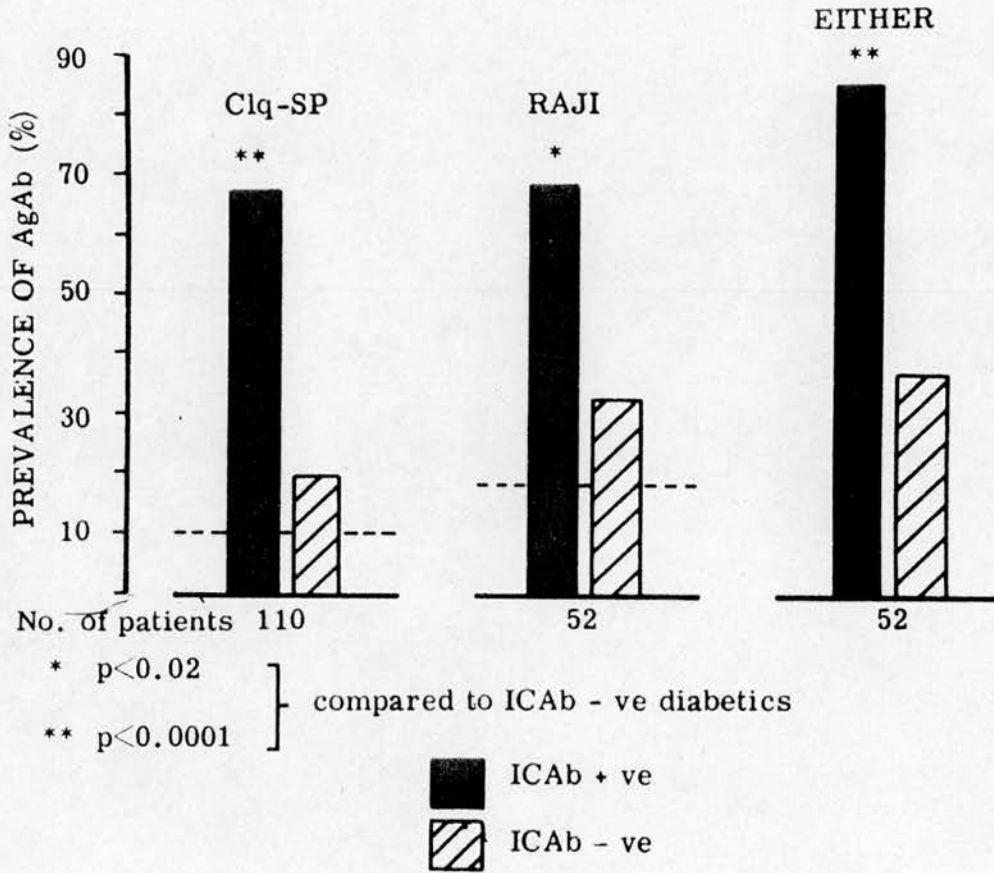
## R e s u l t s

AgAb were found in 43 out of 110 (39%) IDD at or within a week of diagnosis using Clq-SP. The presence of AgAb was significantly higher in comparison with the control population (10% positive) ( $p < 0.0001$ , "C") . 52 subjects of the same group were also tested by RAJI and 26 (50%) were positive compared to the control population (20% positive) ( $p < 0.005$ , "F"). 31(60%) of these 52 patients were positive by one or both methods.

AgAb, studied in IDD at increasing intervals from diagnosis by Clq-SP, were found in 9 patients out of 37 after 1 month of diagnosis ( $p < 0.05$ , "C" vs control population) and in 4 out of 37, 3 out of 33,

FIGURE B1.2/2

The prevalence of AgAb in the serum of newly diagnosed type 1 diabetics as detected by ClqSP and by RAJI or by either method correlated with the presence or absence of ICA.



3 out of 37 respectively after 3,6,12 months from diagnosis (not significant versus control population).

45 out of 110 IDD tested for ICA within a week of diagnosis were positive(41%).Of the same patients studied for immune complexes after diagnosis 41%,36%,27% and 24% remained ICA positive at 1, 3,6, and 12 months, respectively.

Using Clq-SP, 30 of the 45 (67%) ICA positive patients at diagnosis had AgAb whereas only 13 out of 65(20%) of the ICA negative patients were AgAb positive( $p < 0.0001$ , "C").Using RAJI, 17 out of 25(68%) of ICA positive and 9 out of 27(33%) of ICA negative patients at diagnosis had immune complexes in their sera ( $p < 0.02$ , "F"). Combining the results of both methods 84% of ICA positive and 37% of ICA negative patients at diagnosis had immune complexes ( $p < 0.0001$ , "F") (Figure B1.2/2).

At 1 month from diagnosis 47% of ICA positive and 9% ICA negative diabetics had AgAb ( $p < 0.025$ , "F"). At 3,6, and 12 months from diagnosis the differences in prevalence of AgAb between the ICA positive and negative patients did not reach statistical significance.

The results of tests for AgAb in diabetic patients were concordantly positive or negative in 71% ( $p < 0.01$ , "F") of the 52 sera tested.

#### D i s c u s s i o n

The high prevalence of circulating AgAb in newly diagnosed insulin-dependent diabetics and the close correlation between AgAb and ICA, which it has been previously reported in a small number of patients(see B1.2a),has been confirmed by the present study, using

one of the same methods (RAJI) for detecting AgAb and also using a second method (Clq-SP) based on a different principle. Although neither method gives any indication of the nature of the antigens involved, the correlation at diagnosis with the presence of ICA and the comparable decline of the prevalence of both ICA and AgAb with time following diagnosis strongly suggest that islet cell antigen-antibody may well contribute substantially to the AgAb that occur in the sera of type I diabetics at diagnosis and shortly afterwards. The prevalence of AgAb declines to control levels after 6 months in ICA positive patients, while control values are reached before 1 month in ICA negative patients.

AgAb consisting of islet cell antigen-antibody may arise as a consequence of islet cell damage caused by some chemical or virus but it is also conceivable that such complexes may have a primary role in the pathogenesis of islet cell failure. It would appear that the situation pertains at diagnosis, and we surmise also during the early stages of type I diabetes, in which islet cell antigen-antibody complexes exist in the presence of antibody excess. The islet cell antigens involved may well include cell surface antigens (Lernmark et al, 1979) as a correlation has been demonstrated between the presence of cytoplasmic and cell surface islet cell antigens (Freedman et al, 1979). Such complexes could conceivably "arm" K-cells (Calder et al, 1973) to render them specifically cytotoxic to islet cells. Alternatively the presence of AgAb within the islets may activate the complement system with the generation of its biologically active components. The transient nature of lymphocytic infiltration in type I diabetes, which is only observed within the islets during the first month following diagnosis (Gepts, 1979), could fit with the transient



nature of ICA (Irvine et al,1977;Lendrum et al,1976) and of AgAb at this stage of the disease in most type I diabetics.

Impaired clearance of AgAb from the circulation of the reticulo-endothelial system may also contribute to their presence in the circulation as it has been shown that phagocytic function is subnormal during metabolic decompensation in diabetes(Badgage,1976).AgAb in the serum of diabetics at diagnosis and shortly after are probably quite different in the majority of patients from the immune complexes that may be detected sometime after diagnosis in either insulin or OHA treated diabetics and which may be contributory to diabetic microangiopathy (Chapter B3).

B1.2d    I m m u n e   c o m p l e x e s   i n   t y p e   I  
d i a b e t i c s   w i t h   p e r s i s t e n t  
i s l e t   c e l l   a n t i b o d i e s

In this study the prevalence of AgAb in diabetics remaining ICA positive at 3 or more years after diagnosis (ICA persisters) in comparison with ICA negative patients of comparable duration of diabetes has been examined. In addition, the correlation between AgAb and insulin antibodies, HLA antigens and thyroid/gastric cytoplasmic antibodies in ICA persisters has been studied.

M a t e r i a l s   a n d   m e t h o d s

A total of 102 type I (insulin dependent) diabetics were studied. The first group comprised 33 patients with persistent ICA (ICA persisters): that is, patients with detectable ICA in their sera at least three years after diagnosis. The second group comprised 68 diabetics without detectable ICA in their sera at the time of study. The duration of diabetes was comparable in these two groups: the mean duration for ICA persisters was  $13.5 \pm 9.6$  yr (s.d.) and for the second group was  $15.3 \pm 8.0$  yr. Patients with associated clinical autoimmune disease or severe microangiopathy were excluded from the study. Sera from 105 blood donors were included as controls for immune complex measurements.

The indirect immunofluorescence method was used to detect ICA as previously described (chapter A3.5).

The solid phase Clq binding technique of Svehag(1975), adapted by Hay et al(1976) was used to reveal immune complexes. Results were

expressed as ug/ml of aggregated Ig equivalents by reference to preparations of heat aggregated Cohn fraction II immunoglobulin. Those results falling above the 90th percentile of the blood donor controls were considered positive (chapter A3.1).

The method of Ortved Andersen(1972) as modified by Mustaffa et al(1977) was used to detect and titrate insulin antibodies(chapter A3.3).

HLA typing was performed as previously described in B1.2b and for the same range of antigens (Morris et al,1976).

Thyroid and gastric cytoplasmic antibodies were detected by the indirect immunofluorescence technique as previously described (chapter A3.5).

Fisher's exact test and Kendall's test were used as appropriate.

## R e s u l t s

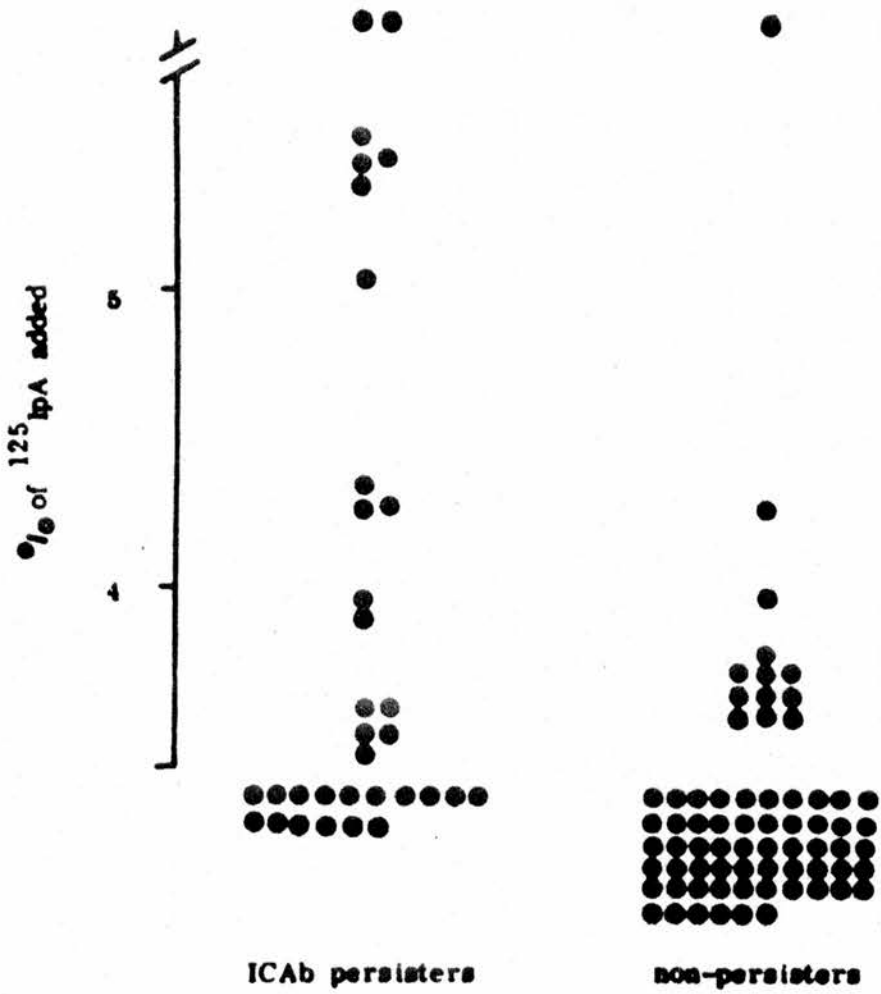
17 (52%) of 33 sera from ICA persisters were found to be positive for AgAb in comparison with 13 (19%) of 69 sera from diabetics without ICA more than 3 years from diagnosis ( $p < 0.001$ ) and 10% of normal blood donors( $p < 0.0005$ ).

16 (67%) of 24 ICA persisters typed for HLA were B8 positive. AgAb were found in 9 (56%) of those 16. In the remaining 8 HLA -B8 negative patients 3 (38%) were positive for AgAb. The difference in the prevalence of AgAb between the B8 positive and B8 negative ICA persisters did not reach statistical significance with the small numbers studied.

There was a significant negative correlation between AgAb values and insulin antibody titres in the sera of 24 ICA persisters tested ( $p < 0.05$ ).

FIGURE B1.2/3

The presence of AgAb according to ICA status (ICAb) of the diabetic patients.



Thyroid and/or gastric cytoplasmic antibodies were found in the sera of 16 of 33 ICA persisters (48%). These autoantibodies were present in 7 of 17 AgAb positive patients (41%) and 9 of 16 AgAb negative patients(56%)(not significant).

#### D i s c u s s i o n

Previous studies on type I diabetics have shown a correlation between circulating AgAb and ICA at the time of clinical presentation of the disease and during the following few months (Chapters B1.2a, B1.2c). In most patients AgAb decline quite rapidly after diagnosis. The prevalence of ICA declines at a more gradual rate so that after about 5 years only 15-20% of diabetics are ICA positive (Lendrum et al,1976;Irvine et al,1977). In ICA persistently positive diabetics there is an increased incidence of the HLA-B8 antigen compared to ICA negative patients, a difference which is not readily apparent at diagnosis(Irvine et al,1977;Morris et al,1976; chapter B1.2b). Moreover, in ICA persisters lower insulin antibody titres were reported in comparison with insulin dependent diabetics without persistent ICA (Chapter B2.2b).

In this study it is reported that ICA persisters also have an increased prevalence of Clq binding AgAb when compared to normal subjects or to ICA negative diabetics of comparable duration of disease. These findings re-emphasise the hypothesis formulated for newly diagnosed type I diabetes: that these complexes may be composed of pancreatic antigens, or that ICA and AgAb are immunologically unrelated but that their formation is related and linked to factors yet unrecognised.

Among ICA persisters the prevalence of AgAb was higher among those who were HLA-B8 compared to those who were not, although the increase was not found to be statistically significant in the small number of patients studied.

In ICA persisters, high insulin antibody titres were not associated with significant amounts of AgAb; a result found also in other insulin treated diabetics selected at random (Chapter B2.4). Indeed, when insulin-anti-insulin complexes are formed in antibody excess their small size (dictated by the bivalency of insulin) would not facilitate Clq activation and hence they may be detected poorly by the Clq-SP technique.

An association between AgAb and thyroid/gastric antibodies was not found in the present study. This is in keeping with the lack of an association between these antibodies and the presence of AgAb as detected by the Raji assay in patients with autoimmune thyroid disease (Al-Khateed & Irvine, 1978). This suggests that either thyroid/gastric cytoplasmic antibodies are different from ICA in terms of immune complex formation, or that the ratio of antibodies to antigens in thyroid autoimmune disease is different from that in insulin dependent diabetes with corresponding differences in relation to the tendency to form AgAb.

At present the biological significance of persistent ICA and AgAb formation is unknown, but persistence of autoantibodies in the serum is characteristic of the "classical" organ-specific autoimmune disorders; e.g. autoimmune Addison's disease (Irvine & Barns, 1975) and pernicious anaemia (Irvine et al, 1965). This is perhaps a reflection of the nature of corresponding immune response genes, rather than any major differences in residual beta cell function of the islets, as

there is no detectable difference in the amount of C-peptide secreted by insulin dependent diabetics with or without ICA (Theophanides et al,1978;Rubenstein et al,1980a).

B1.3 IMMUNOLOGICAL EVENTS IN INSULIN DEPENDENT DIABETICS  
AT DIAGNOSIS AND FOLLOW UP

B1.3a Aim of the study

In this study the aims have been to examine the humoral immune response in IDDM to endogenous and exogenous antigens such as islet cells, heterologous insulin and viruses, and to investigate the presence of antigen-antibody complexes. The relationship of these factors with the HLA antigens was also considered. Therefore AgAb and cytoplasmic islet cell antibodies, as detected by anti IgG (ICA), have been evaluated in a large group of IDDM's at diagnosis and prospectively at intervals up to 3 years following diagnosis. In view of the possible heterogeneity of AgAb in diabetes, immune complexes were detected by three different techniques: the solid phase Clq binding test (ClqSP), the Raji cell radioimmune assay (RAJI) and the conglutinin binding assay (KgBt). Complement fixing islet cell antibodies (CF-ICA) at diagnosis and after 6 months, viral antibodies (VAb) at diagnosis, insulin antibodies (InsAb) at 9 months after diagnosis and HLA antigens B8 and B15 were also evaluated in a number of these patients.



B1.3b      M a t e r i a l s   a n d   m e t h o d s

## Patients

One hundred and fifty-three newly diagnosed, insulin dependent diabetics (IDD) attending an adult clinic were studied within 2 days of diagnosis and of these 88 consecutive patients were studied prospectively for up to 3 yr following diagnosis. Details of patients are shown in Table B1.3/1. ICA were evaluated in all diabetics at diagnosis and in 88,79,76,68,57,31 and 6 patients at 1,3,6,9,12,24, and 36 months after diagnosis respectively. AgAb (Clq) were also measured in all patients at diagnosis but only in 52,58,51,58,53,29 and 5 patients at the above intervals. CF ICA were evaluated in 30 randomly selected diabetics both at diagnosis and after 6 months. AgAb (Kg) and (RAJI) were studied at diagnosis in 34 and 50 insulin dependent diabetics, respectively. A group of normal controls were included in the AgAb studies for the detection of the limit of positivity as described below. Viral antibodies were assayed in 30 insulin dependent diabetics at diagnosis and in 30 age and sex matched normal controls residing in the same geographical region. Samples from the matched pairs of subjects were obtained within 7 days of each other. Insulin antibodies were measured 9 months after diagnosis in 35 diabetics treated with highly purified ("mono-component") insulins from diagnosis. HLA B8 and B15, representing the two HLA diabetogenic series, were evaluated in 115 insulin dependent diabetics.

### Detection of Islet Cell Antibodies

ICA were detected by indirect immunofluorescence on human pancreatic cryostat sections using antihuman IgG conjugated with fluorescein isothiocyanate (Chapter A3.5). CF ICA were detected by an indirect immunofluorescence test using fresh complement and anti C3 conjugated with fluorescein isothiocyanate (Chapter A3.5).

### Detection of Immune Complexes

#### Solid phase Clq radioimmunoassay (ClqSP)

Clq was purified according to Yonemasu and Stroud (1971). The assay was carried out as described by Hay (1976) with minor modifications.  $^{125}\text{I}$  labelled Staphylococcal protein A was labelled according to Dorval et al (1975). One hundred and eighty five blood donors were included in the study for the detection of the limit of positivity as described in chapter A3.1.

#### Conglutinin binding test (KgBt).

Conglutinin was purified and the test performed according to Casali et al (1977).  $^{125}\text{I}$ -labelled protein A was used to reveal conglutinin bound complexes. One hundred and five blood donors were tested for the evaluation of the limit of positivity (Chapter A3.5).

#### Raji Cell Radioimmunoassay (RAJI)

Raji cells were used to detect AgAb by a minor modification of the technique of Theophilopoulos et al (1976). Raji cell bound AgAb were detected by the addition of  $^{125}\text{I}$ -labelled Staphylococcal protein A. The limit of positivity was calculated to be 20 ug AHG (aggregated

human immunoglobulin G) eq/ml on the basis of the results of 68 blood donors (Chapter A3.5).

#### Detection of Virus Antibodies

Inactivated sera (56°C for 30min) were tested in microtitre assay for complement fixing antibodies to clamidia and to a number of viruses and viral antigens, namely coxsackie B1-6, influenza A and B, adenovirus, respiratory syncytial, measles, mumps S and V, herpes simplex and varicella zoster. Sera were screened at a dilution of 1:32 with 3 HD50 guinea pig complement. Sera positive at 1:32 were titrated at further two fold dilutions to 1:512. Antibodies to coxsackie viruses B1 6 were estimated in a quantal neutralisation test. 100 TCID 50 of each virus was reacted with serum dilutions and inoculated into virus cell cultures in tubes. End points were determined by low power microscopy of the viral cytopathic effect. All sera were negative for hepatitis B surface antigen by a reverse passive haemoagglutination test.

#### Detection of Insulin Antibodies

InsAb was evaluated according to the method of Ortved Andersen (1972) as modified by Mustaffa (1977) (chapter A3.3).

#### Statistical Analysis

The Chi-squared (2x2) test ("X") with Yates's correction, Fisher's test ("F") and Cox's test for trend ("C") were used in the analysis of the results.

B1.3c      R e s u l t s

## ICA

ICA were found in 68 of the 153 insulin dependent diabetics studied at diagnosis (table B1.3/1).Forty-four (50%) of the insulin dependent diabetics studied prospectively were ICA positive at diagnosis, the presence of ICA declining to 45,38,36,31,26,19 and 17% at 1,3,6,9,12, 24 and 36 months after diagnosis respectively (table B1.3/2).

## CF-ICA

CF-ICA were found in 30% of the diabetics at diagnosis and in 23% at 6 months. All patients with CF-ICA at diagnosis were ICA positive whilst only 47% of patients with ICA also had CF-ICA in the serum.

## AgAb

ClqSP.AgAb (Clq) were found in 54 of the 153 insulin dependent diabetics studied at diagnosis ( $p < 0.001$  vs. normals, "X").Twenty-nine of the 65 insulin-dependent diabetics studied at diagnosis alone and 25 (28%) of the 88 studied prospectively had AgAb (Clq) in their serum at diagnosis. In the prospective study the AgAb (Clq) positivity declined to 25% ( $p < 0.01$  vs.normals, "X"),22% ( $p < 0.02$ ,"X"),17% (n.s.,"X"),19%,17%,17% and 0% after 1,3,9;12,24 and 36 months from diagnosis respectively.

KgBt.AgAb (Kg) were found in 12 of the 34 diabetics studied at diagnosis ( $p < 0.001$  vs. normals, "X").

#### RAJI

AgAb (RAJI) were found in 27 of 50 diabetics studied at diagnosis ( $p < 0.002$  vs. normals, "X"). Eighty-four patients were studied at diagnosis by more than one AgAb method and of these 57% had at least one positive AgAb result.

#### ICA vs. AgAb

A significant correlation between the presence of AgAb, as detected by ClqSP, and the occurrence of ICA was found in IDDM at diagnosis ( $p < 0.001$ , "X") (Fig. B1.3/1). Fifty-four percent of the ICA positive insulin dependent subjects and 20% of those without ICA showed AgAb in their serum at diagnosis. AgAb (RAJI) were also significantly increased in ICA positive diabetics ( $p < 0.02$ , "F") but there was no significant correlation between AgAb (Kg) and ICA.

#### CF-ICA vs. AgAb (Clq).

There was no significant correlation between CF-ICAb and AgAb (Clq) either at diagnosis or after 6 months ("X").

#### Viral Antibodies

There was no significant difference ("F") in the presence titre of VAb between the diabetics and the controls studied (Fig. B1.3/2). No correlation was found between VAb presence and AgAb or ICA positivity at diagnosis (Table B1.3/3).

### Insulin Antibodies

The mean ( $\pm$  S.D.) InsAb value was 33.6 ( $\pm$ 46.4) uU/ml. No correlation was found between the InsAb levels at 9 months after diagnosis and the presence, both at diagnosis and after 9 months, of ICA or AgAb (table B1.3/4).

### HLA Antigens

Forty-six percent of the insulin dependent diabetics were HLA B8 positive and 15% were HLA B15 positive. No correlation was found between these two diabetogenic series and the presence of ICA, AgAb or VAb at diagnosis. HLA B15 positive patients tend to form significantly higher InsAb levels than B15 negative patients ( $p < 0.05$ , "C")(table B1.3/5).

TABLE B1.3/1

Details of the 153 insulin dependent diabetics studied

| Study                   | ICA status at diagnosis | No. of patients | Sex (% male) | Age at diagnosis (mean $\pm$ SD) years | Associated autoimmune disease* |
|-------------------------|-------------------------|-----------------|--------------|--|--------------------------------|
| Diagnosis and follow up | Positive                | 44              | 63.6         | 25.6 $\pm$ 15.4                        | 0                              |
|                         | Negative                | 44              | 75.0         | 24.8 $\pm$ 14.5                        | 2                              |
| Diagnosis alone         | Positive                | 24              | 41.7         | 19.4 $\pm$ 9.8                         | 0                              |
|                         | Negative                | 41              | 63.4         | 23.7 $\pm$ 12.0                        | 0                              |

\* Thyroid disease in all cases.

TABLE B1.3/2

Islet cell antibody status of the 88 insulin dependent diabetics studied prospectively from diagnosis

| Time after<br>Diagnosis | No. of<br>patients | No. ICA<br>positive (%) |
|-------------------------|--------------------|-------------------------|
| Diagnosis               | 88                 | 44 (50%)                |
| 1 months                | 88                 | 40 (45%)                |
| 3 months                | 79                 | 30 (38%)                |
| 6 months                | 76                 | 27 (36%)                |
| 9 months                | 68                 | 21 (31%)                |
| 1 year                  | 57                 | 15 (26%)                |
| 2 years                 | 31                 | 6 (19%)                 |
| 3 years                 | 6                  | 1 (17%)                 |



TABLE B1.3/3      Antibodies to Coxsackie B(1-6) correlated with AgAb(CIq) and ICA  
in the serum of 30 newly diagnosed insulin dependent diabetics

| Coxsackie B (1-6) antibodies |                  |                                |
|------------------------------|------------------|--------------------------------|
| No. of patients              | No. of positives | ICA Titres                     |
|                              |                  | AgAb (CIq)<br>No. of positives |
| <32 *                        | 6                | 0                              |
| 32-64 †                      | 14               | 4                              |
| >128 §                       | 10               | 2                              |

\* Coxsackie B1-6 titres all < 32.  
 † One or more of the Coxsackie B1-6 titres = 32-64.  
 § One or more of the Coxsackie B1-6 titres ≥ 128

TABLE B1.3/4 Insulin antibodies, measured 9 months after diagnosis, correlated with the presence of AgAb(CIq) and ICA in the serum at diagnosis and at 9 months after diagnosis

| Values (uU/ml) | InsAb | No. of patients | AgAb (CIq)   |             | ICA          |             |
|----------------|-------|-----------------|--------------|-------------|--------------|-------------|
|                |       |                 | At diagnosis | At 9 months | At diagnosis | At 9 months |
| <10            |       | 17              | 7            | 3           | 7            | 6           |
| 10-50          |       | 13              | 7            | 4           | 8            | 6           |
| >50            |       | 5               | 3            | 1           | 2            | 1           |

TABLE B1.3/5

HLA B8 and B15 correlated with serum levels of ICA, AgAb, VAb and InsAb

|                      |                            | No. of patients                 | HLA B8 + patients    | HLA B15 + patients     |       |
|----------------------|----------------------------|---------------------------------|----------------------|------------------------|-------|
| <u>HLA vs. ICA</u>   | ICA at diagnosis           | + 52<br>- 63                    | 23 (44%)<br>31 (49%) | 6 (12%)<br>11 (17%)    |       |
|                      | After 12 months            | + 12<br>- 36                    | 6 (50%)<br>21 (58%)  | 3 (25%)<br>6 (17%)     |       |
|                      | AgAb (Clq) at diagnosis    | + 39<br>- 76                    | 18 (46%)<br>35 (46%) | 7 (18%)<br>10 (13%)    |       |
|                      | AgAb (Clq) after 12 months | + 8<br>- 40                     | 4 (50%)<br>22 (50%)  | 1 (12.5%)<br>7 (17.5%) |       |
| <u>HLA vs. AgAb</u>  | AgAb (Kg) at diagnosis     | + 7<br>- 18                     | 3 (43%)<br>8 (44%)   | 0 (0)<br>2 (14%)       |       |
|                      | AgAb (RAJI) at diagnosis   | + 19<br>- 14                    | 11 (58%)<br>8 (57%)  | 2 (10%)<br>2 (14%)     |       |
|                      | <u>HLA vs. VAb</u>         | Coxsackie B titres at diagnosis |                      |                        |       |
|                      |                            | <32                             | 4                    | 2 (50%)                | 0 (0) |
| 32-64                |                            | 12                              | 4 (33%)              | 2 (17%)                |       |
| >128                 | 9                          | 4 (44%)                         | 1 (11%)              |                        |       |
| <u>HLA vs. InsAb</u> | InsAb (uU/ml)              |                                 |                      |                        |       |
|                      | <10                        | 16                              | 9 (56%)              | 2 (12%)                |       |
|                      | 10-50                      | 10                              | 7 (70%)              | 3 (30%)                |       |
|                      | >50                        | 5                               | 0 (0)                | 3 (60%)                |       |

FIGURE B1.3/1

A comparison of the results of ICA and AgAb (Clq) studies in insulin dependent diabetics at diagnosis and during the 12 months following diagnosis.

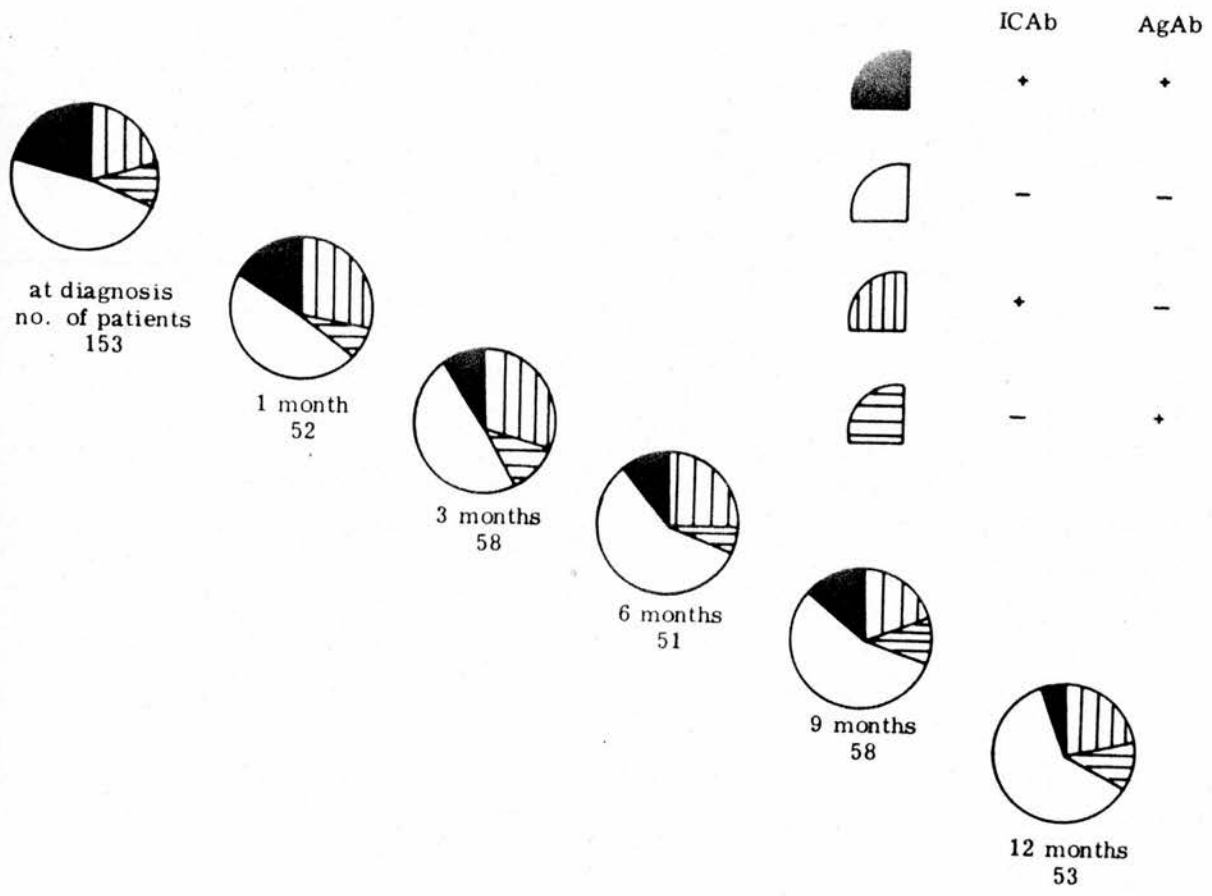
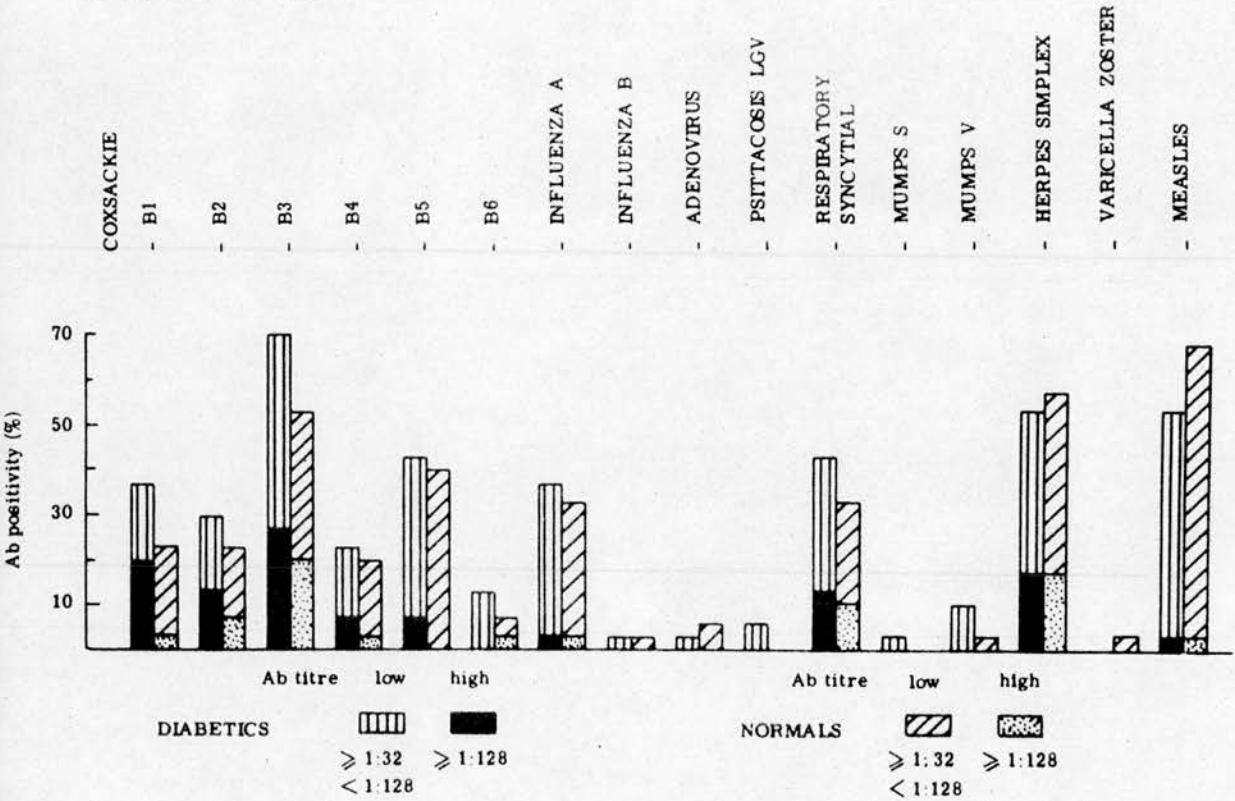


FIGURE B1.3/2

A comparison of the serum viral antibody titres in 30 insulin dependent diabetics at diagnosis and in 30 matched controls.

ANTIBODIES TO VIRUSES



B1.3d      D i s c u s s i o n

The increased presence of circulating immune complexes in Type 1 diabetics at diagnosis is confirmed by this large study. Immune complex positivity tends to decline rapidly following diagnosis and within only a few months their prevalence is similar to that found in the normal population. The methods used to assay AgAb differ in principle and detect different types of complexes. However, with all techniques employed, the increased prevalence of complexed immunoglobulins is similar and complexes are detected that can lead to tissue damage by activating the complement system and by binding to cells with Fc or C3 receptors on their surface. Thus, it has been shown that a heterogenous group of potentially harmful complexes is present at the time of diagnosis of Type 1 diabetes.

ICA were found in approximately half of our newly diagnosed diabetics. This prevalence is lower than that reported by other workers (Lendrum et al, 1975; Bottazzo et al, 1976). The older age of patients in this study and the decision to consider as negatives all doubtful results may account for this apparent discrepancy. There is a need to standardize the detection of ICA since differences in the substrate and in the methodology have sometimes resulted in poorly reproducible findings.

It has been reported that antibodies to certain viruses are increased in Type 1 diabetics at diagnosis (El-Hagrassy et al, 1980). However, in epidemiological studies, where the parameter being investigated is also present in the normal population, there is often difficulty in selecting an appropriate control group. In these viral

studies, each diabetic has been matched with a normal subject of the same age and the sex who resided in the same geographical area. The diabetic and the control subjects were sampled within the same 7 day period to exclude differences resulting from the seasonal variation of viral infections in the community. Although the antibody positivity to certain viruses appeared high in the diabetic patients, there was no significant difference from the results obtained in the control group (Cudworth,1981) and the findings simply reflect the viruses prevalent at the time of sampling.

This finding is not unexpected since it is likely that only in a very few cases does an overwhelming viral infection produce rapid beta cell necrosis. In other cases, a viral infection of the islets may trigger an autoimmune reaction against the islets which in turn produces beta cell damage and leads to the development of diabetes months, or even years, after the initial infection. However, viral antibodies, which represent a positive reaction by the body against an aggressive agent, may not be the most appropriate phenomena to study since our aim is to determine why the host fails to cope adequately with a viral attack and thereby sustains beta cell damage.

Insulin antibody levels generally increase slowly from the time insulin therapy is commenced and assume a steady level after several months. InsAb levels were normal or only modestly elevated in patients studied despite their estimation 9 months after diagnosis. This finding can be explained by the use of purified insulins alone in the patients studied. Furthermore, a correlation between AgAb (CIq) and InsAb was not expected since AgAb presence tends to decline after diagnosis in contrast to InsAb levels. AgAb seem to reflect other

phenomena occurring at diagnosis more than the presence of circulating antibodies to insulin and viruses (see chapter B2).

This study has demonstrated a tendency for HLA B15 positive to form significantly higher IBC levels than B15 negative patients (Chapter B2). This genetic predisposition for some patients to produce higher amounts of insulin antibodies is in accordance with previous work (Bertrams et al, 1976). It is well recognised, at least in animals, that the major histocompatibility genes are very close to genes that regulate the immune response and in particular, the T-cell mediated response. In patients studied this genetic influence was apparent only in the response towards therapeutic insulin since there was no correlation between AgAb, ICA and VAb measured at diagnosis and HLA types. The lack of correlation between ICA at diagnosis and HLA types confirms previous findings (Chapter B1.2b).

Immune complexes (Clq, RAJI) were correlated with the occurrence of ICA at diagnosis but not with the other antibodies studied. Thus, it would appear that AgAb and ICA are related and that the presence of AgAb at diagnosis does not simply reflect a recent viral infection. This is supported by previous reports of a strong correlation between ICA and immune complexes in insulin-dependent diabetics with persistent ICA (Chapter B1.2d) and in newly diagnosed non-insulin dependent diabetics (Chapter B1.4). In the present context, the CF-ICA studies did not provide any additional information regarding the immunological mechanisms which are operative at, and soon after, the onset of IDDM.

It is possible that AgAb and ICA have separate roles in the early stages of Type 1 diabetes or that they may be involved together, either primarily or secondarily. Complexes may reflect damage to the



islets and be present near the clinical onset of the disease together with other immune phenomena such as ICA. Medium or large size antigen-antibody complexes, that fix complement and react with cells having surface receptors for Fc or C3, may produce cell damage either directly or by interacting with specific T-cell subpopulations. AgAb, if present in antibody excess, may arm K cells against a specific antigen(s) in or the pancreatic islets and thereby result in the development of diabetes mellitus. The correlation between the presence of AgAb and ICA in the serum, at the time IDDM is diagnosed, suggests that some of the AgAb may be comprised of islet cell Antibody/islet cell Antigen.

B1.4 IMMUNOLOGICAL EVENTS IN DIABETICS NOT REQUIRING  
INSULIN AT DIAGNOSIS AND FOLLOW UP

B1.4a Aim of the study

In this study, the presence of soluble AgAb in diabetic patients who did not require insulin at the time of clinical diagnosis has been investigated. Among them, particular attention was focused on the minority who did not have ketonuria but showed ICA in the serum at diagnosis. In this relatively uncommon group of patients, ICA, complement fixing islet cell antibodies (CF-ICA) and AgAb were tested at diagnosis and at regular intervals thereafter. Clinical and subclinical associations with other autoimmune phenomena were also studied. Aims of this study were to highlight the presence of AgAb in patients who were ICA-positive but not ketotic, the modifications with time of ICA, CF-ICA and AgAb and the correlation between the presence of ICA, CF-ICA or AgAb at diagnosis and in the following months with the tendency to progress to insulin dependency.

Bl.4b      M a t e r i a l s   a n d   M e t h o d s

## Patients

Over a period of more than a year, most patients newly diagnosed at a few Edinburgh Diabetic Clinics as non-insulin dependent diabetic subjects, according to the National Diabetes Data Group Classification(1979), were tested for ICA at their first attendance. Two hundred and sixty patients were tested and 106 of these were included in the study: all those(17) who were found to be ICA-positive, together with 89 who were randomly selected from the newly diagnosed ICA-negative diabetic patients.

In the ICA-positive group, there were five males and 12 females with a mean age at diagnosis of  $55.4 \pm 16.1$  years. In the ICA-negative group, 42 were male and 47 were female and the mean age at diagnosis was  $61.6 \pm 12$  years. Two of the 17 ICA-positive and two of the 89 ICA-negative patients had other associated autoimmune diseases, (Hashimoto's thyroiditis, pernicious anaemia and primary hypothyroidism). Almost all the 17 ICA-positive patients could be considered Type 2a patients(National Diabetes Data Group,1979),the median of the ideal body weight percent being 98.5 (interquartile range 89.8, 104.5).No other substantial difference in the symptoms preceding diagnosis or in the clinical and metabolic signs at the onset of overt diabetes was noticed between ICA-positive and ICA-negative patients.

All the patients were followed clinically for approximately 3 years. ICA titres tend to decline after clinical diagnosis and there have so far been no reports of ICA-negative diabetic patients later

becoming ICA-positive, attention has been focussed on the 17 initially ICA-positive patients. They were also followed serologically at regular intervals for approximately 1 year from diagnosis and serum antibody studies were performed twice between 2 and 4 months after diagnosis and 10 or more months after diagnosis.

The clinicians did not know the result of the ICA test, at least in the first months, and in any case the ICA status was not among the parameters taken into account when the decision to put patients on insulin was taken.

#### Immune complexes

AgAb were evaluated by the solid phase Clq radioimmunoassay according to Hay et al(1976), with minor modifications. Details of the technique used, the limit of positivity, the expression of results and the reproducibility of the test have been described previously (chapter A3.1).

#### Autoantibodies

ICA, thyroid cytoplasmic and gastric parietal cell antibodies were determined by indirect immunofluorescence using fresh frozen cryostat sections (Chapter A3.5). CF-ICA were evaluated with the addition of fresh normal serum as source of complement, and of fluorescein conjugated anti C3 antibody (Chapter A3.5).

#### Statistical evaluations

Statistical evaluation was done by chi squared test, with Yates' correction when appropriate, and by the two tailed Cox's test for trends in proportions when indicated.

Bl.4c      R e s u l t s

## Islet cell antibodies

In the ICA-positive patients, ICA were still present in 14 out of 17 between 2 and 4 months after diagnosis and in 10 out of 16 after 1 year or more of clinical diabetes (Fig.Bl.4/1).

## Immune complexes

AgAb were found in eight of the 17 ICA-positive diabetic patients ( $p < 0.01$  versus normal subjects) and in 16 of the 89 ICA-negative patients (NS versus normal subjects,  $p < 0.02$  versus ICA-positive patients), both groups being studied at diagnosis.

## Complement fixing islet cell antibodies

CF-ICA were found in 11 of the 17 ICA-positive diabetic patients. At the end of the first 3 months, ten were positive. Five were still positive 10 months or more from diagnosis (Fig. Bl.4/1). None of the CF-ICA-negative patients at diagnosis became CF-ICA positive with time.

## Thyrogastric antibodies

Autoantibodies to thyroid or to gastric parietal cells, studied in all the patients at diagnosis, were found in five of the 17 ICA-positive and in 16 of the 89 ICA negative diabetic patients (NS).

## ICA,CF-ICA,AgAb and clinical tendency

## to progress to insulin dependency

Ten of the 17 ICA positive and two of the 89 ICA-negative diabetic patients required insulin within 3 years of diagnosis ( $p < 0.0001$ ) either because of the presence of ketonuria and/or because of a metabolic derangement (glycaemia constantly  $> 12$  mMol/l) non-controllable with other therapy. When the patients were divided according to tendency to progress to insulin dependency (Fig.-B1.4/2), five were still on oral hypoglycaemic agents more than 3 years from diagnosis, six went on to insulin after 20 months of clinical diabetes, four between 6 and 10 months, and two died from vascular complications after 5 and 21 months, respectively. There was a significant trend for the presence of CF-ICA at diagnosis to be associated with earlier development of insulin dependency ( $p < 0.05$ ). The occurrence of AgAb and of thyro-gastric antibodies were not correlated with the tendency to progress to insulin dependency.

FIGURE B1.4/1

ICA (●-----●), CF-ICA (■-----■) and AgAb (▲-----▲) in the 17 ICA-positive diabetic patients initially treated without insulin, at diagnosis (A), between the second and the fourth month of the disease (B), and after 10 months or more of clinical diabetes (C).

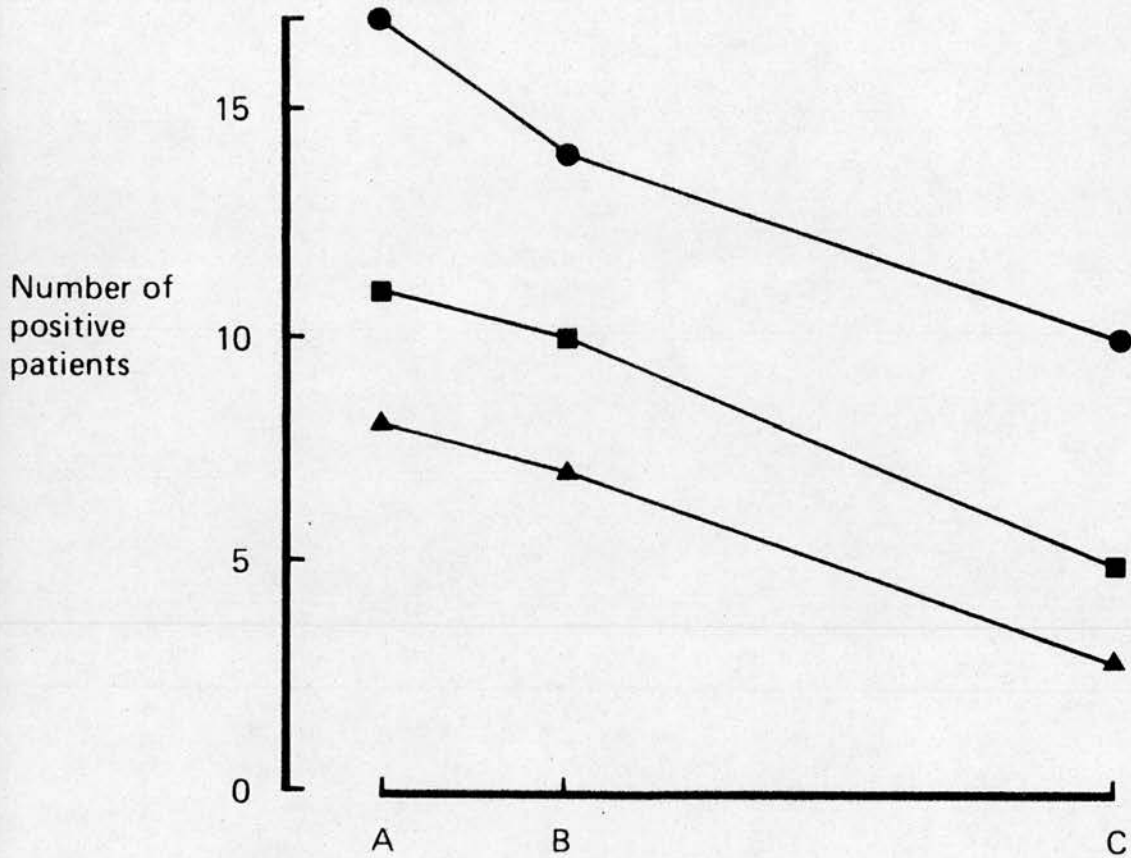
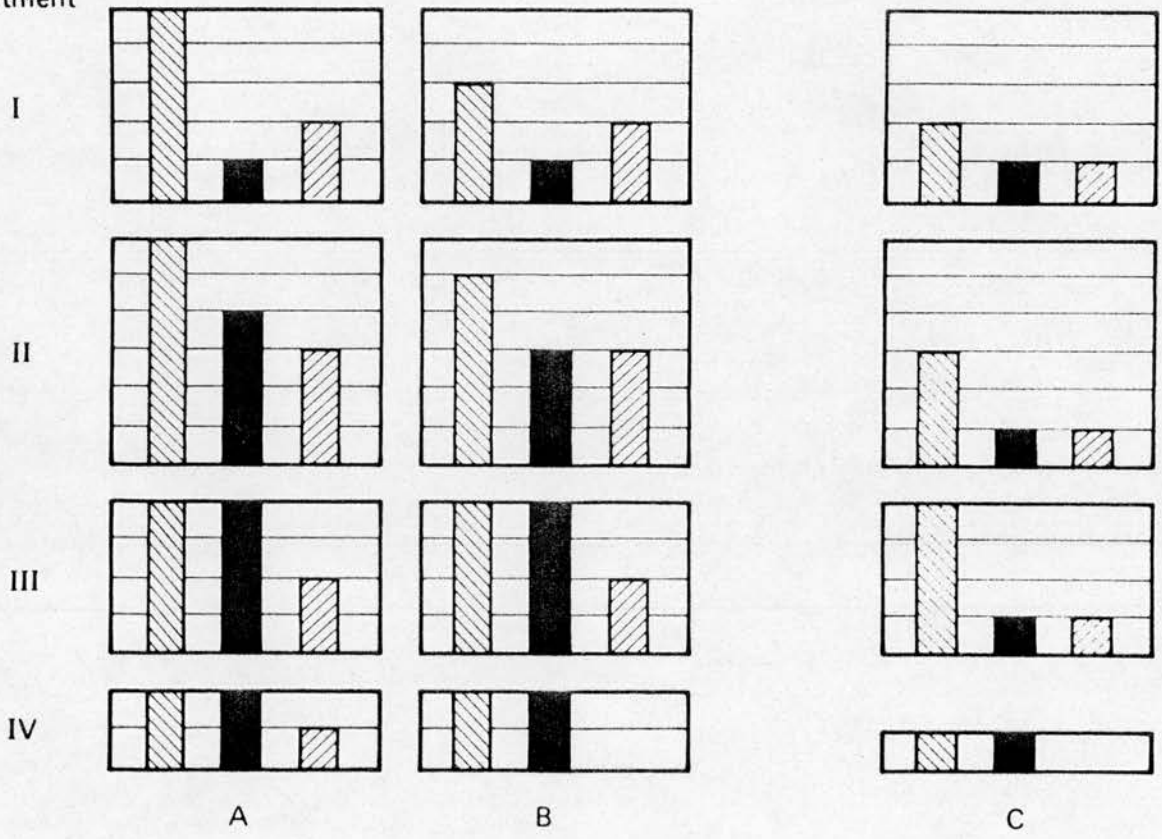


FIGURE B1.4/2

ICA (▨), CF-ICA (■), AgAb (▩) and tendency to progress to insulin dependency. The horizontal lines within the rectangular frames indicate the number of patients. Group 1 patients (n=5) were still on oral hypoglycaemic agents 3 years from clinical diagnosis, Group 2 (n=6) required insulin between 20 and 36 months from diagnosis, Group 3 (n=4) needed insulin within 10 months and the two Group 4 patients died during the follow-up study.

Treatment





B1.4d      Discussion

While an increased prevalence of immune complexes has been shown to be present in the serum at diagnosis of Type 1 diabetes (Chapters B1.2a, B1.2c, B1.3), this is not the case here where a substantial number of randomly selected Type 2 diabetic patients have been studied at diagnosis and compared with control subjects. However, the present study demonstrates that the prevalence of AgAb is increased in the minority of diabetic patients who have ICA in the serum but do not require insulin at diagnosis or during the first months of the disease. A correlation between serum AgAb and ICA has also been demonstrated in Type 1 diabetic patients close to diagnosis (Chapters B1.2c, B1.3) and in those Type 1 diabetic patients in whom ICA persists for some years (Chapter B1.2d). The correlation between serum ICA and AgAb suggests that islet cell antigen-antibody complexes may be part of the AgAb detected in the subjects. Whether AgAb are involved in islet cell damage in these patients has not yet been established, but they could be involved in serum cytotoxic activity or they could interact in several ways with cells which have receptors for the Fc portion of immunoglobulins or for the third component of complement (WHO Scientific Group Report, 1977). As ICA-positive diabetic patients in this study, not requiring insulin at diagnosis, were not ketotic, severe metabolic derangement would not seem to be a major factor in the development of serum AgAb in these subjects. Furthermore, hyperglycaemia is unlikely to be responsible as an increased presence of AgAb was seen in ICA positive non-insulin diabetic patients but not in the majority of Type 2 diabetes.

The present study demonstrates that a small proportion of non insulin dependent diabetic patients shows the same immunopathological phenomena that occur in classical Type 1 diabetes at diagnosis; i.e. ICA, CF-ICA and AgAb. It has been demonstrated previously that the presence of serum ICA in diabetic patients initially treated with diet and/or oral hypoglycaemic agents is a useful marker for predicting subsequent insulin dependency (Irvine et al,1977). The findings of this study suggest that when serum CF-ICA are present, the development of insulin dependency tends to occur earlier. A similar predictive value for CF-ICA was recently shown by other workers in different situations, i.e. Type 1 diabetes, relatives of patients with Type 1 diabetes or patients with autoimmune disorders (Bottazzo et al, 1980;Gorsuch et al,1981;Betterle et al,1980).

ICA-positive diabetic patients, not requiring insulin at diagnosis, who may also have CF-ICA and AgAb at diagnosis, clearly do not fit logically into the broad clinical subdivision of the majority of idiopathic diabetic patients according to the presence or absence of insulin dependency (Gorsuch et al,1981).At clinical diagnosis,Type 1 diabetic patients are ketosis prone and have islet cell autoimmunity. Type 2 diabetic patients classically have neither of these characteristics. The small group of subjects included in the present study showed no ketonuria and did not require insulin at diagnosis but had evidence of islet cell autoimmunity and tended to progress to insulin dependency. The type of diabetes in ICA positive non-insulin requiring diabetic patients should be regarded as a subgroup of Type 1 diabetes, probably representing a stage earlier in the same disease process (or group of disease processes) that tends to culminate in classical Type 1 diabetes.

B1.5 SEARCH FOR CIRCULATING SPECIFIC ANTIGENS IN THE EARLY  
STAGES OF TYPE I DIABETES BY MONOCLONAL ANTIBODIES

B1.5a Aims of the study

The presence of circulating islet antigens, either free or complexed with antibody, in the early stages of type 1 diabetes has been investigated with monoclonal anti-islet cell antibodies. The presence of such antigens, released into the circulation, might be a good indicator of islet cell damage occurring in this form of diabetes. Their presence was correlated to the occurrence of other immunological factors, such as islet cell antibodies and different types of complexes.

B1.5b Materials and methods

Patients

33 recently diagnosed type 1 diabetics, 18 patients with non-immunological non-endocrine disorders and 26 normal controls were

included in the study. 15 type 1 diabetics were studied at diagnosis, 10 within one month and 8 within 6 months of diagnosis.

The mean age of diabetic patients studied was  $20.4 \pm 8.3$  years. The 18 patients with non-immune and non-endocrine disorders suffered from heart or gastrointestinal disorders and acted only as controls.

#### Circulating islet cell antigens

Circulating islet cell antigens, as detected by the monoclonal antibodies A2B5, HISL5, HISL19 and 3G5, were investigated by a fluid-phase technique as described in chapter A3.6.

#### Islet cell antibodies

Islet cell antibodies were detected with an indirect immunofluorescence test (Chapter A3.5).

#### Immune complexes

Circulating immune complexes were detected with two methods differing in principle, the solid phase Clq binding test (Hay et al, 1976 with minor modifications) and the conglutinin radioimmune assay (Casali et al 1977, with minor modifications) as described in details in chapter A3.1.

#### Statistical analysis

Student's unpaired t test was used to evaluate the results.

Bl.5c      R e s u l t s

## HISL 19

The mean value of circulating islet cell antigens, as detected by the monoclonal antibody HISL 19, in type 1 diabetics was  $18.5\% \pm 1.8$  whereas in control patients and in normal controls the values were  $12.1\% \pm 1.3$  and  $13.7\% \pm 1.6$  respectively (Figure Bl.5/1). The difference in circulating islet cell antigens between type 1 diabetics and control patients or normal controls was significant ( $p < 0.001$  and  $p < 0.001$ ).

## HISL 5

The mean value of circulating islet cell antigens, as detected by the monoclonal antibody HISL5, in type 1 diabetics were  $19\% \pm 2.5$  whereas in normal controls was  $13.2\% \pm 1.4$  ( $p < 0.001$ ) (Figure Bl.5/2).

## 3G5

The mean value of circulating antigens, as detected by the monoclonal antibody 3G5, was  $17.2\% \pm 2.8$  in type 1 diabetics,  $14.9\% \pm 2.4$  in control patients and  $18.1\% \pm 3.4$  in normal controls. The differences were not significant (Figure Bl.5/3).

## A2B5

The mean value of circulating antigens, as detected by the monoclonal antibody 3G5, was  $22.9 \pm 2.9$  in type 1 diabetics,  $17.05 \pm 1.5$  in control patients and  $17.6 \pm 1.2$  in normal controls. The presence of A2B5 circulating antigens was significantly increased in

type 1 diabetics in comparison with control patients and normal controls ( $p < 0.001$  and  $p < 0.001$ ) (Figure B1.5/4).

Circulating islet antigens  
and islet cell antibodies

There was no significant difference in the values of circulating antigens between islet cell antibody positive and negative subjects (Table B1.5/1).

Circulating islet antigens  
and immune complexes

There was no significant difference in the values of circulating antigens between immune complex positive and negative subjects, both when the Clq method and the conglutinin method were used (Table B1.5/1).

TABLE B1.5/1

Circulating islet antigens, islet cell antibodies and immune complexes

|          | HISL 19 |      | HISL 5 |     | 3G5  |     | A2B5 |     |
|----------|---------|------|--------|-----|------|-----|------|-----|
|          | mean    | SD   | mean   | SD  | mean | SD  | mean | SD  |
| ICA      |         |      |        |     |      |     |      |     |
| positive | 18.7    | 0.53 | 19.2   | 3   | 17.6 | 2   | 23.6 | 1.7 |
| negative | 18.5    | 1.9  | 19     | 2.4 | 17.7 | 2.8 | 24.7 | 3.2 |
| Clq-AgAb |         |      |        |     |      |     |      |     |
| positive | 19.9    | 2.2  | 20.3   | 2.3 | 16.1 | 2   | 26.5 | 3.2 |
| negative | 17.2    | 2.3  | 18.1   | 2.1 | 18.5 | 2.1 | 23.8 | 2.9 |
| Kg-AgAb  |         |      |        |     |      |     |      |     |
| positive | 18.9    | 2.3  | 19.9   | 1.8 | 17.4 | 2.3 |      |     |
| negative | 18.1    | 1.4  | 19     | 1.7 | 17.9 | 2.1 |      |     |





FIGURE B1.5/2

Circulating islet cell antigens, as detected by the monoclonal antibody HISL 5, in type 1 diabetics and normal controls and in patients with non-immunological and non-endocrine disorders.

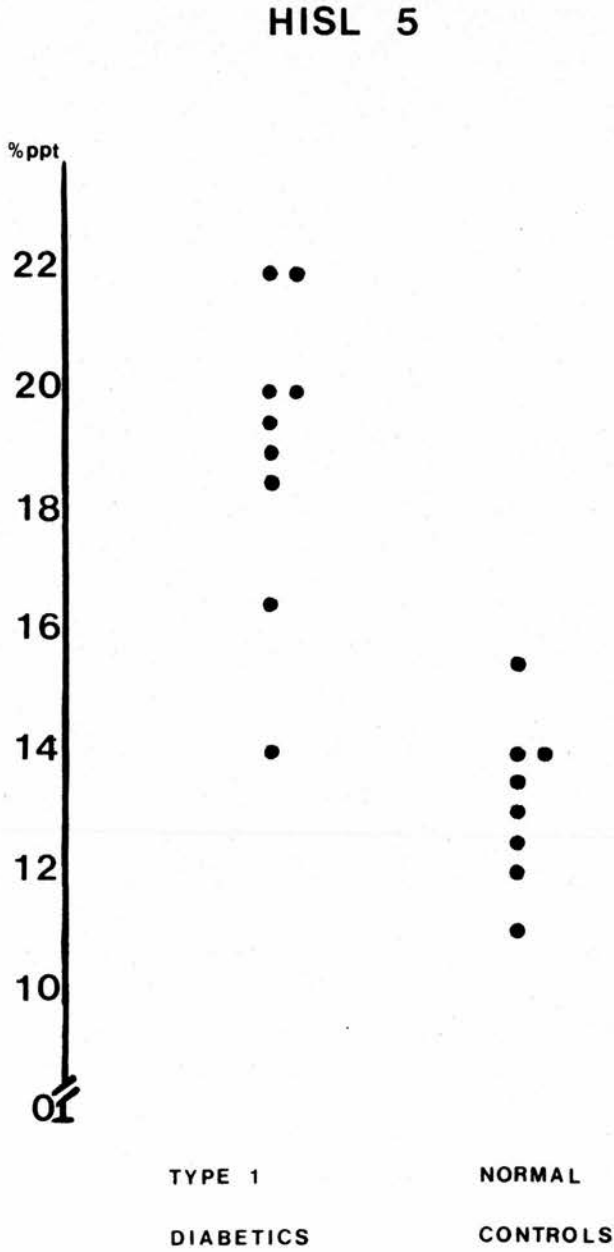


FIGURE B1.5/3

Circulating islet cell antigens, as detected by the monoclonal antibody 3G5, in type 1 diabetics, in normal controls and in patients with non-immunological and non-endocrine disorders.

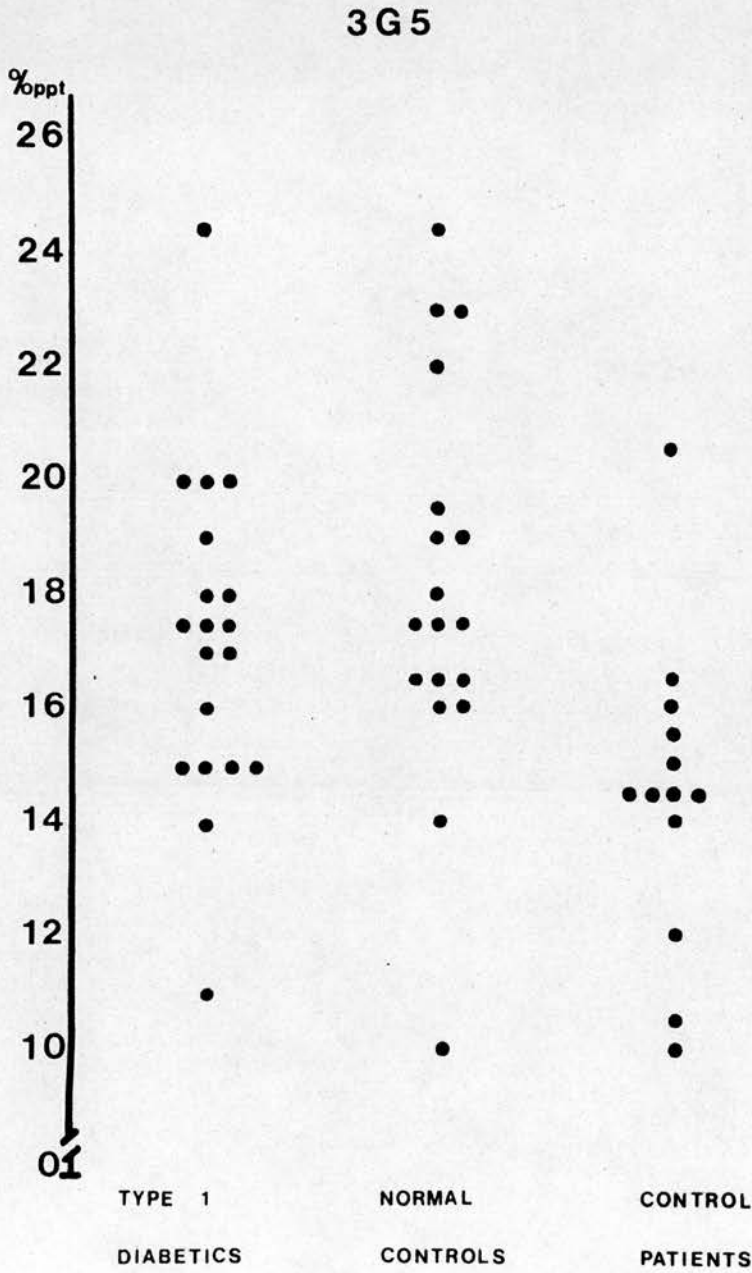
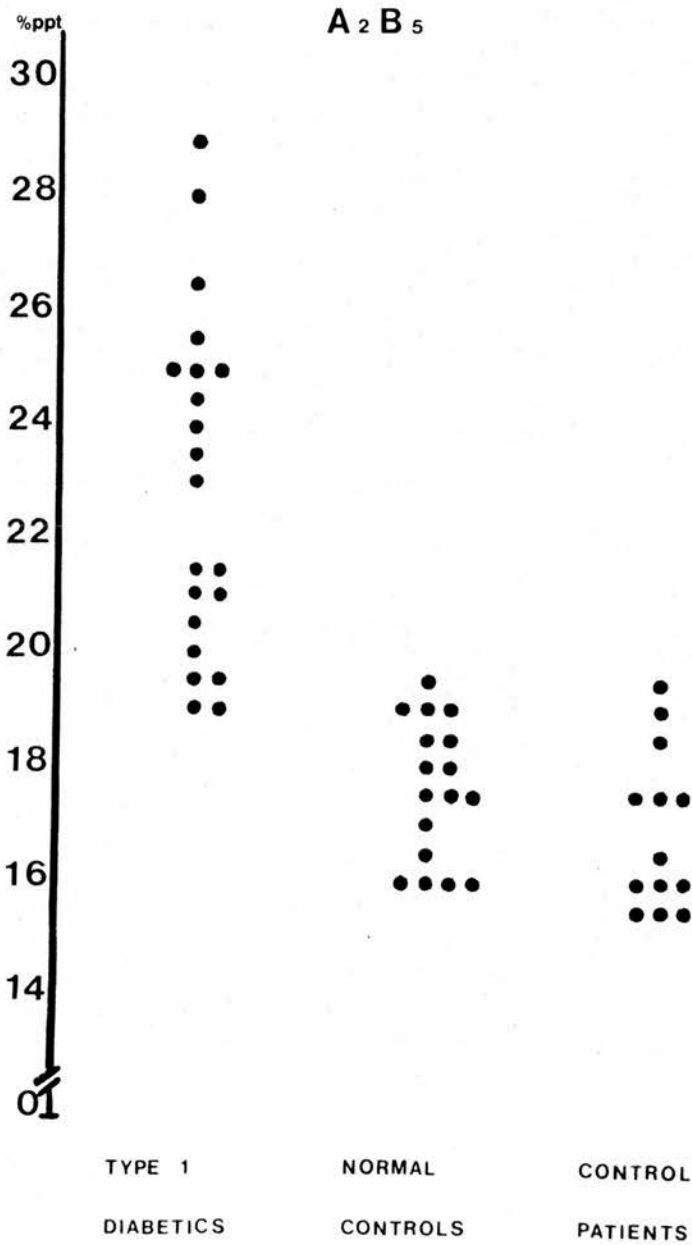


FIGURE B1.5/4

Circulating islet cell antigens, as detected by the monoclonal antibody A2B5, in type 1 diabetics, in normal controls and in patients with non-immunological and non-endocrine disorders.



B1.5d      D i s c u s s i o n

During the last decade a great deal of evidence has accumulated to support the role of immunological phenomena in the pathogenesis of insulin dependent (type I) diabetes mellitus (Chapter A1.3). Different types of islet cell antibodies have been found in the circulation of newly diagnosed type I diabetics (Bottazzo et al,1974; MacCuish et al,1974; Lendrum et al,1975). The author has been particularly interested in circulating immune complexes since the original report in 1977 of their increased presence in some insulin dependent diabetics at diagnosis (Chapter B1.2a). The presently available methods for the assay of immune complexes reveal different types of complexes and the concordance between these techniques is low. The above mentioned methods are antigen non specific and consequently do not differentiate between complexes related to specific disease processes and those found in commoner conditions, such as viral infections. The presence of Clq immune complexes was found to correlate with the occurrence of islet cell antibodies at diagnosis in type I diabetics, at least during the first year of the disease, in type I ICA positive diabetics but not requiring insulin at least in the first year of diagnosis (Chapters B1.2c, B1.3, B1.4). This suggests that both islet cell antigens and their related antibodies are in circulation in type I diabetes in the early stages following diagnosis.

The pathologic hallmark of type I (insulin-dependent) diabetes is the selective destruction of the pancreatic islet beta cells. In the early stages of the disease the islets may be infiltrated by

mononuclear cells (Gepts & LeCompte,1981). This phenomenon,"insulinitis", has been found in a substantial proportion of newly diagnosed cases and may be compatible with a recent viral infection or with a cell mediated immune response causing beta cell destruction. It is likely that islet cell antigens are released into the circulation as a consequence of islet cell damage.

Prospective population studies have suggested that the initiation of the pathological processes may precede the abrupt clinical onset of diabetes by several years (Gorsuch et al,1982). Anti islet cell antibodies are found several years before the diagnosis. It is also likely that antigens from the damaged tissues are slowly released in circulation during this long prediabetic period. This has important implications both for research and for possible future prophylaxis. However there is no satisfactory investigation, so far, on islet cell antigens, or on other antigens, possibly released in circulation before and during the clinical onset of diabetes. Thus this study was carried out with the attempt of filling this gap with the development of methods to identify circulating antigens by the use of monoclonal antibodies and the study of the role of these humoral immune factors in the pathogenesis of type I diabetes. One of the most important points in this kind of study is the availability of a good panel of monoclonal antibodies. Only very few antibodies, raised against antigens specific to the islets, have been reported (Chapter A3.6). In this study two antibodies raised following immunization of mice with human islets and two monoclonal antibodies raised against other tissues but reactive with pancreatic islet cell determinants have been used.

In this study an increase in the circulation of those antigens detected by the monoclonal antibodies HISL5, HISL19 and A2B5, is reported in the sera of recently diagnosed type 1 diabetics. This finding seems to confirm that islet cell antigens do circulate after islet damage.

Some aspects remain to be elucidated.

The lack of correlation between islet cell antibodies and circulating antigens, or immune complexes and circulating antigens may be due to the relatively small number of patients studied. However other immune factors also present in the early stages of the disease, do not show a clear statistical correlation. For example there is no clear correlation between islet cell antibodies and abnormalities of k cells function or islet cell surface antibodies and complement fixing islet cell antibodies.

The fact that the monoclonal antibody A2B5 was raised against gangliosides and thus recognizes antigens in the neuroendocrine system and not only in the islet, does not permit the conclusion that the A2B5 antigen found in the circulation is of islet cell origin. However, the increase in circulating antigens detected by this antibody parallels that found with the two islet specific monoclonal antibodies. Further more no increase in circulating A2B5 antigens was found in other patients without immune or endocrine disorders.

The differences in circulating antigens, as detected by these monoclonal antibodies, between diabetics and controls are clear but not very large. In fact the islet cell antigens in circulation are expected to be in rather small amounts. Future research must concentrate on the development of highly sensitive techniques to reveal very small quantities of pancreatic antigen released in circulation.

The availability of sensitive techniques to study and monitor the release and presence in circulation of islet antigens may be of paramount importance to describe and follow with time the islet cell damage in the early events of diabetes, to screen the normal subjects at risk to develop diabetes, monitor the effectiveness of possible pathogenetic antidiabetic treatment and to understand more clearly the significance of several pathogenetic events in type I diabetes.

## B1.6 CONCLUSIONS

The results of humoral immune studies in the first stages of type 1 diabetes suggest a number of conclusions.

Islet cell antibodies are present in about half of the newly diagnosed diabetics at diagnosis and decline thereafter. In about 20% of the patients these antibodies in the circulation can be found for at least a few years following diagnosis.

The occurrence of complement fixing islet cell antibody, which is found in about one third of the patients studied at diagnosis, declines thereafter. All the patients who showed complement fixing islet cell antibodies in their serum at diagnosis also had circulating islet cell antibodies.

Immune complexes, as detected by the Clq methods are increased at diagnosis in type 1 diabetics. The decline in their occurrence parallels that of islet cell antibodies but not of the other factors studied. Other types of complexes, such as those detected by conglutinin binding assay or Raji cells assay, are also increased at diagnosis.

Viral antibodies do not seem to be increased in newly diagnosed type 1 diabetics when compared with matched normal controls.

Thus the pattern of the decline of the presence of islet cell antibodies, complement fixing islet cell antibodies and Clq-immune complexes seems to reflect the immunological events related to islet cell damage.



The second aim of the studies in this section was to evaluate the possible presence and the clinical prognostic value of different immunological markers in diabetic patients not requiring insulin at diagnosis. Attention has been focussed on that minority of patients who are initially treated with diet or oral agents but show islet cell antibodies in their serum.

In most of the non insulin dependent diabetics studied, none of the immunological factors evaluated were found to be increased. However, immune complexes and complement fixing islet cell antibodies were found to be increased in most of those rare islet cell antibody-positive, non-insulin dependent diabetics.

When islet cell antibodies and complement fixing islet cell antibodies are both found in the circulation of diabetic patients not requiring insulin at diagnosis, this is valuable prognostically as an index of early insulin dependency.

In conclusion the type of diabetes in islet cell antibody positive patients not requiring insulin at diagnosis has strong immunological and clinical similarities to classical type 1 (insulin dependent) diabetes.

The search for circulating pancreatic islet in newly diagnosed type 1 diabetics lead to the detection in serum of some antigens present also in the pancreatic islet. The potential value of this finding is high. An islet cell antigen in the circulation may be a good marker to monitor the pathogenetic events in diabetics and, more interestingly, in subjects at risk to become diabetics.

The production of specific anti islet cell monoclonal antibodies and the development of highly sensitive techniques to study and

monitor the release and presence in circulation of islet antigens may be of paramount importance:

- to describe and follow with time the islet cell damage in the early events of diabetes.
- to screen the normal subjects at risk of developing diabetes
- to monitor the effectiveness of possible pathogenetic anti-diabetic treatment
- to better understand the significance of several pathogenetic events in type I diabetes correlating the antigens studied with other immunological and genetic parameters.

C H A P T E R    B 2

IMMUNOLOGICAL CONSEQUENCES OF INSULIN TREATMENT

Chapter B2      IMMUNOLOGICAL CONSEQUENCES OF INSULIN TREATMENT

- B2.1    Experimental design of the studies on the consequences of insulin treatment
- B2.2    Earlier studies by the author
  - B2.2a    Insulin antibodies and immune complexes in insulin-treated diabetics: a preliminary observation
  - B2.2b    Insulin antibodies in relation to HLA antigens in type 1 diabetes
- B2.3    Immune complexes in relation to the type of treatment, duration of diabetes, sex and age in diabetic patients
  - B2.3a    Aim of the study
  - B2.3b    Materials and methods
  - B2.3c    Results
  - B2.3d    Discussion
- B2.4    Insulin antibodies and immune complexes in randomly selected diabetics treated with conventional insulins
  - B2.4a    Aim of the study
  - B2.4b    Materials and methods
  - B2.4c    Results
  - B2.4d    Discussion
- B2.5    Insulin antibodies and immune complexes in newly diagnosed type 1 diabetics treated with conventional or mono-component insulins: a 1 year follow up
  - B2.5a    Aim of the study

- B2.5b Materials and methods
- B2.5c Results
- B2.5d Discussion
- B2.6 Insulin antibodies and immune complexes in type 1 and 2 diabetics treated for the first time with conventional, monocomponent and human insulins: a six month follow up
  - B2.6a Aim of the study
  - B2.6b Materials and methods
  - B2.6c Results
  - B2.6d Discussion
- B2.7 Conclusions

## B2.1      EXPERIMENTAL DESIGN OF THE STUDIES ON THE CONSEQUENCES OF INSULIN TREATMENT

It is well known that the administration of heterologous insulin induces an immune reaction in most patients. The best known immunological consequence is the formation of insulin antibodies. These antibodies are a consequence of impurities present in the preparations, of differences of the aminoacid composition and spatial configuration of heterologous insulin molecule and of physico-chemical changes of the molecule before or after injection.

It is also known that the formation of insulin antibodies is not the only immunological consequence of the insulin treatment. An immediate-type and a delayed-type hypersensitivity are likely to occur in some patients at the site of insulin injection. However other humoral immune reaction are also present. Insulin-anti-insulin complexes have been proved to be present in several insulin treated patients, and data on these complexes will be presented in chapters B3 and B4. It is mentioned elsewhere (chapter A2 and B2.2) that increased levels of circulating immune complexes are observed in some insulin treated diabetics. It remains to be shown that there is a correlation between this increase and the administration of insulin. (For details and references see chapter A1).

Attention has been focussed in this study on two aspects. The first is the relationship between insulin antibodies and immune

complexes. In this way the consequences of exogenous insulin on immune complex formation is indirectly investigated. The second is the relative influence of impurities, of species differences and of physico-chemical changes in the insulin preparations on the immunological response.

The main variables that have been taken into account are : levels of insulin antibodies, types of complexes, type of antidiabetic treatment, purity of insulin preparations, species of exogenous insulin, and the type of diabetes.

a) The types of immune complexes already found to be increased in some diabetics have been studied in a large group of patients selected at random to evaluate the influence of the antidiabetic treatment, insulin or oral agents, on their formations (B2.3). Other variables have also been considered.

b) The presence of the above studied types of immune complexes has been correlated to the levels of insulin antibodies in insulin treated diabetic patients selected at random (B2.4)

c) The types of complexes studied above and other types of complexes have been assayed and correlated to the presence of insulin antibodies in diabetics treated with insulins of different purity. To avoid the interference of other variables, such as the duration of diabetes, the type of diabetes and previous insulin treatment the study was done in newly diagnosed type 1, diabetics who were regularly followed up for one year after diagnosis (B2.5).

d) Different types of complexes and the levels of insulin antibodies have been studied in diabetics treated with human or heterologous insulins. The investigation was performed in both type 1 and 2 diabetics who had never before been treated with insulin and the study was done at the beginning of insulin treatment and for a 6 month follow up period.

The clinical part of the studies was done in collaboration with the Out-Patient Diabetic Department, Royal Infirmary, Edinburgh, UK and with the Diabetic Unit, University of Rome, Italy.

HLA studies were performed in the Nuffield Department of Surgery, University of Oxford, UK.



## B2.2 EARLIER STUDIES BY THE AUTHOR

B2.2a I n s u l i n a n t i b o d i e s a n d i m m u n e  
c o m p l e x e s i n i n s u l i n - t r e a t e d  
d i a b e t i c s : a p r e l i m i n a r y  
o b s e r v a t i o n

In the present study, the presence of antibodies to insulin and the occurrence of immune complexes have been investigated in the sera of treated diabetics and compared to those in controls. The significance of their possible correlation is discussed.

## M a t e r i a l s a n d m e t h o d s

63 diabetic patients have been included in the study. Sera were obtained from thirty-two diabetics who had been treated with bovine or porcine insulin for a mean duration of 16 years (range 2-42 years), from thirty-one diabetics who had been treated with oral hypoglycaemic agents (OHA) for a mean duration of 6 years (range 0-14 years), and from eighty-four control subjects. All sera were stored in 500 ul aliquots at  $-40^{\circ}\text{C}$ .

The Raji cell radioimmunoassay as described by Theofilopoulos et al, 1976, was used. Purified human IgG, was diluted to 10 mg/ml in PBS and then aggregated by heating at  $63^{\circ}\text{C}$  for 15 min. The amount of

soluble immune complexes in each test serum was expressed as being equivalent to so many ug aggregated human gamma globulin (AHG) per ml serum. For the limit of detection of the assay and other details see chapter A3.1.

Antibodies to insulin were estimated by a minor modification of the method of Ortved Andersen et al,1972, according to Mustaffa et al,1977. Insulin-binding of 10 uU/ml serum was regarded as indicative of the presence of insulin antibodies. Insulin-antibody levels between 10 and 300 uU/ml were regarded as indicating moderate insulin antibody titres, while binding of >300uU/ml was interpreted as being due to high titres of insulin antibodies (chapter A3.3).

All statistical analysis were done using Fisher's exact test, except where indicated.

## R e s u l t s

Immune complexes were found in seventeen out of thirty-two (53%) insulin-treated diabetics ( $P < 0.001$ ;  $\chi^2$  test), in three out of thirty-one (10%) patients requiring OHA (not significant); while in the eighty-four normal age- and sex-matched control subjects immune complexes were detected in five (6%) of them.

The sera of all the thirty-two diabetics treated with heterologous insulin had increased binding activity, indicating the presence of antibody to insulin. The titres of insulin antibodies varied in different patients from 26 to 586 uU/ml irrespective of the duration of insulin therapy (between 2 and 42 years), dosage of heterologous insulin used or sex. The sera of all the insulin-independent diabetics were negative for insulin antibodies. Immune complexes occurred more commonly in patients treated with insulin for 19 years or more

(sixteen out of twenty- three) than in those receiving insulin for less than 10 years (one out of nine) ( $P < 0.001$ ). There was no correlation between the presence or amounts of insulin antibodies, or of immune complexes, with the patients' insulin requirements. All patients with high titres of insulin antibodies ( $> 300$  uU/ml) had soluble immune complexes.

When the insulin-treated diabetics were subdivided according to whether they developed diabetes before or after the age of 30 years, immune complexes occurred more frequently in the presence of moderate titres of insulin antibodies in the early onset, compared to the late onset diabetics ( $P < 0.05$ ). The mean duration of diabetes and of insulin treatment in those diabetics with moderate insulin antibody titres who developed diabetes before the age of 30 and in those who developed diabetes after 30 were closely similar. When all the patients in each of these two group were compared, there were no significant differences in the prevalence of immune complexes.

#### D i s c u s s i o n

Insulin antibodies were only detected in the insulin-treated subjects, and their titres appeared to be a characteristic of the patient rather than the dosage or duration of insulin therapy. Indeed, it has been shown that patients who are HLA-B15 tend to have higher titres of insulin antibodies consequent upon insulin treatment (Bertrams et al, 1976). A correlation was observed in the present study between high titres of insulin antibodies and the presence of immune complexes, suggesting that in these patients the antigen involved in such complexes may be derived from heterologous insulin. The presence of low or moderate titres of insulin antibodies did not show any

correlation with the amount of complexes present. This may be due to technical reasons, to the varying affinities that insulin antibodies may have in different patients (Dixon, et al. 1975) or to the varying rates at which immune complexes of different sizes are removed from the circulation, or it may be that other antigens are involved in such complexes. The possibility exists that the late complications of diabetes may be related, at least in part, to the production of immune complexes, and it is therefore noteworthy that juvenile-onset insulin-dependent diabetics tended to have more immune complexes in relation to moderate or low titres of insulin antibodies than did subjects who developed insulin-dependent diabetes after the age of 30 years, even although the duration of diabetes and of insulin treatment was comparable in the two groups. Insulin-dependent diabetes developing in young people is considered to be associated with a greater risk for developing severe late diabetic complications over a given time than diabetes developing later in life (Bradley & Ramons, 1971).

B2.2b      I n s u l i n   a n t i b o d i e s   i n  
                 r e l a t i o n   t o   H L A   a n t i g e n s  
                 i n   t y p e   1   d i a b e t e s

Evidence is accumulating that the immune response against exogenous insulin is under genetic control in animals as well as in man(Keck,1975;Bertrams et al,1976).HLA antigens involved in the regulation of the immune response towards heterologous insulin are among those associated with type I diabetes(Bertrams et al,1976; Ludvigsson et al,1977).To analyse this more closely the relationship between insulin antibodies, ICA and the HLA types in insulin-treated type I diabetics has been investigated.

P a t i e n t s

Insulin binding capacity (InsAb) and islet cell antibodies (ICA) were studied in 191 diabetics divided into three groups. Because in most cases ICA persist for only a short period after diagnosis it was decided to study only patients near diagnosis and patients with ICA detectable at more than 3 years from diagnosis together with a suitable control group.

Group I: 59 insulin dependent diabetics (age at diagnosis 21.8 yr,SD 9.2), of whom there was a specimen of serum available for ICA detection sampled within a week of diagnosis, were studied for InsAb. 34 patients were found to be ICA positive (group Ia) and 25 ICA negative (group Ib). As InsAb titres show small variations with the duration of insulin treatment,44 patients after a mean of 15 months (SD 5.2) and 15 after a mean of 10.4 years (SD 2.3) from diagnosis

were tested for InsAb. None of these patients had any clinical evidence of other organ specific autoimmune disease.

Group II: Another 59 insulin treated diabetics (age at diagnosis 32.4 yr., SD 19.9), who were ICA positive at more than 3 years from diagnosis, were tested for InsAb. The 40 patients in group IIa (age at diagnosis 27.5 yr, SD 16.4; interval from diagnosis when InsAb was tested 13.6 yr, SD 8.6) did not show any clinical organ specific autoimmune disease; 19 patients in group IIb (age at diagnosis 41 yr, Sd 22.9; interval from diagnosis when InsAb was tested 19 yr, SD 9.7) had one of the following autoimmune diseases (AID): Addison's disease, thyrotoxicosis, Hashimoto thyroiditis, primary hypothyroidism, pernicious anaemia.

Group III: 73 insulin treated diabetics (age at diagnosis 29.9 yr, SD 15.2), in whom ICA was not detectable after 3 years of diagnosis were studied for InsAb. These patients were matched as closely as possible with patients of group II. The 51 patients in group IIIa (age at diagnosis 26.6 yr, SD 15.9; interval from diagnosis when InsAb was tested 14.2 years, SD 8.2), were without any AID; the 22 patients in group IIIb (age at diagnosis 32.4 yr, SD 13.7; interval from diagnosis when InsAb was tested 19.6 yr, SD 8.3) had one of the above mentioned AID.

All these patients were Caucasians and were attending the same clinic. None had been treated with highly purified insulin preparations at the time of the study. No patient was in a remission phase. 93 patients were recalled for HLA typing, 52 of group I and 41 of group II. 300 healthy Caucasians from the Oxford area served as HLA typing controls.

## M e t h o d s

Insulin binding capacity was estimated by a minor modification of the method of Ortved Andersen, Brunfeldt and Albigard (1972) according to Mustaffa, Dagget and Nabarro (1977) (chapter A3.3). Insulin binding of more than 10 uU/ml of serum was regarded as indicative of the presence of insulin antibodies. Insulin binding levels less than 100, between 100 and 300, and above 300 uU/ml were considered as indicating low, medium and high levels of insulin antibodies respectively.

Islet cell antibodies were detected by the indirect immunofluorescence test as previously described (chapter A3.5).

HLA typing was performed as described by Morris et al, 1976 for the following 40 antigens:

A series: 1,2,3,9,10,11,25,26,28,29 and W 23,24,30,31,32,33.

B series: 5,7,8,12,13,14,15,17,18,27,37,40 and W 16,21,22,35,  
38,39,41.

C series: W 1,2,3,4,5.

The chi-squared test ("C"), Wilcoxon's two sample test ("W") and Cox's test for trend in proportions ("T") (Cox, 1970) were used as indicated in the analysis of the results.

## R e s u l t s

In patients without other clinical autoimmune disease and tested for ICA at more than 3 years from diagnosis, InsAb titres were significantly lower ( $p < 0.05$ , "W") in the 40 patients who were ICA positive (group IIa) compared to those in the 51 patients who were ICA negative (group IIIa). No significant difference was found between InsAb titres in patients in group IIa compared to those in patients in group I or Ib.

There was no significant difference between the InsAb titres in 34 ICA positive patients in group Ia compared to the 25 ICA negative diabetics in group Ib. Again the InsAb titres were not significantly different in ICA positive or negative patients in group I either after 15 months from diagnosis (InsAb median in ICA positive patients 191 uU/ml, interquartile range or IR 92-294; in ICA negative 89 uU/ml, IR 60-214) or after 10 years from diagnosis (InsAb median in ICA positive 94 uU/ml, IR 19-19; in ICA negative patients 80 uU/ml, IR 31-130). There also no significant difference in InsAb titres in patients in group II or III between those without (IIa, IIIa) and with (IIb, IIIb) other clinical autoimmune disease.

The prevalence of HLA-A1 and B8 in the 93 diabetics HLA typed was significantly increased in comparison with the normal population ( $p < 0.005$  and  $p < 0.0001$  respectively, "C").

Diabetics who were B15 and/or CW3 positive showed significantly higher titres of InsAb. The median of InsAb titres for the B15 patients was 233 uU/ml (interquartile range or IR 133-370) and for CW3 patients was 169 uU/ml (IR 98-305). These values are significantly higher than those found in the total patients (107 uU/ml, IR 35-213) ( $p < 0.001$  for B15,  $p < 0.01$  for CW3, "W") (Fig. B2.2/1). The difference in InsAb levels in the B15 compared to the B8 patients was also statistically significant.

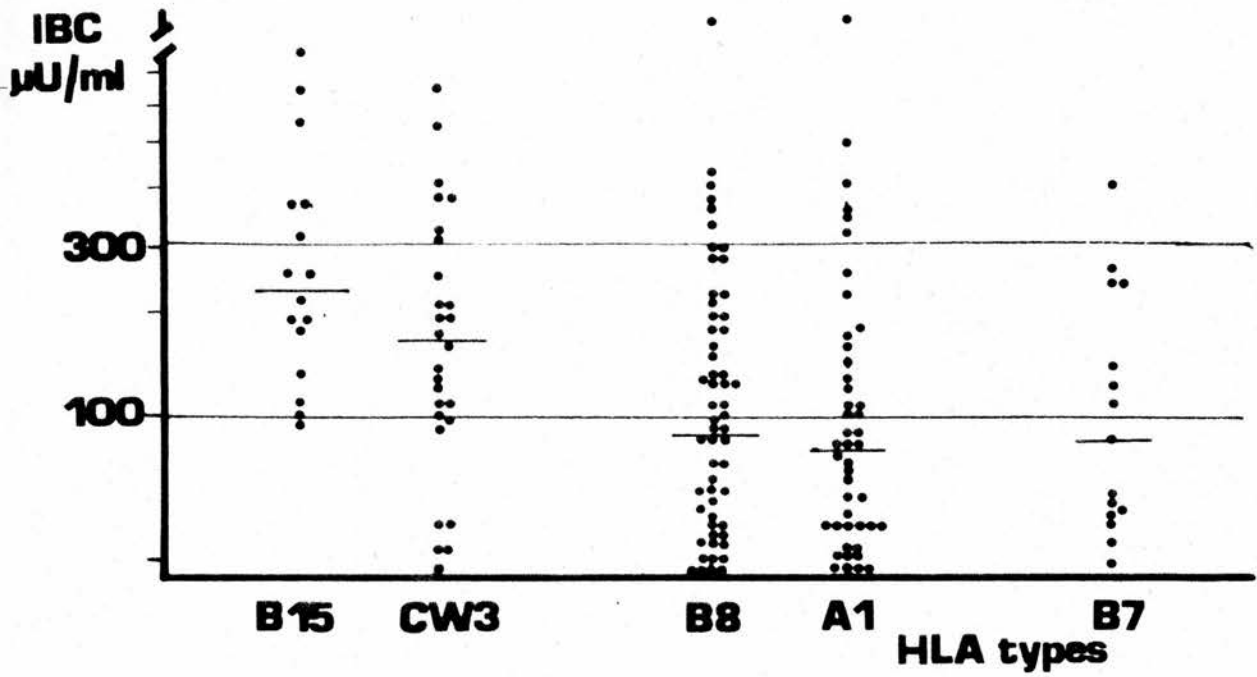
Dividing the patients into negative and low-, medium-, and high-responder groups according to the levels of insulin antibodies, there was a significant trend for B15 and/or CW3 positive patients to have InsAb levels in the medium- and high-responder range ( $p < 0.001$  and  $p < 0.005$ , "T") and B8 and/or positive diabetics to have levels in the negative and low-responder range ( $p < 0.05$  and  $p < 0.02$ , "T"). There was



FIGURE B2.2/1

Insulin antibodies levels and HLA antigens in 93 insulin treated type 1 diabetics.

### insulin antibodies in relation to HLA types



no significant correlation between the presence of B7 and InsAb titres.

#### D i s c u s s i o n

The findings in the present paper emphasize that there are at least two separate immune response (Ir) genes operative in type I diabetes that are associated with HLA. It has previously shown (chapter B1.2) that type I diabetics with what might be conveniently referred to as Ir1 (and which is in linkage disequilibrium with A1, B8, DW3 and DR3) tend to have persistent ICA or other clinical autoimmune disorders. It has now been shown that such patients tend to form low levels of insulin antibodies. However, type I diabetics with Ir2 (which is in linkage disequilibrium with A2, B15, CW3, DR4) tend to have high levels of insulin antibodies following treatment with conventional heterologous insulin. The phenomenon of linkage disequilibrium in relation to these two Ir genes is further illustrated in that patients who are CW3 have similar insulin antibody levels as those who are B15 and patients who are A1 have similar insulin antibody titres as those who are B8.

The observation that insulin treated diabetics with persistent ICA have lower insulin antibody levels than a matched group of ICA negative diabetics confirms the difference in the immune response to these endogenous and exogenous antigens. There is suggestive evidence that patients who are B15 may have higher titres of antibody to Cocksackie virus types B1-B4 (Cudworth et al, 1977).

It would thus appear that Ir1 is concerned with organ-specific autoimmunity, while Ir2 is concerned with the immune response to certain exogenous antigens. This would indicate that the association

of Irl with autoimmunity is not merely a manifestation of a heightened immune response in general to all antigens, be they autoimmune or otherwise.

Why B7, and presumably the HLA antigens A3, DW2 and DRW2 in linkage disequilibrium with it, should be "protective" against the development of type I diabetes is not understood. The observation that B7 is associated with cutaneous allergic reactions to heterologous insulin (Bertrams & Gruneklee, 1977) and also the humoral response to monomeric flagellin (Morris et al, 1977) suggests that it is in linkage disequilibrium with a third immune response gene Ir3. However, it must be borne in mind that the HLA antigens mentioned above may also be in linkage disequilibrium with other genes that are not related to immune response.

Hopefully DR antigens will show even stronger associations with the different type of immune response studied as illustrated by persistent islet cell autoimmunity and high levels of insulin antibodies in type I diabetics.

B2.3 IMMUNE COMPLEXES IN RELATION TO THE TYPE OF TREATMENT,  
DURATION OF DIABETES, SEX AND AGE IN DIABETIC PATIENTS

B2.3a Aim of the study

In this study the influence of sex, age, duration of disease and type of antidiabetic treatment (insulin or oral agents) on immune complex levels has been investigated.

B2.3b Materials and Methods

Patients

Two hundred and seventy-six diabetics attending the Diabetic Clinics of Rome and of Edinburgh were selected at random. One hundred and forty-seven were males and 129 were females. Twenty-five diabetics were less than 20 years old, 42 were in the third decade, 29 in the fourth, 32 in the fifth, 53 in the sixth and 95 were more than 60 years old. In 67 patients the duration of diabetes was between 1 and 5 yr, whereas in 61, 62, 42 and 44 patients the duration of diabetes was between 6 and 10, 11-15, 16-20 and over 20 yr, respectively. One

hundred and forty-eight were treated with insulin at the time of the study and 128 with diet or oral hypoglycaemic agents. Insulin treated patients received ordinary commercial insulins (Lente, NPH, crystalline). None of those was treated with purified insulins. One hundred and thirty blood donors were included in the study as normal controls.

#### Immune complexes

Circulating AgAb were assayed by the solid phase Clq binding test according to Hay et al(1976) with minor modifications (chapter A3.1) The limit of positivity was chosen as the 90th percentile of blood donor values(Lambert et al,1978)

#### Statistical analysis

The Chi square test was used for the statistical analysis of the results.

B2.3c      R e s u l t s

## Immune complexes

AgAb levels were above the limit of positivity in 65 out of the total 276 diabetics studied and in 13 out of the 130 blood donors. Therefore AgAb were significantly increased in the diabetics ( $p < 0.002$ ).

## AgAb and sex of patients

Among the diabetics, AgAb were present in 24.5% of males and in 22.5% of females.

#### AgAb and age of patients

When the diabetics were divided according to age, there was no significant difference in AgAb levels (Table B2.3/1).

#### AgAb and duration of diabetes

When the duration of diabetes was taken into account considering all the patients together, there was no significant correlation between AgAb and duration of disease (table B2.3/2).

#### AgAb and type of treatment

However, when patients were divided according to the type of treatment, insulin treated diabetics showed a significantly higher prevalence of AgAb compared to the non-insulin treated diabetics (30% vs 16%  $p < 0.005$ ). The difference in AgAb positivity between insulin treated diabetics and normal controls was highly significant ( $p < 0.0001$ ), whereas the difference between non-insulin treated diabetics and normals failed to reach statistical significance ( $x = 1.9$ ).

Within the insulin treated group there is a statistically significant peak in the prevalence of AgAb in diabetics of 11-20 years duration ( $p < 0.001$ ) (Table B2.3/2) (Fig. B2.3/1), but there was no correlation with age of patients.

TABLE B2.3/1

The influence of age on AgAb

|                           |  | Age of patients (years) |       |       |       |       |     |
|---------------------------|--|-------------------------|-------|-------|-------|-------|-----|
|                           |  | <20                     | 21-30 | 31-40 | 41-50 | 51-60 | >60 |
| (A)                       |  |                         |       |       |       |       |     |
| No. of patients           |  | 25                      | 42    | 29    | 32    | 53    | 95  |
| AgAb positive             |  | 8                       | 12    | 7     | 11    | 11    | 16  |
| (B)                       |  |                         |       |       |       |       |     |
| Insulin treated           |  | 25                      | 37    | 26    | 16    | 24    | 20  |
| AgAb positive             |  | 8                       | 12    | 6     | 7     | 5     | 7   |
| Non-insulin treated (no.) |  | 0                       | 5     | 3     | 16    | 29    | 75  |
| AgAB positive             |  | 0                       | 0     | 1     | 4     | 6     | 9   |



TABLE B2.3/2

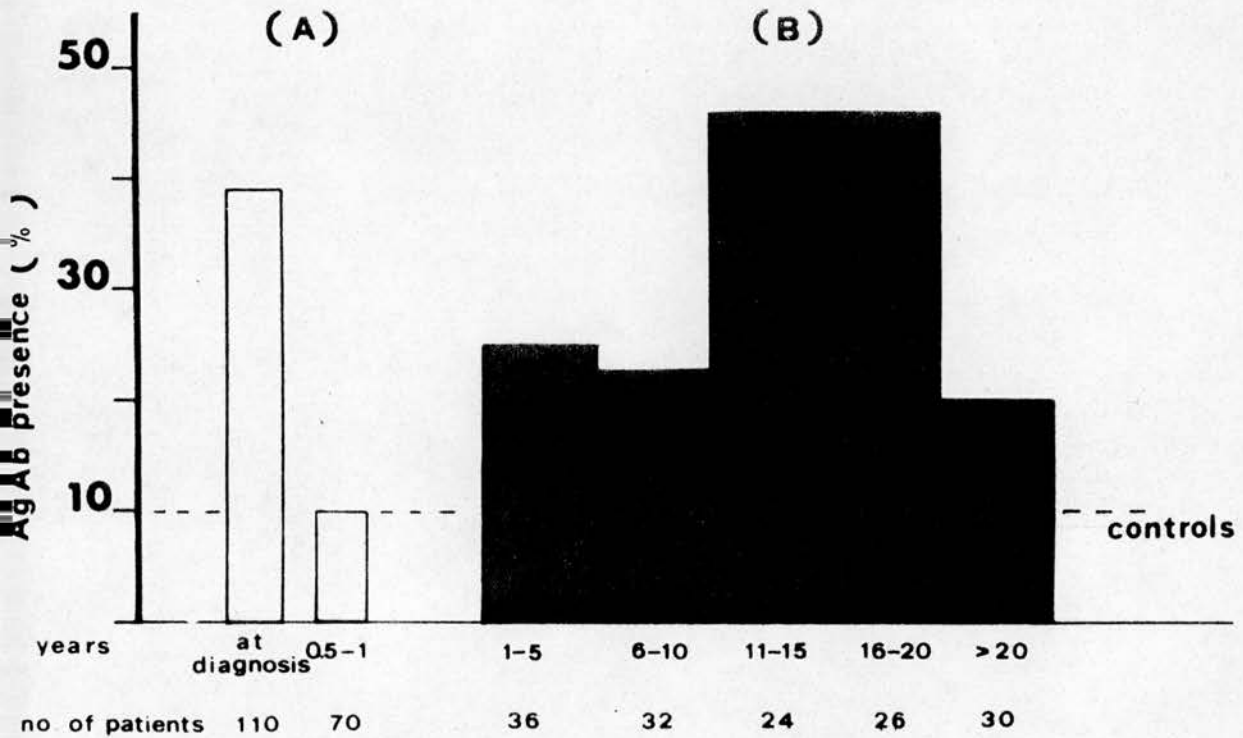
The influence of the duration of diabetes on AgAb

|     |                           | Duration of diabetes (years) |      |       |       |     |
|-----|---------------------------|------------------------------|------|-------|-------|-----|
|     |                           | 1-5                          | 6-10 | 11-15 | 16-20 | >20 |
| (A) |                           |                              |      |       |       |     |
|     | No. of patients           | 67                           | 61   | 62    | 42    | 44  |
|     | AgAb positive             | 15                           | 13   | 16    | 13    | 8   |
| (B) |                           |                              |      |       |       |     |
|     | Insulin treated (no.)     | 36                           | 32   | 24    | 26    | 30  |
|     | AgAb positive             | 9                            | 7    | 11*   | 12*   | 6   |
|     | Non-insulin treated (no.) | 31                           | 29   | 38    | 16    | 14  |
|     | AgAb positive             | 6                            | 6    | 5     | 1     | 2   |

\*p &lt; 0.001 vs the remainder.

FIGURE B2.3/1

The prevalence of AgAb in diabetes. (A) The percentage positivity for AgAb in recently diagnosed patients (see text). (B) The percentage positivity in diabetics according to the duration of clinical diabetes.



B2.3d      D i s c u s s i o n

In this study on a randomly selected population of diabetics, AgAb were found to be increased in insulin treated diabetics in comparison with non-insulin treated diabetics and normal controls. As the nature of AgAb has not been analyzed in the present study it is possible only surmise as to what the antigen components of these complexes might be. This correlation between insulin treatment and AgAb can be explained either as an intrinsic difference between type I and type II diabetes or as the effect of heterologous insulin administration. In some type I diabetics the increase in AgAb positivity may be explained as a consequence of a genetically determined increased immunoresponsiveness to exogenous agents present in some insulin dependent diabetics (Chapter B2.2b; Scherthaner et al, 1979). On the other hand the administration of exogenous insulin may induce the formation of insulin-anti-insulin complexes and several clinical and experimental studies have already shown the presence of these complexes in insulin treated subjects (Folling, 1976; Jayarao et al, 1974 chapter B3.4).

It is however by no means certain that insulin-anti-insulin complexes are detected by the Clq technique because of the immunological characteristics of insulin. Considering the bivalency of insulin, most insulin-anti-insulin complexes tend to be of very small size and so are not detectable by the techniques which use complement to reveal circulating AgAb. Only a small proportion of insulin-anti-insulin complexes form polymers and aggregates of detectable size. Nevertheless, in a randomly selected diabetic population it is possible

that some of the complexes found may be a consequence of an increased formation of such complexes induced by exogenous insulin.

This seems to be true especially when insulin-anti-insulin complexes are formed in antigen excess, whereas in the presence of antibody excess these complexes are not detected by the method used. Indeed an inverse correlation has been found between increasing levels of insulin antibodies and higher titres of AgAb in randomly selected diabetics (chapter B2.4).

Within the insulin treated diabetics there is a pronounced peak in the prevalence of AgAb in diabetics of 11-20yr duration. Thus in insulin treated diabetics it is possible so far to describe two peaks of AgAb positivity according to the duration of diabetes. The first peak in AgAb levels is found within a few days of diagnosis with a sharp decline in positivity thereafter (Fig.B2.3/1), as already described in chapter B1.3. Since the techniques used to detect AgAb are antigen non-specific it is likely that the two peaks in AgAb positivity are due to different types of AgAb. AgAb in the first peak seem to be part of the immunopathological phenomena occurring at diagnosis and are statistically correlated with the presence of islet cell antibodies (ICA). In the second peak, AgAb may be induced by other factors even though in that minority of long standing insulin treated diabetics with persistently positive ICA, AgAb were reported to be increased (chapter B1.2d). While the highest titres of insulin antibodies tend to occur at 9-12 months of insulin treatment, the highest amounts of AgAb in the second peak (as detected by ClqSP) occur at 11-20 yr. It is therefore likely that antigens other than insulin are involved in the second peak of AgAb.

This conclusion is also supported by the fact that, when severe microangiopathy is present, AgAb are increased in insulin as well as in non-insulin treated diabetics. This suggests that insulin-anti-insulin AgAb may not be the main component of these complexes, but that there may be a decreased clearance of miscellaneous types of complexes resulting from an impaired phagocytic system (chapters B3.2b B3.2c, B3.2d)

The decline in AgAb positivity in diabetics with more than 20 yr of diabetes may be explained by the presence of milder complications in this group of patients or by the presence of some insulin treated type II diabetics. This study shows that the age of patients cannot explain this decline.

These results, together with findings reported in other chapters, suggest that a heterogeneous population of immune complexes is present in diabetics. A careful selection of patients and the identification of antigens involved in complexes are important to understand their significance in diabetes.

B2.4        INSULIN ANTIBODIES AND IMMUNE COMPLEXES IN RANDOMLY SELECTED  
              DIABETICS TREATED WITH CONVENTIONAL INSULINS

B2.4a        A i m   o f   t h e   s t u d y

This study was planned to investigate the correlation between the presence of insulin antibodies and the occurrence of immune complexes in randomly selected insulin treated diabetics. To detect circulating immune complexes, a method detecting medium sized complexes in antigen excess through the Fc portion of complexed immunoglobulins was used.

B2.4b        M a t e r i a l s   a n d   M e t h o d s

Patients

Ninety-seven randomly selected consecutive patients treated with insulin for more than 1 year were included in the study 51 were males and 46 females. Their mean age was  $33.8 \pm 17.4$ . One hundred and eighty-nine blood donors were used as the control population in immune complex studies.

### Immune complexes

ClqSP as described by Hay, Nineham and Roitt (1976) with some personal modifications (chapter A3.1) was used to detect immune complexes. The characteristics of the methods, the limit of positivity, the expression and analysis of the results for AgAb the limit of positivity of IC have been discussed in chapter A3.1.

### Insulin antibodies

Andersen's radioimmune assay (Orteved Andersen, Brunfeldt and Albigard, 1972) as modified by Mustaffa, Dagget and Nabarro (1978) was used to detect insulin antibodies. The results were expressed in uU/ml of insulin binding capacity (chapter A3.3).

### Statistical analysis

Fisher's exact test, the  $\chi^2$  test, Cox's test, the t-unpaired test and Kendall's test were used for the statistical evaluation of the results.

B2.4c      R e s u l t s

## Immune complexes

In randomly selected diabetics, immune complexes were found in 26 out of 97 patients (27%,  $P < 0.01$  vs normal population).

## Insulin antibodies

In the same patients, InsAb, as evaluated by Andersen's method, showed a mean value of  $116.4 \pm 113.2$  uU/ml.

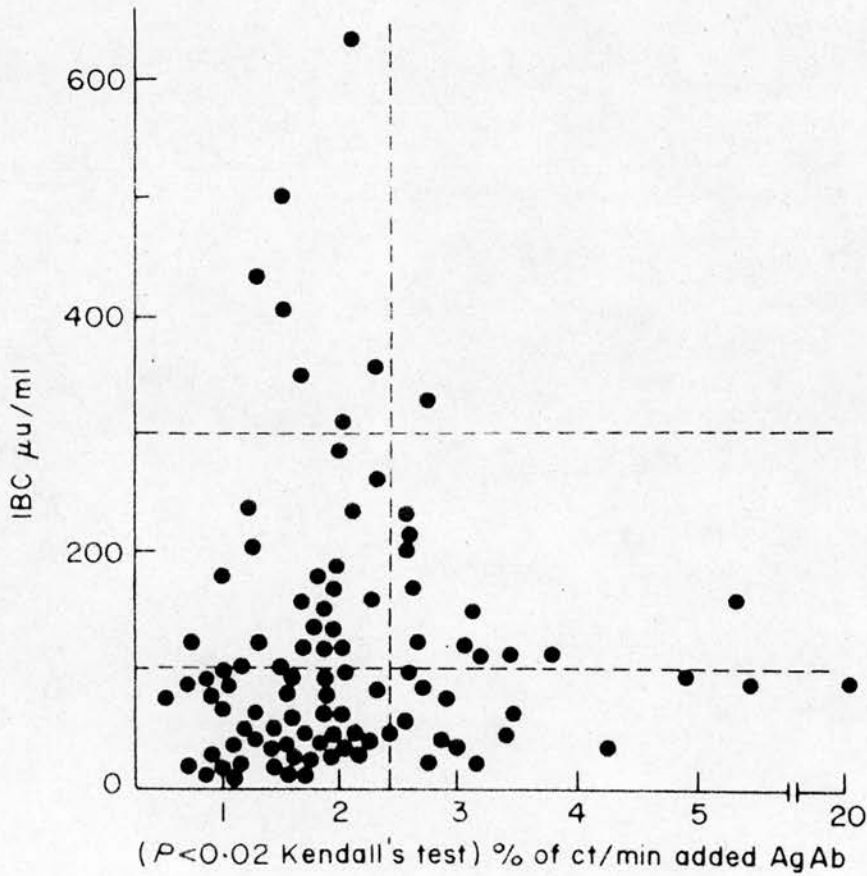
## Immune complexes vs insulin antibodies

When InsAb were correlated with Clq-IC values, an inverse correlation was found between InsAb levels and Clq-IC values ( $P < 0.02$ ) and patients with high levels of InsAb tended to be Clq-IC negative (Fig.B2.5/1).



FIGURE B2.4/1

Immune complexes (AgAb), as detected by Clq-SP, and insulin antibodies (IBC), as assayed by Andersen's method, in insulin treated diabetics. The horizontal dotted lines divide insulin antibody levels into negative low, medium and high levels. The vertical dotted line divided immune complex values into negative and positive.



B2.4d      D i s c u s s i o n

In insulin treated diabetics selected at random, when InsAb were present in high amounts, immune complexes were not usually detectable. Since ClqSP does not adequately detect complexes in antibody excess (Scullion, Balint and Whaley, 1979), in patients with high levels of InsAb, insulin antibody complexes would not be expected to be detected. It is conceivable that in patients with lower InsAb levels some immune complexes may be related to insulin treatment. In fact, in the study described in chapter B2.3, Clq-immune complexes were found to be higher in randomly selected insulin treated patients than in comparable diabetics not treated with insulin.

B2.5        INSULIN ANTIBODIES AND IMMUNE COMPLEXES IN NEWLY DIAGNOSED  
TYPE 1 DIABETICS TREATED WITH CONVENTIONAL OR MONOCOMPONENT  
INSULINS : A ONE YEAR FOLLOW-UP

B2.5a        A i m   o f   t h e   s t u d y

The aim of the present study was to attempt to correlate circulating immune complexes (detected by Clq and conglutinin) and the presence of insulin antibodies in type I diabetics during the first year of disease bearing in mind the type and duration of the diabetes and antigenicity of the insulin used in the treatment. The presence of islet cell antibodies was taken into account.

B2.5b        M a t e r i a l s   a n d   M e t h o d s

Patients

Forty-one newly diagnosed Type 1 diabetic patients, with age at onset <30 years, were studied before insulin treatment was started. Twenty of these patients (12 males and eight females, mean age 9 years, range 5-11 years) were treated with conventional insulins (group 1), whereas 21 (15 males and 6 females, mean age 13 years,

range 822 years) were treated with monocomponent porcine insulins (group 2). Patients were randomly assigned to each group. Blood samples were collected at diagnosis and 1,3,6 and 12 months later in all patients.

A series of 189 blood donors was also studied to determine the normal range of the immune complex assays.

#### Insulin antibodies

Anti-insulin antibodies, evaluated as insulin binding capacity, were measured by an immunoelectrophoretic method (Christiansen 1972); the sensitivity of the method is 0.05 mU/ml, the interassay variation 3%. (chapter A3.3).

#### Immune complexes

Immune complexes were determined by the Clq solid phase method according to Hay et al. (Casali et al.). The characteristics of the methods are described elsewhere (chapter A3.1).

#### Islet cell antibodies

Islet cell antibodies (ICA) were assayed by indirect immunofluorescence on cryostat sections of blood group 0 human pancreas. The presence of islet cell antibodies was evaluated by two independent observers using a Leitz Dialux microscope (Chapter A3.5).

#### Statistical analysis

The  $X^2$  test, Fisher's exact test, Cox's test, Student's t-test for independent variables and the binomial test were used for statistical evaluation of the results.

B2.5c      R e s u l t s

## Insulin antibodies

Anti-insulin antibodies were detectable after the first month of treatment, being significantly higher in group 1 than in group 2 at each stage of follow-up (table B2.5/1).

## Immune complexes

Clq immune complexes were present in 19 of the 41 diabetic patients at diagnosis (46%) and progressively decreased thereafter (table B2.5/2). Prevalence at 12 months' follow-up was 12%. This decrease was statistically significant ( $p < 0.01$ ). No difference was found between groups 1 and 2. Conglutinin immune complexes were present in 12 of the 41 diabetic patients at diagnosis (29%), prevalence varying during follow-up from a minimum of 26% to a maximum of 38% (NS). Although at 12 months the prevalence was 40% in group 1 and 20% in group 2, the differences between the two groups were not significant.

## Islet cell antibodies

Islet cell antibodies were detected in 19 of the 41 diabetic patients at diagnosis (46%). During the follow-up period the prevalence of ICA decreased progressively to 10% after 12 months. This decrease was statistically significant ( $p < 0.001$ ). No difference in occurrence of islet cell antibody was found between the two groups of patients.

## ICA vs AgAb

Islet cell antibodies and Clq immune complexes showed similar decreases in frequency from diagnosis to 12 months in all patients together and in the two groups examined separately. Clq immune complexes were detected in 58% of the 19 islet-cell-antibody-positive subjects at diagnosis. The agreement between the two tests (both positive and negative) was significant (1 month  $p < 0.05$ , 6 months  $p < 0.001$ , 12 months  $p < 0.001$ ).

Islet cell antibodies and conglutinin immune complexes showed different profiles during follow-up (Fig.B2.5/1). Analysis of the relationship between these two immunological factors demonstrated a difference in prevalence at diagnosis (46% versus 29%), and only four out of the 19 islet-cell-antibody positive subjects also showed conglutinin immune complexes. No significant agreement was found between the two tests.

## AgAb vs Insulin antibodies

No significant correlation was found between Clq immune complexes and anti-insulin antibodies, either in the 41 diabetic patients or in groups 1 and 2 considered separately. The presence of conglutinin immune complexes, on the other hand, showed a tendency to parallel the increase in anti-insulin antibody levels in the 41 diabetic patients ( $p < 0.001$ ). This trend was still present when the two groups were examined separately ( $p < 0.001$ ). However, whereas in group 1 the maximum prevalence of conglutinin immune complexes (85%) was reached when anti-insulin antibody levels were above 2 mU/ml ( $p < 0.002$ ), the maximum (75%) in group 2 was reached at lower anti-insulin antibody levels (0.5-2mU/ml) ( $p < 0.002$ ).

TABLE B2.5/1

Anti-insulin antibodies in Type 1 diabetic patients  
at diagnosis and during 1 year's treatment

|         |                 | Duration of treatment (months) |                 |                 |    |
|---------|-----------------|--------------------------------|-----------------|-----------------|----|
|         |                 | 1                              | 3               | 6               | 12 |
| Group 1 | $0.8 \pm 0.8^a$ | $1.7 \pm 1.5^b$                | $1.8 \pm 1.9^b$ | $1.9 \pm 1.3^b$ |    |
| Group 2 | $0.08 \pm 0.11$ | $0.3 \pm 0.4$                  | $0.3 \pm 0.5$   | $0.2 \pm 0.3$   |    |

Results are presented as mean  $\pm$  SD;a  $p < 0.02$ ;b  $p < 0.001$ .

TABLE B2.5/2 Immune complexes assayed with Clq solid phase method and with conglutinin binding test in Type 1 diabetic patients at diagnosis and during 1 year's treatment

|                                 |       | Duration of treatment (months) |       |       |       |    |
|---------------------------------|-------|--------------------------------|-------|-------|-------|----|
|                                 |       | 0                              | 1     | 3     | 6     | 12 |
| <b>Clq solid phase method</b>   |       |                                |       |       |       |    |
| Group 1                         | 9/20  | 5/17                           | 4/16  | 2/18  | 3/20  |    |
| Group 2                         | 10/21 | 6/19                           | 3/15  | 3/19  | 2/20  |    |
| Total                           | 19/41 | 11/36                          | 7/31  | 5/37  | 5/40  |    |
| <b>Conglutinin binding test</b> |       |                                |       |       |       |    |
| Group 1                         | 6/20  | 6/17                           | 6/17  | 7/19  | 8/20  |    |
| Group 2                         | 6/21  | 4/18                           | 5/18  | 7/18  | 4/20  |    |
| Total                           | 12/41 | 10/35                          | 11/35 | 14/37 | 12/40 |    |

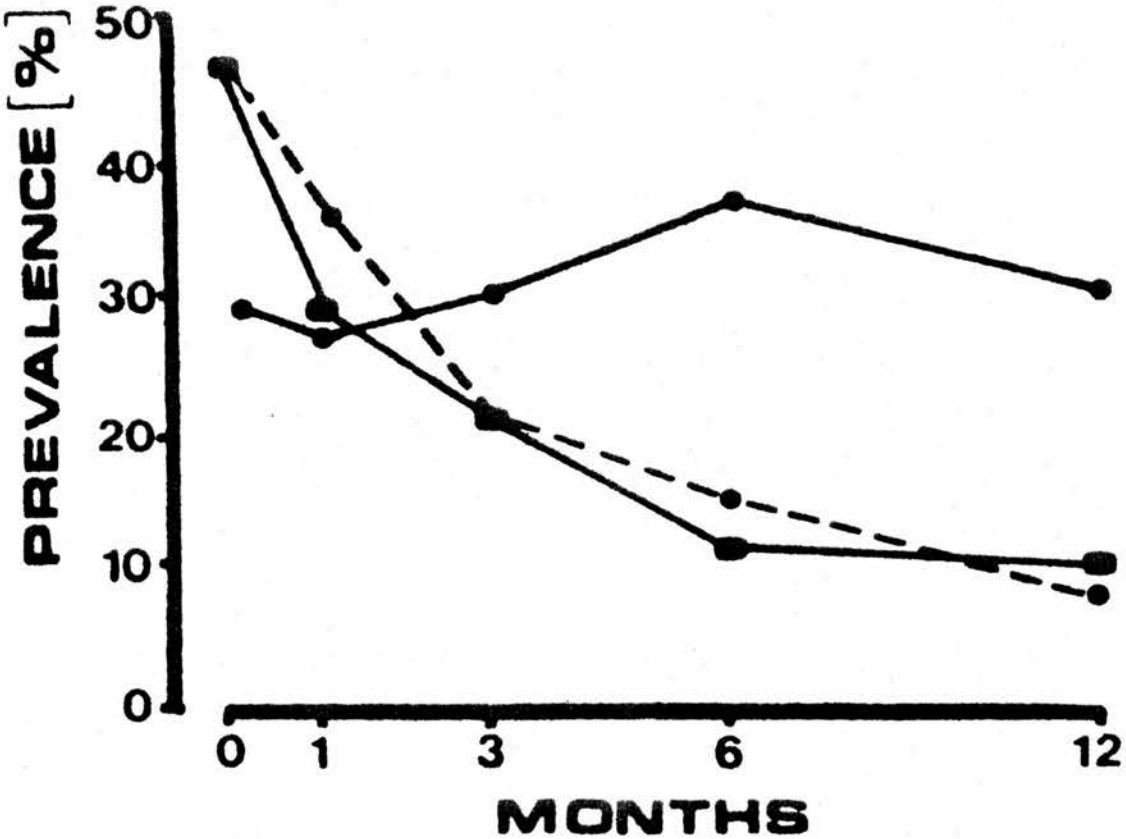
Results are presented as positive/total subjects

p<0.01



FIGURE B2.5/1

Prevalence of immune complexes assayed with Clq solid phase method (●-----●) and with conglutinin binding test (●——●) and of islet cell antibodies (■——■) in 41 Type 1 diabetic patients at diagnosis and during 1 year follow-up.



B2.5d      D i s c u s s i o n

These results demonstrate a correlation between Clq immune complexes and islet cell antibodies, and between conglutinin immune complexes and anti-insulin antibodies, whereas no correlation was found between Clq immune complexes and anti-insulin antibodies, or between conglutinin immune complexes and islet cell antibodies. Indeed, Clq and conglutinin detect different populations of immune complexes (Chapter A3.1)

It is possible that islet cell antibodies and Clq immune complexes are present at the time of diagnosis independently of each other and are different expressions of the immune response to the same aetiological agent. On the other hand, the correlation between Clq immune complexes and islet cell antibodies observed in the present study suggests that the islet cell antibodies might circulate in the form of soluble complexes bound to antigens of islet origin. In this case immune complexes detected by Clq assay at diagnosis may be a reflection of such a phenomenon.

Of interest in the present study is the relationship observed between conglutinin-immune complexes and insulin binding capacity. A relationship between this type of immune complex and insulin treatment is described in chapter B3.2c: these complexes were significantly more frequent in patients treated with insulin than in others. The present data, showing the existence of a significant trend in the prevalence of circulating conglutinin-immune complexes with increasing levels of insulin binding capacity, appear to suggest that the conglutinin method may detect some of the insulin-anti-insulin complexes present

in diabetic patients following treatment. On the other hand, assay of immune complexes using conglutinin could be affected by the anti-insulin antibodies and by the aggregates of these antibodies. It is more difficult to offer an explanation for the observation that the relationship between conglutinin-immune complexes and the levels of insulin binding capacity in patients treated with conventional insulins (group 1) differs from that in patients treated with monocomponent insulins (group 2). It would, in fact, appear that while the former tend to produce immune complexes at high levels of insulin binding capacity, the latter form these complexes at low or medium levels of insulin binding capacity. This finding may be due to the difference in immunogenicity of the two types of insulin. Even though, because of their purity, monocomponent insulins produce low overall levels of insulin-binding capacity, the antibodies induced may have a relatively high affinity for insulin. In contrast, conventional insulins, which contain extractive polypeptides with a greater molecular weight and immunogenic capacity than pure insulin, tend to produce larger families of antibodies only some of which display marked affinity for insulin, even though all may be detectable as insulin binding capacity by Christiansen's method (Berson and Yalow, 1959; Wehner & Larsen, 1969). It is thus possible that for the same insulin-binding capacity level, patients treated with monocomponent insulin produce a larger quantity of specific complexes than those using conventional insulins.

In conclusion, the present findings confirm the presence of circulating immune complexes in patients with Type 1 diabetes mellitus, some of which may be related to factors present at diagnosis, others to anti-insulin antibodies. On the other hand conglutinin-

immune complexes might provide a useful method with which to follow the pathological events associated with circulating anti-insulin antibodies.

B2.6        INSULIN ANTIBODIES AND IMMUNE COMPLEXES IN TYPE 1 AND 2  
DIABETICS TREATED FOR THE FIRST TIME WITH CONVENTIONAL,  
MONOCOMPONENT AND HUMAN INSULINS : A SIX MOUNTH FOLLOW UP

B2.6a        A i m   o f   t h e   s t u d y

Insulin antibodies and immune complexes were studied in type 1 and 2 diabetics receiving insulin for the first time. A six month follow up was carried out.

The aim of the present investigation was to study human monocomponent insulin and its biologic and immunologic activity, and to draw a comparison with porcine monocomponent insulin and other conventional types of insulin preparations.

B2.6b        M a t e r i a l s   a n d   m e t h o d s

Sixty-eight diabetic Patients receiving insulin for the first time were examined. None of these patients had evidence of other diseases likely to involve the immune system, and none exceeded 10% of mean body weight values. No pregnant women were included in the series.

Patients comparable for sex, age, and duration of disease were divided into the following three groups according to the type of insulin received.

Group I (15 subjects):

Eight men and seven women comprised group 1 (mean age,  $28.2 \pm 6.3$  yr; mean duration of diabetes,  $4.4 \pm 2.1$  yr). Ten were newly diagnosed insulin-dependent diabetic patients (IDDM) and five had been treated previously with oral antidiabetic drugs. Patients in this group were treated with human monocomponent insulins; 5 received only the Monotard (Novo Industri, Copenhagen, Denmark) preparation and 10 the combination Monotard + Actrapid (Novo).

Group II (28 subjects):

Sixteen male and 12 female subjects comprised group 2 (mean age,  $22.5 \pm 3.3$  yr; mean duration of diabetes,  $2.6 \pm 1.2$  yr). Twenty-one were newly diagnosed IDDM and seven had been treated previously with oral antidiabetic drugs. Patients in this group were treated with porcine monocomponent insulins; 10 received only the Monotard (Novo) preparation and 18 the combination Monotard + Actrapid (Novo).

Group III (25 subjects):

Fourteen male and 11 female subjects comprised group 3 (mean age,  $21.4 \pm 4.1$  yr; mean duration of diabetes,  $3.3 \pm 1.4$  yr). Nineteen were newly IDDM and five had been treated previously with oral antidiabetic drugs. Patients in this group were treated with conventional insulins; 9 received only long-acting preparations and 16 the regular plus long-acting. Blood samples were collected before insulin treatment and 1, 2, 3 and 6 months after the beginning of insulin treatment. Serum from blood samples was stored at  $-20$ .

### Insulin antibodies

Insulin antibodies were measured according to the immuno-electrophoretic method described by Christiansen 1970 (Chapter A3.3).

### Immune complexes

Immune complexes were evaluated by two different methods : the solid phase Clq method (Clq-AgAb) described by Hay et al 1976 with minor modifications (chapter A3.1) and the conglutinin binding assay (Kg-AgAb) described by Casali et al, 1977, with minor modifications (chapter A3.1)

### Islet cell antibodies

Antibodies to pancreatic islet cells were detected using the indirect immunofluorescence technique (chapter A3.5)

### Haemoglobin A1

Metabolic control was evaluated by assay of glycosylated haemoglobin (HbA<sub>1</sub>) with column chromatography using the Bio Rad kit (Bio-Rad, Richmond, California). The upper limit of normal values in our laboratory was 9%, intrassay variation was 0.3% (HbA<sub>1</sub><7%) and 0.6% (HbA<sub>1</sub>>7%), and interassay variation was 0.8% and 1.4%, respectively.

### Statistical analysis

Student's test and Cox's trend test were used for statistical evaluation of the data.

## B2.6c      R e s u l t s

The prevalence of ICA at the beginning of the investigation was comparable in the three groups of patients (52% in group 1, 57% in group 2, and 52% in group 3). The metabolic control after 6 months of treatment was similar in all three groups: the mean HbA<sub>1</sub> was 9.4%, 9.2%, and 10.1%, respectively, in groups 1, 2, and 3. The insulin requirement (table B2.6/1) was similar in all three groups 15 days after the beginning of insulin treatment, whereas after 6 months a progressive rise was observed from group 1 to group 3, but these differences were not statistically significant.

### Insulin antibodies

Anti-insulin antibodies (InsAb)(table B2.6/2) at 6 months were significantly lower ( $p < 0.05$ ) in patients in group 1 than in group 2. The levels were significantly higher ( $p < 0.05-0.002$ ) in group 3 than in groups 1 and 2 from the end of the first month onward. No statistical difference was found in insulin antibody levels between type 1 and 2 diabetics.

### Immune complexes

The prevalence of Clq-AgAb was similar in the three groups of patients before the beginning of insulin treatment; thereafter values decreased progressively to reach, by 6 months values ranging between 13% and 16%(table B2.6/3).



The prevalence of Kg-AgAb was similar in the three groups before the beginning of insulin treatment (table B2.6/4). At 6 mo the presence of Kg-AgAb remained practically constant in groups 1 and 2, and rose significantly ( $p < 0.001$ ) from 24% at the beginning of treatment to 44% at 6 months of observation in group 3.

The immune complex results were not statistically different between type 1 and 2 diabetics.

TABLE B2.6/1 Insulin requirement (U/kg/day) in 68 newly treated IDDM from 2 weeks to 6 months after the beginning of insulin treatment

| Type of insulin | No. of subjects | 0*     | Months |      |      |      |
|-----------------|-----------------|--------|--------|------|------|------|
|                 |                 |        | 1      | 2    | 3    | 6    |
| Human           | 15              | 0.41+  | 0.48   | 0.44 | 0.42 | 0.44 |
|                 |                 | 0.03++ | 0.03   | 0.04 | 0.02 | 0.05 |
| Porcine         | 28              | 0.42+  | 0.46   | 0.50 | 0.43 | 0.52 |
|                 |                 | 0.02++ | 0.04   | 0.05 | 0.04 | 0.03 |
| Conventional    | 25              | 0.40+  | 0.45   | 0.51 | 0.55 | 0.62 |
|                 |                 | 0.03++ | 0.05   | 0.05 | 0.07 | 0.07 |

\* Fifteen days after the beginning of insulin therapy  
+ Mean; ++ SEM

TABLE B2.6/2 Insulin antibody (mU/ml) in 68 newly treated IDDM from the first to the sixth month after the beginning of insulin therapy.

| Type of insulin | N. of subjects | Months |         |         |         |
|-----------------|----------------|--------|---------|---------|---------|
|                 |                | 1      | 2       | 3       | 6       |
| Human           | 15             | 0.07*  | 0.1     | 0.1     | 0.05    |
|                 |                | 0.03+  | 0.07    | 0.05    | 0.02    |
| Porcine         | 28             | 0.07*  | 0.2     | 0.3     | 0.4     |
|                 |                | 0.02+  | 0.08    | 0.07    | 0.09    |
| Conventional    | 25             | 0.5*   | 0.8     | 1.2     | 1.7     |
|                 |                | 0.2+   | 0.2     | 0.3     | 0.3     |
| H-P             |                | NS     | NS      | NS      | p<0.05  |
| H-C             |                | p<0.05 | p<0.05  | p<0.025 | p<0.01  |
| P-C             |                | p<0.05 | p<0.002 | p<0.002 | p<0.005 |

H= human insulin; P= porcine insulin; C= conventional insulin.  
 Statistical evaluation by Student's test. \* Mean; + SEM

TABLE B2.6/3 Prevalence of circulating immune complexes (Ciq-AgAb) (%) in 68 newly treated IDDM from the beginning of insulin treatment to the sixth month of therapy.

| Type of insulin | N. of subjects | Months |    |    |    |    |  |
|-----------------|----------------|--------|----|----|----|----|--|
|                 |                | 0      | 1  | 2  | 3  | 6  |  |
| Human           | 15             | 40     | 40 | 27 | 20 | 13 |  |
| Porcine         | 28             | 53     | 42 | 22 | 22 | 16 |  |
| Conventional    | 25             | 46     | 33 | 25 | 25 | 14 |  |

TABLE B2.6/4 Prevalence of circulating immune complexes Kg-AgAb (%) in 68 newly treated IDDM from the beginning of insulin treatment to the sixth month of therapy.

| Type of insulin | N. of subjects | Months |     |     |     |     |  |
|-----------------|----------------|--------|-----|-----|-----|-----|--|
|                 |                | 0      | 1   | 2   | 3   | 6   |  |
| Human           | 15             | 27     | 27  | 33  | 27  | 20  |  |
| Porcine         | 28             | 25     | 27  | 24  | 24  | 28  |  |
| Conventional    | 25             | 29*    | 33* | 33* | 38* | 44* |  |

\*  $p < 0.001$  (Cox's trend test).

B2.6d      D i s c u s s i o n

Before the beginning of insulin treatment the three groups of patients displayed similar prevalence of ICA, Clq-AgAb and Kg-AgAb. Two weeks after the beginning of insulin treatment, insulin requirement was also comparable in the three groups. The present study confirms a previous report (Scherthaner et al, 1983) indicating that human monocomponent insulin produces a lower insulin antibody response than porcine monocomponent insulin in subjects never treated previously with insulin.

The observed differences may be due to the primary structure of these insulins. However, it should be noted that the amino acid in position 30 of the B-chain, which differentiates human from porcine insulin, does not appear to be directly involved in the immunogenic activity of the hormone. On the other hand, since not only the primary structure but also the stereoisometric configuration define the immunogenic characteristics of a polypeptide, the difference in only one amino acid may result in differences in the three-dimensional structures of proteins and alter the capacity to induce the formation of specific antibodies.

The difference in insulin antibody formation between groups 1 and 2 could also be explained by a difference in the genetic background of the diabetic population studied. Subjects with HLA-B15 and/or HLA-DR4 antigens produce more IgG insulin antibodies to animal insulin than patients with other DR-types (chapter B2.2b; Scherthaner & Mayr, 1981; Bertrams, 1981). Of interest, however, Scherthaner et al (1981) found a high prevalence of HLA-DR3 in patients producing IgG insulin

antibodies against human monocomponent insulin. The relevance of these observations to the current study remains to be determined.

Circulating immune complexes assayed with Clq, which have been previously shown to be correlated with the onset of type 1 diabetes (chapter B1.2) and with the severe forms of microangiopathy in both types of diabetes (chapter B3.2), do not appear to be influenced by the type of insulin treatment employed. This observation would confirm earlier findings indicating that insulin is not a substantial component of Clq-AgAb (chapters B2.3,B2.3,B2.4,B2.5). The prevalence of circulating immune complexes determined with the KgBt technique appears, in contrast, to correlate with on the type of insulin treatment used. This pattern is probably due to the positive correlation between Kg-AgAb and anti-insulin antibodies demonstrated elsewhere (chapter B3.5). However there is no definite evidence to show that insulin is found among the antigens present in the Kg-AgAb complexes.

Whatever mechanism is involved, human insulins injected into man appear to induce less immune complexes in comparison with the levels of immune complexes induced following administration of heterologous conventional insulins.

The clinical importance of Kg-AgAb in diabetes mellitus still remains to be defined, in as much as the presence of severe microangiopathy has been shown to be correlated only with the Clq-AgAb.

In conclusion, the present 6 month trial suggests that equivalent glycemic control may be achieved with human monocomponent insulins at similar doses to those required with porcine monocomponent insulins. Furthermore, human insulin is the least immunogenic of the present

available insulins. No evidence of undesired side effects of human monocomponent insulin was found.



## B2.7 CONCLUSIONS

The first aim of these studies was to investigate the relationship between insulin antibodies and different types of complexes in diabetes.

Some types of complexes have been found to be correlated, with insulin antibodies.

The presence of immune complexes detected by conglutinin, apparently medium-large size complexes with complement bound, parallels that of insulin antibodies. This is confirmed also by other studies described in chapters B1 and B3. The correlation of insulin antibodies with this type of complexes was not hypothesized at the beginning of the study. The complexes that insulin is likely to form are expected to be of very small size with a scanty complement fixation due to the characteristics of the antigen. On the contrary the complexes detected by conglutinin are of a large size and have the third fraction of the complement already bound. It is possible that those complexes detected by conglutinin are related to the immunological response induced either by polymers of insulin or by physicochemical modifications of the antigen that occur in the insulin preparations or in tissues following injection. Another likely possibility is that conglutinin complexes are related to the impurities present in the insulin preparations. This is substantiated by the fact that conglutinin complexes were lower in patients treated with highly purified or human insulins.

The immune complexes detected by Clq, apparently of small-medium size in antigen excess, do not seem to correlate with the levels of insulin antibody. The Clq complexes appear to correlate with other immune phenomena occurring in the early stages of type 1 diabetes, namely with the presence of islet cell antibodies (chapter B1). Furthermore Clq complexes seem to be increased when severe signs of microangiopathy are present, as will be discussed in chapter B3. The higher presence of immune complexes, as detected by Clq method, found in insulin treated diabetics and described in the study B2.3 is confined to those patients with a duration of diabetes of more than 10 years. In these patients it is possible that other factors, including microangiopathic processes, may be operating.

The second aim of these studies was to evaluate the immunogenicity of insulins differing in purity and of homologous and heterologous insulin molecules.

Newly diagnosed type 1 diabetics, treated with highly purified insulin, formed less insulin antibody after one year of treatment than comparable diabetics treated with conventional insulins. The presence of immune complexes, as detected by conglutinin, correlated with that of insulin antibodies at one year of diagnosis.

Patients treated for the first time with human insulin showed after six months a much smaller insulin antibody formation and lower presence of complexes detected by conglutinin than patients in comparable conditions and treated with conventional insulins. Even though the humoral immunological factors studied were not significantly different in patients treated with human insulins and in those treated with insulins from animal sources but highly purified, it appeared

that human insulin is the least immunogenic of the present available insulins.

C H A P T E R    B 3

IMMUNOLOGICAL PHENOMENA IN DIABETIC VASCULAR COMPLICATIONS

Chapter B3      IMMUNOLOGICAL PHENOMENA IN DIABETIC VASCULAR  
COMPLICATIONS

- B3.1    Experimental design of the studies on diabetic vascular complications
- B3.2    Earlier studies by the author
  - B3.2a    Insulin antibodies, immune complexes and diabetic microangiopathy: a preliminary observation
  - B3.2b    Immune complexes in diabetics with various degrees of microangiopathy : a pilot study
  - B3.2c    Immune complexes in diabetics with severe microangiopathy: evaluation by two different methods
  - B3.2d    Immune complexes and phagocytic clearance in diabetics with severe microangiopathy
- B3.3    Insulin antibodies and immune complexes in patients with and without microangiopathy
  - B3.3a    Aim of the study
  - B3.3b    Materials and methods
  - B3.3c    Results
  - B3.3d    Discussion
- B3.4    Immune complexes, insulin antibodies, insulin-anti-insulin complexes related to platelet and coagulation factors in patients with proliferative retinopathy

- B3.4a Aim of the study
  - B3.4b Materials and methods
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  - B3.4d Discussion
- B3.5 Immune complexes and lymphocyte subsets in patients with microangiopathy
- B3.5a Aim of the study
  - B3.5b Materials and methods
  - B3.5c Results
  - B3.5d Discussion
- B3.6 Conclusions

B3.1        EXPERIMENTAL DESIGN OF THE STUDIES ON DIABETIC VASCULAR  
              COMPLICATIONS

Much controversy still exists regarding the mechanism and factors involved in the pathogenesis of diabetic microangiopathy. Whilst various processes have been shown to be involved in diabetic microvascular lesions, certain findings appear to be of particular interest: vascular lesions present in immune complex diseases share morphological aspects with those observed in diabetes, diabetic-like vascular lesions can be experimentally induced by immune mechanisms, biochemical changes occurring in the vascular basement membrane increase the binding or trapping of circulating macromolecules.

(For details and references see Chapters A1.5 and B3.2).

Circulating immune complexes have been described by the author to be increased in diabetics with microangiopathy (B3.2a). After this preliminary observation other studies in selected groups of patients (B3.2b, B3.2c) have been conducted to confirm the increase of some types of complexes in patients with microangiopathy and to determine whether an impairment of the reticuloendothelial system could result in a decreased clearance of such complexes (B3.2d).

In diabetic patients with microangiopathy the influence of insulin treatment on immune complex formation and on progression of vascular lesions remained to be determined. The possible interaction

between these complexes, with other factors or cellular systems, such as platelets, coagulation factors and lymphocytes was considered to be of interest. In fact, the presence of immune complexes in the circulation or in the tissue "per se" may be simply a normal biological phenomenon. The quantity, type and destructive potentiality of these complexes, and the way in which the host deals with them, are the main factors that give a pathological significance to the presence of such complexes.

The aims of the studies on diabetic microangiopathy are :

- a) To evaluate the presence of different types of complexes and the levels of insulin antibody in patients with severe microangiopathy and with no clinical sign of it.
- b) To relate the presence of immune complexes, insulin antibodies and insulin-anti-insulin complexes to the abnormalities of platelet and coagulation factors in patients with proliferative retinopathy.
- c) To investigate the possible correlation between some types of complexes, subsets of lymphocytes and the presence of microangiopathy.

Insulin treated diabetic patients were selected so as to have patients in opposite conditions, i.e. with severe microangiopathy and without any clinical signs of microangiopathy, and included in the study B3.3. Two methods detecting immune complexes and differing in principle and a method to detect insulin antibodies were used.

The presence of these immunological factors was correlated to the presence of vascular lesions.



Since several abnormalities of endothelial and platelet function have been reported in diabetic microangiopathy, the interaction between circulating immune complexes and platelets in particular appears to be extremely interesting. Two main aspects deserve attention. On the one hand, immune complexes may activate platelet function, while on the other, platelet factors may modify the deposition of complexes in the vessel walls. In the study B3.4 immune complexes, insulin-anti-insulin complexes and insulin antibodies have been evaluated and correlated to a number of coagulation and platelet factors in patients with proliferative retinopathy. Patients without retinopathy and normal subjects acted as controls.

It is known that lymphocyte bearing receptors for the Fc portion of immunoglobulins or for complement may bind and remove some types of complexes; in study B3.5, the presence of some types of complexes was correlated to the relative presence of different subsets of lymphocytes in patients with and without microangiopathy.

The clinical part of these studies was done in collaboration with the Diabetic Clinic, Royal Infirmary, Edinburgh, UK and with the Diabetic Unit, University of Rome, Italy.

Platelet and coagulation factors were assayed in the Blood Transfusion Service, Royal Infirmary, Edinburgh, UK.

## B3.2 EARLIER STUDIES

B3.2a Insulin antibodies, immune  
complexes and diabetic micro-  
angiopathy: a preliminary  
observation

In the present study, the presence of antibodies to insulin and the occurrence of immune complexes have been investigated in the sera of treated diabetics and compared to those in controls. Their association with the occurrence of diabetic complications and their possible role in microangiopathy are discussed.

M a t e r i a l s   a n d   m e t h o d s

(See chapter B2.2a)

R e s u l t s

(See chapter B2.2a)

Sixteen of the thirty-two insulin-treated diabetics had diabetic complications that were clinically manifest, including diabetic retinopathy (defined by the presence of more than 3 microaneurisms, retinal haemorrhages, exudates or new blood vessel formation) or diabetic nephropathy (defined by a permanent proteinuria with or without abnormal kidney function). Twelve out of the sixteen had

soluble immune complexes in the serum while only five of the remaining sixteen patients did so. The presence of immune complexes in the serum was associated with late diabetic complications only in patients who had had diabetes for more than 13 years. Four of the six patients with insulin antibodies in the serum in a titre of  $>300$  uU/ml had late diabetic complications and had had diabetes for 15 years or more. In the other two the duration of diabetes was 2 and 13 years, respectively.

#### D i s c u s s i o n

That immune complexes in relation to insulin may be implicated in diabetic complications is suggested by the observation that renal tissue from diabetics shows fine granular deposits on the basement membrane, resembling those found in certain types of glomerulonephritis (Bloodworth, 1968), while the glomeruli in diabetic nephropathy have insulin-binding capacity and also contain immunologically detectable insulin (Berns et al., 1962). However, immuno-histopathological studies have shown that the basement membrane in diabetic glomerulosclerosis contains not only insulin, IgG, IgM, and components of complement, but also 'non-immunological' plasma proteins deposited in a linear fashion (Westberg & Michael, 1972), which suggests that there may be a non-specific lesion involving the trapping (binding) of serum proteins. These two possible mechanisms for the production of diabetic complications may not be mutually exclusive. While there was no clear correlation between the titres of insulin antibodies and the late complications of diabetes, these preliminary findings suggest that there is a tendency for immune complexes to occur after prolonged insulin therapy and at a time when

such complications are likely to become clinically common (i.e. after 10 years).

Clearly a long-term prospective study is required in order to determine whether the development and persistence of soluble immune complexes in insulin-dependent diabetes is correlated with the development of late diabetic complications and whether the use of monocomponent insulin will avoid the development of immune complexes, and whether or not this will be associated with a reduction in late complications.

B3.2b Immune complexes and insulin antibodies in diabetics with various degrees of microangiopathy: a pilot study

The purpose of the present study was to investigate the possibility that soluble immune complexes, not necessarily comprised of insulin, may play a role in diabetic microangiopathy. Thus the levels of complexes in circulation were assayed in groups of diabetics with different degrees of microangiopathy.

Materials and methods

Ninety-three insulin or oral hypoglycaemic agent (OHA) treated diabetics attending the same diabetic clinic of the University of Rome were selected, and grouped according to the fluoroangiography findings and the duration of diabetes.

The patients in groups A, B and C had had diabetes for many years. Patients in group A were without any sign of diabetic retinopathy and had no clinical evidence of other diabetic complications. Patients in group B had non proliferative retinopathy with or without other diabetic complications, while patients in group C had proliferative retinopathy with or without other diabetic complications.

The patients in groups D and E had had diabetes for only a few years. Those in group E had developed retinopathy within 3 years of diagnosis (precocious retinopathy) and they were compared with an otherwise comparable group of diabetics without retinopathy (group D).

Fifty-three of the patients were treated with insulin while 40 were treated with OHA. None of the patients had laser treatment for retinopathy. Diabetics with other AgAb associated diseases were not included in the study.

One hundred and fifty blood donors were included as controls.

Because of the heterogeneity of AgAb two sensitive methods differing in principle were used to detect AgAb: the solid phase Clq binding test (Clq-SP) (Hay et al, 1976) based on the method of Svehag (Svehag, 1975), and the Raji cell radioimmune assay (RAJI) (Theofilopoulos et al, 1976). Quantitation in both methods was obtained by reference to the uptake of serially diluted heat aggregated human IgG (AHG) in normal serum (EDTA inactivated in Clq-SP) and the results were expressed in ug AHG equiv/ml undiluted serum. In the Clq-SP, values greater than the 90th percentile of a series of 80 blood donors were considered as positive. In the RAJI assay, values greater than 20 ug AHG equiv/ml were considered positive. Further details of the two methods for AgAb are given in the chapter A3.1.

Insulin antibodies, as measured by plasma binding capacity InsAb, were detected and titrated by Christiansen's method (Christiansen, 1970) (see chapter A3.3).

## R e s u l t s

Considering all the patients together, the presence of AgAb was significantly increased in diabetics compared to the control population using either Clq-SP (27% vs 10%) or RAJI (41% vs 24%). When the results of the two tests were combined, 53% of the diabetics had evidence for AgAb in their serum.

In diabetics of more than 15 years duration (group A,B,C), patients with proliferative retinopathy (group C) had a higher prevalence of AgAb as detected by Clq-SP than did the control population, while those without retinopathy (group A) did not. The prevalence of AgAb showed an increase in group B compared to group A and in group C compared to group B. Applying the test for a trend in proportions as proposed by Cox(1971), the trend for increased prevalence of AgAb as detected by Clq-SP according to increasing severity of retinopathy is statistically significant at the 5% level (normal deviate=2.13,  $p=0.03$ ).

In diabetics with onset of retinopathy within 3 years of diagnosis (group E) the prevalence of AgAb was higher than in the control population but no such difference compared to controls could be shown in diabetics of similar duration without retinopathy (group D).

The prevalence of AgAb in group D and E can be summarized in 2x2 contingency table and analysis by means of Fisher's Exact Test gave statistically significant differences at the 10% but not at the 5% level for both Clq-SP and RAJI (using a two-tailed test) assessed individually. When the same analysis was made in longer established diabetics by comparing group C and A (ignoring B), the Clq-SP technique also gave statistically significant results at the 10% level, while no significant difference was obtained with RAJI.

A test for combining the information from two or more 2x2 contingency tables of the same type (e.g. group A vs C and D vs E) has also been given by Cox(1971). Applying this to the data from the Clq-SP technique gave a significantly higher prevalence of AgAb in patients with severe or early retinopathy (normal deviate =3.25,

$p=0.001$ ) compared to those without retinopathy, while with the RAJI data, statistical significance at conventional levels was not found (normal deviate=1.62,  $p=0.11$ ).

Instead of using the prevalence, it is possible to apply non-parametric tests of significance (randomization tests) which utilize the levels of AgAb observed. Applying these tests gave precisely the same conclusions that were obtained by considering prevalences.

There was no statistically significant differences in the prevalence of AgAb when insulin treated and OHA treated diabetics in groups A, B and C were compared using either Clq-SP or RAJI. Similar negative results in relation to the form of treatment were found in patients with or without precocious onset of retinopathy (groups D, E), or when all the patients were considered together.

There was no statistical correlation between the titres of insulin antibodies determined by insulin binding capacity and the occurrence of AgAb in insulin treated diabetics.

#### D i s c u s s i o n

In the past, the detection of soluble immune complexes (AgAb) was difficult because of the absence of a sensitive, reproducible and simple method. In the seventies the methods for detecting circulating AgAb were substantially improved (Lambert et al, 1978), though they are still antigen non-specific and do not differentiate true AgAb from immunoglobulin aggregates. Methods based on different principles detect soluble complexes with different characteristics (Lambert et al, 1978), and it is therefore not surprising that the two methods used in the present study, give somewhat different results although showing the same overall trends (see chapter A3.1).



Both methods indicate that AgAb are increased in treated diabetics. This could be due to either an increased rate of production of AgAb or to a decreased rate of clearance by the reticulo-endothelial system. The composition of AgAb and change in phagocytic function may influence the rate of clearance (Mannik et al,1974). The methods used in the present study detect AgAb of medium to large size and one would expect that such complexes would be rapidly removed by a normally functioning phagocytic system (Mannik et al,1974). An impairment of phagocytic function has been described in poorly controlled diabetics (Badgale,1976) and a positive correlation between poor metabolic control and microangiopathy in diabetes is widely believed to exist (Colwell,1966;Job et al,1976). Conceivably, therefore, poor metabolic control could lead to the accumulation of AgAb in the circulation and this may contribute to the development of microangiopathy.

The methods used for detecting AgAb in the present study do not characterize the antigen(s) or the immunoglobulin(s) involved, but only tell us that the immunoglobulin(s) belongs to IgG1,IgG2 or IgG4. However it can be surmised that most of the AgAb detected would have a medium/high degree of lattice formation, would be of medium/large size, would activate the complement system and would react with cells possessing Fc or C receptors (WHO Scientific Group,1977). AgAb with these characteristics have the greatest pathogenic potential (WHO Scientific Group,1977) and they may act as harmful agents by complement activation and subsequent chemotaxis of leucocytes after passive vascular trapping or binding.

Increased production and accumulation of AgAb may be due to recurrent infections, which are likely to be related to poor

metabolic control, irrespective of whether the patient required insulin or not.

The presence of AgAb does not seem to be related to the type of treatment or to insulin antibody titres.

It is probable that most AgAb related to diabetic retinopathy are not comprised of insulin. Although a pathogenic role for insulin anti-insulin complexes cannot be excluded, their involvement might be a relatively minor one. Thus, on account of the bivalency of insulin (Folling, 1976), AgAb involving insulin would tend to have a low degree of lattice formation and would therefore bind complement poorly (Jaton et al, 1976). Such complexes would be detected poorly by the methods used in the present study and would not be expected to have much pathogenic potentiality. These arguments would be in keeping with the clinical observation that microangiopathy is present with similar characteristics in insulin and non-insulin treated diabetics.

In conclusion these preliminary findings suggest that the study of soluble immune complexes provides a new approach to old and still unsolved problem of the pathogenesis of diabetic microangiopathy.

B3.2c      I m m u n e   c o m p l e x e s   i n   d i a b e -  
t i c s   w i t h   s e v e r e   m i c r o a n g i o -  
p a t h y : e v a l u a t i o n   b y   t w o  
d i f f e r e n t   m e t h o d s

In this study the presence of circulating immune complexes (AgAb) in serum of diabetics has been investigated in order to establish the correlation between AgAb and severe microangiopathy, either proliferative retinopathy or advanced nephropathy. Attention has been focused especially on patients with long standing diabetes not presenting with microangiopathy. The influence of the type of diabetes and of the antidiabetic treatment on AgAb levels was also studied. Since AgAb are heterogenous and different methods detect different types of complexes, two sensitive methods differing in principle to detect them were used.

M a t e r i a l s   a n d   M e t h o d s

One hundred and fifty-one diabetics were selected according to the presence of microangiopathy and the duration of disease. Since many factors theoretically might influence the levels of AgAb in diabetics, such as the duration of diabetes and the type of treatment, to minimize the interference of other variables, the relation between the presence of microangiopathy and AgAb positivity was studied in diabetics in two quite different, well defined and rather uncommon conditions.

At first patients were studied who, after many years of diabetes, showed no sign of diabetic complications and they were

compared them with diabetics who, at the same time after diagnosis, showed proliferative retinopathy or severe nephropathy. For this purpose, 92 diabetics at more than 15 years after diagnosis were studied (Group I): 31 had no detectable sign of diabetic microangiopathy whereas 30 showed proliferative retinopathy without marked nephropathy, and 25 had severe nephropathy with various degrees of retinopathy.

Thereafter diabetics with apparently a precocious onset of severe retinopathy were studied and their levels of AgAb were compared with those found in diabetics with no sign of diabetic complications at a similar time from diagnosis. Fifty-nine patients were assembled for this investigation (Group II): 19 diabetics manifested the onset of severe retinopathy within 3 years of diagnosis whereas 40 had no sign of microangiopathy at comparable time from diagnosis.

Diabetics presenting mild microangiopathy, or patients with chronic disorders with primary or secondary immune aspects, were excluded from the investigation.

Part of the patients were type I diabetics and the remainder type II diabetics. Type II diabetics with an overweight of more than 20% of their ideal body weight were not included in the study in order to minimize the number of variables.

One hundred and eighty-nine blood donors, age range 18-59, were controls for the evaluation of the limit of positivity in AgAb assays.

The presence and degree of retinopathy were assessed in all the diabetics by retinal fluoroangiography and the results were evaluated

by an ophthalmologist. Patients with severe retinopathy, included in the study, showed either proliferative retinopathy or vitreal haemorrhages. Nephropathy was evaluated on the basis of Blood Urea Nitrogen (BUN) serum creatinine levels and proteinuria. BUN > 50 mg/100 ml, serum creatinine levels > 2 mg/100 ml, and/or proteinuria > 2 g/l were considered as an index of kidney involvement.

The solid phase Clq binding test (ClqSP) (Hay et al. 1976) and the conglutinin binding test (KgBt) (Casali et al. 1977) were used to detect circulating AgAb (see chapter A3.1).

The selection of patients and the laboratory assays were done by two independent researchers and so the sera were in a random order in the experiments. If a difference of more of 10% in results from duplicate tubes was found, the sample was re-tested. The same batch of immunoglobulin divided in aliquots and aggregated at 63°C for 20 min, was used throughout the study. Values greater than 6 ug AHG Eq/ml were considered above the limit of positivity in the ClqSP whereas 4 ug AHG Eq/ml was the limit in the KgBt (chapter A3.1).

The chi square test (X), with Yates' correction when indicated (Y), Fisher's exact test (F), Student's t-test (S) and Cox's test (C) (Cox 1971) were used in the statistical evaluations of the results as indicated. The quantities of AgAb were expressed as mean  $\pm$  SEM of values.

## R e s u l t s

When the results in long standing diabetics (Group I) were examined, the prevalence of AgAb, using ClqSP, was similar in

patients without microangiopathy (16%) and in the normal population (10%), whereas the prevalence of AgAb was significantly higher in patients with severe microangiopathy (51%) than both in the normal population ( $P < 0.001$ , Y) and in the patients without complications ( $P < 0.005$ , Y). Also the quantities of AgAb in diabetics with severe microangiopathy were significantly higher ( $P < 0.01$ , S) when compared to those found in patients without microangiopathy ( $17.7 \pm 3.2$  vs  $5.7 \pm 2.8$  ug AHG Eq/ml).

There was a trend for severe microangiopathy to go with medium-high levels of AgAb ( $P < 0.002$ , C, performed on AgAb values divided as indicated in Fig.B3.2/1).

The presence of AgAb studied by ClqSP, was not correlated with the type of diabetes. The AgAb prevalence in type I diabetics with microangiopathy was 52% and in those without microangiopathy 20%. In type II diabetics with microangiopathy the prevalence was 50% and in those without microangiopathy 13%.

The prevalences and the quantities of AgAb were similar in patients with proliferative retinopathy alone (50% and  $21.3 \pm 6.3$  ug AHG Eq/ml, respectively) and in patients with nephropathy and various degrees of retinopathy (52% and  $15.2 \pm 3.2$  ug AHG Eq/ml).

When the KgBt method was used in patients in Group I, an increased prevalence of AgAb was found both in patients with (39%) and without microangiopathy (36%) in comparison with the normal population ( $P < 0.0001$  and  $P < 0.0005$ , Y). Taking into account the type of diabetes, AgAb presence detected by KgBt was higher in type I diabetics ( $P < 0.02$ , X). The AgAb prevalence in type I diabetics with microangiopathy was 48% and in those without microangiopathy 47%. The AgAb prevalence detected by KgBt in type II diabetics was

respectively 25 and 19%, AgAb presence was the same in diabetics with retinopathy alone (35%) and in those with nephropathy(35%).

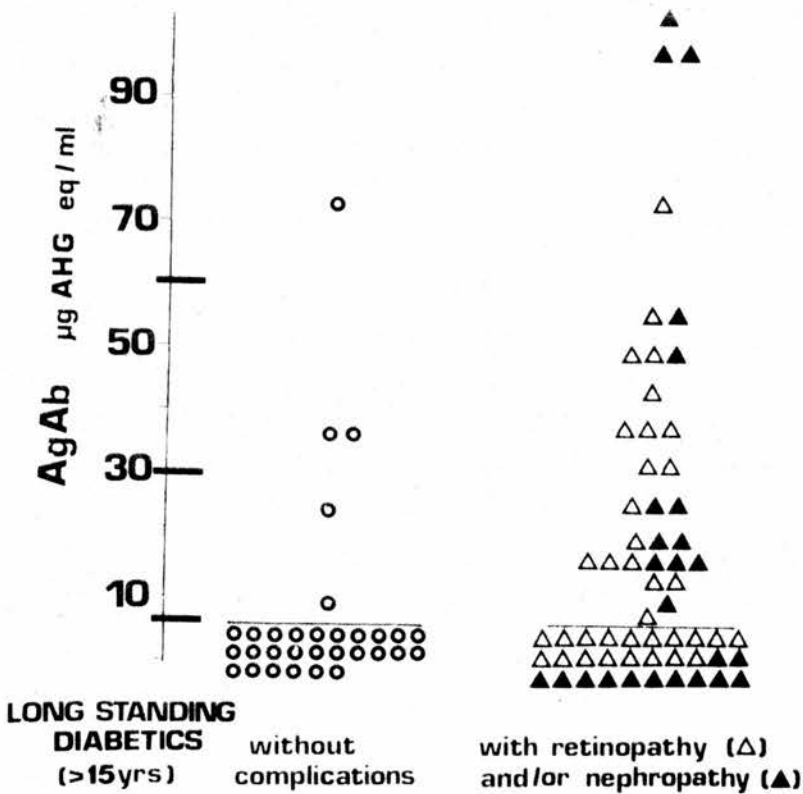
When patients were examined a short time after diagnosis (Group II), using ClqSP method for the analysis of AgAb, the prevalence of AgAb appeared to be significantly higher in patients with early retinopathy (63%) than in normal subjects ( $P < 0.001, Y$ ), or in diabetics without microangiopathy (20%) ( $P < 0.005, F$ ). Also the quantities of AgAb were different between diabetics with ( $15.5 \pm 4.2$  ug AHG Eq/ml) and without retinopathy ( $3.1 \pm 1.1$  ug AHG Eq/ml) ( $p < 0.001, S$ , and  $p < 0.005 C$ , on AgAb values divided as previously described). Taking into account the type of diabetes, the AgAb prevalence using ClqSP was 67% in type I diabetics with microangiopathy and 25% in those without microangiopathy, whereas in type II diabetics with and without microangiopathy was respectively 62 and 15%. There is no difference in AgAb prevalence using ClqSP between type I and II diabetics.

Using the Kgbt method in patients in group II, differences in the prevalence of AgAb in patients with early retinopathy (52%) and in those without microvascular involvement (30%) were not statistically significant, though the prevalence of AgAb in all diabetics (37%) was higher than in the control population ( $p < 0.005, Y$ ). No difference in AgAb prevalence was found between type I and II diabetics.

As previously shown (Lambert et al, 1978) the Clq-Sp and Kgbt techniques detect different types of AgAb and also in this work there was not a significant concordance between AgAb values obtained by these two methods: AgAb values were concordantly positive in 15% of the cases and concordantly negative in 41%. 24% of sera showed an AgAb presence only by ClqSP and 20% only by Kgbt.

FIGURE B3.2/1

Immune complexes, detected by Clq-SP, in 31 long standing diabetics without complications (o) and in 62 patients with microangiopathy (proliferative retinopathy and/or severe nephropathy ). The horizontal line at 6 ug AHG Eq/ml represents the limit of positivity (see text). The heavy bars on the y-axis divide AgAb values in negative, low, medium and levels (see text).





## D i s c u s s i o n

In agreement with other reports (Kumar & Quismorio,1978; Kumar et al,1979;Ludwig et al,1979) circulating AgAb were found to be increased in some diabetics. The presence of AgAb in several diabetic conditions, namely type I diabetics at diagnosis (Chapters B1.2a, B1.2c, B1.3) and long standing insulin treated diabetics (Chapters B2.2a, B2.3, B2.4; Charlesworth et al,1979;Delespesse et al,1980), has previously been described. In the present study an increase in circulating AgAb was found in diabetics with severe microangiopathy.

AgAb detected by ClqSP are increased both in diabetics with proliferative retinopathy and in those with severe nephropathy, irrespective of the organ overtly damaged.

AgAb (ClqSP) are also increased in patients with early retinopathy, including patients with the so-called malignant microangiopathy (Andreani,1980), and long standing diabetics without microangiopathy of comparable duration of disease.

The entity of microangiopathic changes, therefore, seems related to AgAb levels and not to the duration of the illness.

In patients with severe complications, AgAb levels were increased irrespective of the type of anti-diabetic treatment; therefore, in the presence of complications, the type of treatment seems to be of no relevance.

All these findings suggest that increased levels of AgAb, accumulated as a consequence of modification of the dynamics of formation, clearance and tissue deposition of complexes, are related to microvascular damage in proliferative retinopathy and diabetic nephropathy, and probably also to the course of complications, being

more marked in patients with rapid development of the pathological changes.

Our selection of diabetics with extreme and unusual conditions, i.e. severe retinopathy within a few years of disease and absence of microangiopathy even after many years of disease, highlights the correlation between AgAb and microangiopathy. This correlation is perhaps not so obvious in less severe microangiopathy, and this may explain the apparent discrepancy with other reports (Ludwig et al, 1979; Balestrieri et al, 1979).

When KgBt method was used, a higher prevalence of AgAb in diabetics was demonstrated, but partially discordant results were found. Conglutinin and Clq bind different types of AgAb through unrelated mechanisms. It is likely that the AgAb present in diabetes are heterogeneous and, from the characteristics of the two methods used, it may be inferred that complexes associated with microangiopathy are formed in antigen excess (Casali et al, 1977), have a low degree of lattice formation (Di Mario et al, 1980), may be readily trapped or bound in blood vessel walls and thus are potentially harmful (WHO 1977).

Since only some diabetics with severe microangiopathy have an obvious increase in circulating immune complexes, the true importance of their presence remains to be established.

Nevertheless, this correlation between the presence of circulating AgAb and the occurrence of severe microangiopathy should be kept in mind when attempting to establish the pathogenesis of diabetic microangiopathy.

B3.2d      I m m u n e c o m p l e x e s a n d p h a g o c y t i c  
c l e a r a n c e i n d i a b e t i c s w i t h  
s e v e r e m i c r o a n g i o p h a t y

The occurrence of circulating AgAb may conceivably be related to the histologic abnormalities observed in diabetics with microangiopathy. It was therefore considered of interest to study the mechanisms of the increase in AgAb in diabetics to throw some light on the pathogenesis of late diabetic complications. AgAb may be increased in the circulation either because of increased formation or decreased clearance (or both). Since medium-sized AgAb are cleared from the circulation by phagocytes, Mannik et al (1974) the function of these cells in diabetics with various degrees of microangiopathy, utilizing a colloid clearance test has been studied. The principle of the test is that above a critical dose of colloid injected, the rate of its clearance adequately reflects phagocyte activity (Mannik et al, 1974; Biozzi et al, 1958)

The relationship between colloid clearance rate and the presence and concentration of AgAb was examined. Due to the heterogeneity of circulating AgAb, two methods were used to detect them: the Clq-SP and the conglutinin binding test (KgBT), which define different populations of AgAb.

P a t i e n t s a n d m e t h o d s

Thirty patients with overt diabetes in whom informed consent for the study was obtained, were the subjects of the study. Eighteen patients were insulin-dependent (type I) and twelve were non-insulin-

dependent (type II) diabetics. Of the latter, three were treated with insulin and nine with sulfonylureas. To limit some of the possible variables, only patients not exceeding ideal body weight by more than 20% and with a fasting blood glucose (BG) ranging between 5 and 11 mM before the test were included.

The patients were divided into three groups according to the degree of microangiopathy. Retinopathy was chosen as an index of microangiopathy because of easy assessment and quantification of the lesions. Kidney function was also evaluated.

Twelve patients with severe (proliferative) retinopathy were classified as group 1; seven of these also showed impaired kidney function. Group 2 comprised seven patients with moderate (background) retinopathy and five of these also showed impaired kidney function. Group 3 consisted of 11 patients with no sign of retinopathy or nephropathy.

The presence and the degree of retinopathy were established by retinal fluoroangiography. The occurrence of nephropathy was assessed by routine laboratory tests. Normality of kidney function was defined as BUN (Blood Urea Nitrogen) < 23 mg/dl; serum creatinine < 1.5 mg/dl, and absence of proteinuria. Metabolic control was similar in all three groups of patients during the 3 months before testing (as assessed by glycosylated hemoglobin ( $HbA_{1c}$ ), fasting BG, and glycosuria).

Forty normal volunteers (26 males and 14 females) were included in the study as controls for the colloid clearance test. Their ages ranged from 22 to 55 yr and all were more than 20% above ideal body weight. In addition, the sera of 198 blood donors were studied as normal controls in AgAb assays.

The colloid clearance test

The colloid used for clearance studies was human serum albumin, which was heat aggregated and iodinated according to the procedure described by Ilio & Wagner(1963) and as modified by Drivas et al(1975).The test, which has been used by various authors (Biozzi et al,1958 and Drivas & Wardle,1978) was performed as follows. The day before the experiment, thyroid uptake was blocked by the administration of Lugol's solution. The following day, after an overnight fast, microaggregated human serum albumin (delta-HSA) was injected intravenously, within 1 min, at a dose of 3.5 mg/kg body weight, to saturate the function of fixed phagocytes. After 10 min, microaggregated and iodinated human serum albumin ( $^{125}\text{I}$ -delta-HSA), 3.5 mg/kg body weight, was injected intravenously within 1 min. Blood samples were collected every 5 minute up to the 45th minute. Serum radioactivity was measured in each sample to study the disappearance from the circulation of injected  $^{125}\text{I}$ -delta-HSA. Radioactivity was plotted semilogarithmically against time, and the half-life ( $t_{1/2}$ ) was determined by graphic resolution of the straight line present between 8 and 20 min. Colloid clearance was considered reduced when the  $t_{1/2}$  values were greater than 2 standard deviations above the mean of the values in normal subjects. Radioactivity was also measured in serum after precipitation with 10% trichloroacetic acid (TCA) to detect the presence of non-protein-bound radioactivity (free iodine) in the supernatant. Possible phagocytosis of  $^{125}\text{I}$ -delta-HSA by circulating leucocytes was examined in white cell buffy coat. BG,HbA<sub>1c</sub>, BUN, creatinine levels, and AgAb were assayed in blood samples collected immediately before the colloid clearance test.

AgAb were measured in duplicate both by Clq-SP(Hay et al,1976) and KgBt(Casali et al,1977). Quantitation of circulating AgAb was obtained by reference to the uptake of serially diluted human aggregated immunoglobulins (AHG). Results were expressed as micrograms of aggregate equivalents per ml of undiluted serum. Uptake greater than the 90th percentile of the blood donor population assayed in the same experiment was considered positive(see chapter A3.1).Patients were selected and laboratory tests were performed by two independent research workers. If a difference of more than 10% in duplicates was found, the sample was retested. The same batch of immunoglobulin G, divided into aliquots and aggregated at 63°C for 20 min, was used throughout the study.Values greater than 6 ug AHG eq/ml were considered above the limit of positivity in the Clq-SP, whereas the limit in KgBt was 4 ug AHG eq/ml.

Cox's test for trend in proportion (C) and Fisher's exact test (F) were used to evaluate the results.

#### R e s u l t s

The half-life of  $^{125}\text{I}$ -delta-HSA was 23.4 $\pm$ 8 min in group 1, 17.7 minutes  $\pm$  5.3 in group 2, 17.9 minutes  $\pm$  4.2 in group 3, and 15.2 minutes  $\pm$  2.4 in normal subjects. Ten of the 12 diabetics in group 1, 2 of the 7 in group 2, and 2 of the 11 in group 3 showed reduced clearance (Figure B3.2/2), suggesting a significant trend toward reduced  $^{125}\text{I}$ -delta-HSA clearance with increasing severity of microangiopathy (C -  $P < 0.002$ ). Radioactivity found in circulating phagocytes up to the 45th min and the amount of free iodine in the supernatant after serum TCA precipitation up to the 25th min of the test

were negligible. These findings confirm the absence of uptake by circulating phagocytes or breakdown of microaggregates.

Clq-SP revealed a 50% prevalence of AgAB in group 1, 28% in group 2, and 18% in group 3. Using KgBt, AgAb positivity was 42%, 28%, and 36%, respectively. The prevalence of circulating AgAb both with Clq-SP and KgBt was 10% in normal subjects.

No differences in BG and in HbA<sub>1c</sub> concentrations were found between the three groups of diabetics.

There was significant difference between type I and II diabetes in the results of colloid clearance and AgAb.

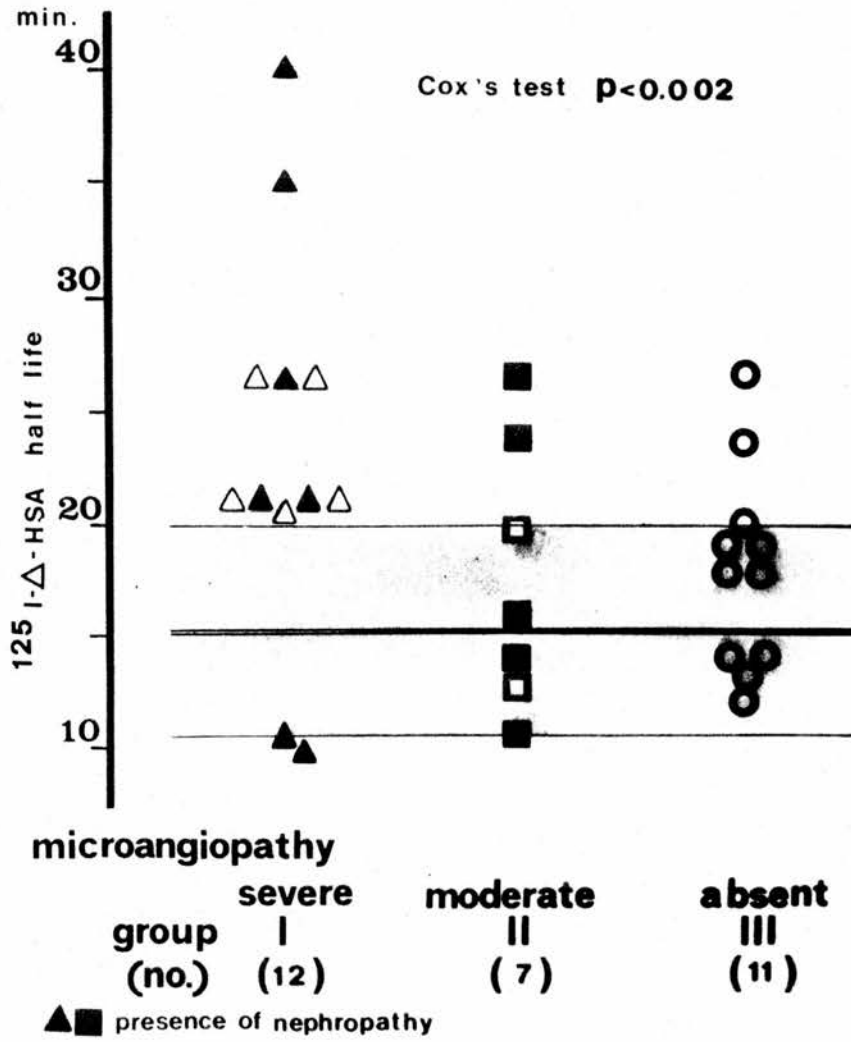
Attempts were made to analyse a possible relationship between the presence of circulating AgAb and phagocytic function. Using Clq-SP, AgAb were found in 8 out of the 14 diabetics with reduced <sup>125</sup>I-delta-HSA clearance (a half-life of over 20 min), and in 2 out of the 16 patients showing a <sup>125</sup>I-delta-HSA half-life below 20 min. AgAb prevalence was significantly higher in the former group ( $F=P<0.05$ ). Using KgBt, no significant correlation was found between presence of circulating AgAb and reduced <sup>125</sup>I-delta-HSA clearance (43% vs. 31%, respectively).

#### D i s c u s s i o n

In the present study, impaired function of fixed phagocytes (mainly Kupffer cells) was found in diabetics with severe microangiopathy. The colloid clearance test is one of the most reliable tests presently available to study phagocyte function in vivo and, as the results therein show, is not affected by phagocytosis of microaggregates by circulating monocytes. It may be preferable to in vitro assays of phagocytic function. The present study confirms the

FIGURE B3.2/2

$^{125}\text{I}$ -HSA-clearance in diabetics with various degrees of microangiopathy.





presence of increased concentrations of circulating AgAb in diabetics with severe microangiopathy compared with diabetics who show no sign of microangiopathy, and with normal subjects. The percentage of diabetic sera found positive for AgAb in this investigation is similar to that previously discussed (Chapters B3.2a, B3.2c). As in these previous studies, differences in the prevalence of AgAb were found when AgAb were measured using the Clq-SP and Kgbt tests. As stated previously, it should be stressed that these two methods differ in principle and detect different types of complexes. As in previous studies (Chapter B3.2c), only AgAb detectable by Clq-SP appear to be correlated with the presence of severe microangiopathy. The data confirm that AgAb are a population with differing biological properties and suggest that only a part of them are related to the microvascular damage.

Data in this study also confirm that the percentage of sera positive for circulating AgAb is similar in type I and type II diabetics when severe microangiopathy is present. Furthermore, the data indicate that colloid clearance is similar in the two types of diabetes.

The finding that the prevalence of circulating AgAb is significantly increased in diabetics showing impaired fixed phagocyte function is of considerable importance. Since the Clq-SP method detects medium-sized AgAb, which are removed by fixed phagocytes, the present data suggest that the increase in circulating AgAb may be a consequence, at least in part, of a decreased clearance due to impaired function of fixed phagocytes.

The reason for this impairment in diabetics with severe microangiopathy is presently not clear. Poor metabolic control over a

prolonged period of time, which seems the most relevant factor correlated with the occurrence of diabetic microangiopathy, could influence the function of phagocytes. It has already been reported that hyperglycemia impairs the function of circulating phagocytes (Badgade,1976;Badgade et al,1978) and lymphocytes(MacCuish et al, 1974)in diabetics as well as in normal subjects(Pozzilli et al,1976). High glucose concentrations, also influence circulating cell function in diabetics and in normal subjects (MacCuish et al,1974; Pozzilli et al,1976) , while lowering of the blood glucose reverses the abnormalities(Badgade et al,1972).Similar data are not available for fixed phagocytes.

In the present investigation, metabolic control was similar in patients with various degrees of microangiopathy; in previous studies no correlation was found between metabolic control and circulating AgAb concentrations (Di Mario et al,1979).Since the methods used here to evaluate the metabolic control (BG and HbA<sub>1c</sub>) provide information only over relatively short periods of time, one cannot ignore the suggestion that prolonged metabolic derangement not detectable by these methods might have modified fixed phagocytic function.

In conclusion, this study suggests that impaired fixed phagocytic function could contribute to long-lasting accumulation of AgAb in plasma. It may be speculated that these AgAb are bound and trapped in the vessel walls and contribute to the development of diabetic microangiopathy.

B3.3        INSULIN ANTIBODIES AND IMMUNE COMPLEXES IN PATIENTS WITH  
              MICROANGIOPATHY

B3.3a        A i m   o f   t h e   s t u d y

This study was planned to investigate the correlation between the presence of insulin antibodies and the occurrence of immune complexes in patients with and without microangiopathy. Since there are substantial differences in the results of different techniques for the detection of circulating immune complexes, two methods were employed, one detecting medium sized complexes in antigen excess through the Fc portion of complexed immunoglobulins - the solid phase Clq binding test (ClqSP) and the other detecting C3 binding complexes near the equivalence point - the conglutinin radioimmune assay (KgBt).

Patients were selected so as to have patients in opposite conditions, i.e. with severe microangiopathy and without any clinical signs of microangiopathy.

### B3.3b Materials and methods

#### Patients

140 diabetics treated with insulin for more than 1 year were selected according to the presence and degree of microangiopathy: 58 showed severe microangiopathy, 82 had no sign of vascular lesions. The clinical and personal data of the patients are shown in table B3.4/1. One hundred and eighty-nine blood donors were used as the control population in AgAb studies. The evaluation of the presence and degree of microangiopathy was performed as follows: retinopathy was assessed by retinal fluorangiography. Patients with proliferative retinopathy or recidivant vitreous haemorrhages were considered to have severe retinopathy and patients with the absence of any sign of retinopathy were considered free of ocular complications. Patients with intermediate forms of retinal involvement were excluded from the study. Blood urea  $>16$  mmol/l, serum creatinine levels  $>2$  mg/dl and/or proteinuria  $>2$  g/l were considered the index of kidney involvement.

#### Immune complexes

ClqSP as described by Hay, Nineham and Roitt(1976) and KgBt according to Casali et al.(1977), with some personal modifications, were used to detect immune complexes; The characteristics of the methods, the limit of positivity, the expression and analysis of the results for AgAb are discussed in chapter A3.1.

#### Insulin antibodies

Christiansen's electrophoretic method (Christiansen,1970) was used to detect InsAb respectively (chapter A3.3).The results were expressed in U/ml of insulin binding capacity

#### Statistical analysis

Fisher's exact test, the  $\chi^2$  test, Cox's test, the t-unpaired test and Kendall's test were used for the statistical evaluation of the results.

### B3.3c      R e s u l t s

#### Immune complexes

Clq-AgAb were found in 29 out of 58 patients with severe microangiopathy (50%,  $P < 0.0001$  vs normals) and in 20 out of 82 of those without microangiopathy (24%,  $P < 0.01$  vs normals,  $P < 0.001$  vs complicated diabetics). Kg-AgAb were found in 21 out of 58 patients without vascular lesions (36%,  $P < 0.0001$  vs normals) and in 20 out of 53 diabetics with severe microangiopathy (38%,  $P < 0.0001$  vs normals, N.S. vs uncomplicated diabetics).

#### Insulin antibodies

Mean InsAb levels, as evaluated by Christiansen's method, were 1.33 mU/ml in group 2 patients. There was no significant difference in InsAb levels between those with severe microangiopathy ( $1.27 \pm 1.47$  mu/ml) and those without complications ( $1.38 \pm 1.88$  mu/ml).

#### Immune complexes vs insulin antibodies

The correlation between Clq-AgAb and InsAb was significantly different in patients with and without microangiopathy Figure B3.3/1. In microangiopathic patients the positivity of Clq-AgAb did not vary significantly at increasing levels of InsAb, whereas in patients without microangiopathy, when InsAb levels were high, Clq-AgAb positivity was significantly reduced in comparison with that found in those with lower InsAb levels ( $P < 0.05$ ). Diabetics without microangiopathy having medium/high levels of InsAb ( $> 0.5$  mU/ml) showed a significantly reduced AgAb positivity in comparison with diabetics

with microangiopathy showing similar InsAb levels. A different correlation was found between Kg-IC and InsAb Figure B3.3/2. In group 2 there was a positive trend for Kg-IC positivity to go with increasing levels of InsAb ( $P < 0.05$ ). This trend was even more evident when only patients without microangiopathy were considered ( $P < 0.01$ ; Figure B3.3/2b).

TABLE B3.3/1

Clinical characteristics of the patients

|                             | Sex |    | Age<br>(mean $\pm$ s.d.) | Type of diabetes |    | Complications* |    |       |    |
|-----------------------------|-----|----|--------------------------|------------------|----|----------------|----|-------|----|
|                             | No  | F  |                          | I                | II | R              | N  | R + N |    |
| Diabetic patients           |     |    |                          |                  |    |                |    |       |    |
| with severe microangiopathy | 58  | 25 | 33                       | 49.5 $\pm$ 16.1  | 38 | 20             | 36 | 12    | 10 |
| without microangiopathy     | 82  | 44 | 38                       | 37.8 $\pm$ 16.7  | 67 | 15             |    |       |    |

\* R = proliferative retinopathy;  
 N = advanced nephropathy;  
 R + N = both complications.



FIGURE B3.3/1

The association between AgAb, as detected by Clq, and insulin antibodies (InsAb) in patients with (a) and without (b) complications. InsAb are indicated in the Y axis and divided into negative, low (0.05-0.5 mu/ml), medium (0.5-2 mu/ml) and high (>2 mu/ml) levels.

FIGURE B3.3/2

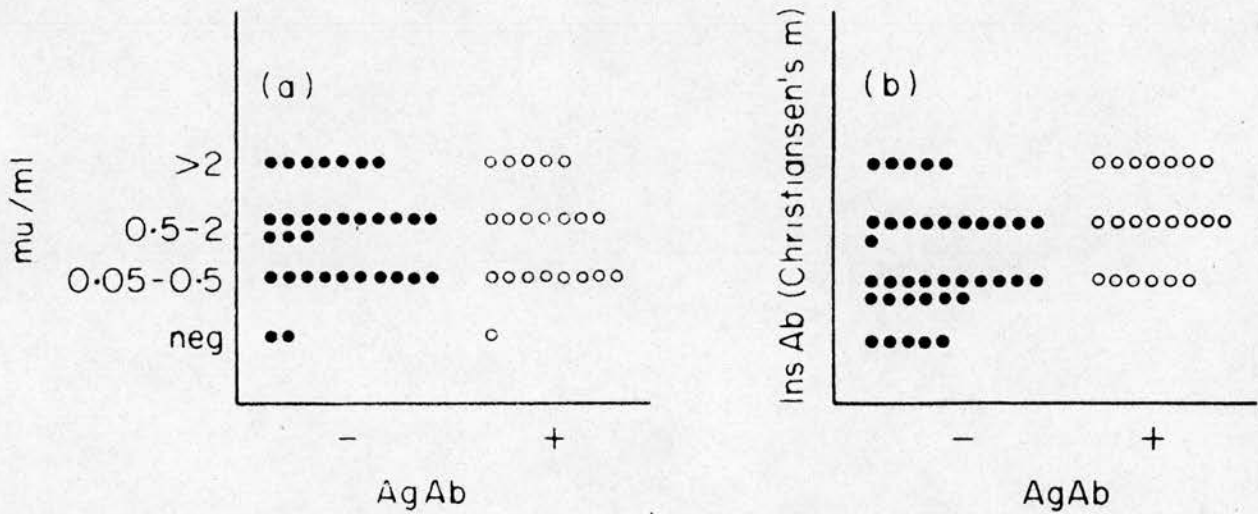
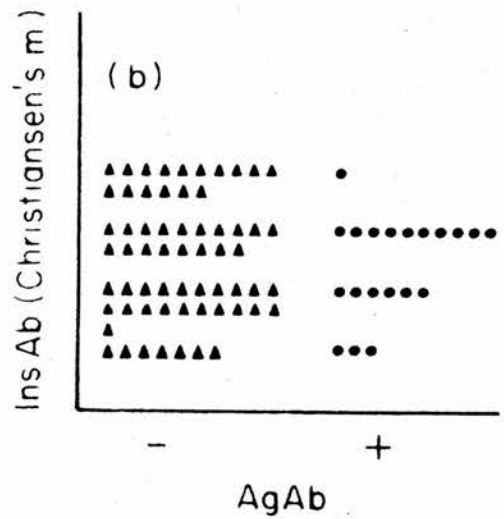
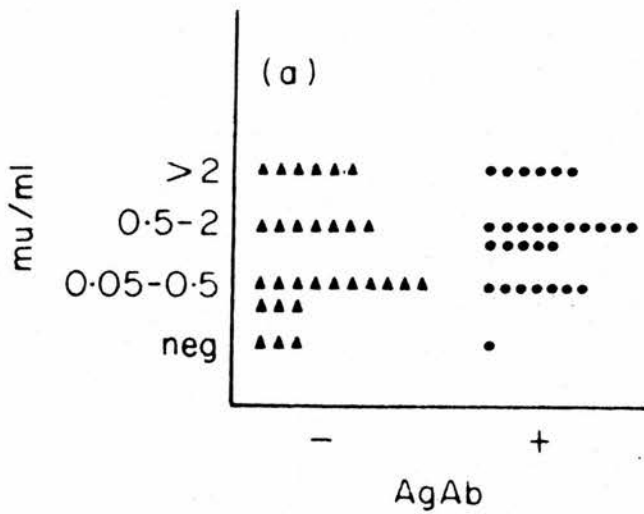


FIGURE B3.3/2

The association between immune complexes (AgAb), as detected by conglutinin (Kg-IC), and insulin antibodies (InsAb) in patients with (a) and without (b) complications. InsAb as in Fig. B3.3/1.

FIGURE B3.3/4



B3.3d      D i s c u s s i o n

The relationship between InsAb and immune complexes is different when Clq-AgAb or Kg-AgAb are studied.

In insulin treated diabetics selected at random, when InsAb are present in high amounts, Clq-AgAb are not usually detectable. Since ClqSP does not readily detect complexes in antibody excess (Scullion et al,1979),in patients with high levels of InsAb, insulin antibody complexes would not be expected to be detectable by Clq-SP. It is conceivable that in patients with lower InsAb levels some immune complexes may be related to insulin treatment. In fact, in a previous study, Clq-AgAb were found to be higher in randomly selected insulin treated patients than in comparable diabetics not treated with insulin (Chapter B2.3). Similar results were found in patients without microangiopathy. It is interesting to note that when microangiopathy is present, Clq-AgAb levels are much higher and not correlated with the levels of InsAb. This study confirms the increase in Clq-AgAb in diabetics with microangiopathy observed in previous studies (Chapters B3.2b, B3.2c) . When microangiopathy is present, some factors appear to modify the dynamics of formation and clearance of medium-large sized complexes, leading to an increase in circulation of these macromolecules. These factors do not seem to be directly related to insulin treatment since Clq-AgAb were increased both in type I and in type II diabetics with microangiopathy (Chapter B3.2c).It has been suggested that impairment of phagocytosis, described in patients with microangiopathy (Drivas & Wardle, 1978;Bagdade,1976),might result in an increase of circulating

macromolecules. Indeed in diabetic patients with an impaired phagocytic clearance it has also been shown an increase in circulating Clq-AgAb (Chapter B3.2d). It is suggested that in diabetics with microangiopathy an impairment of the phagocytic system leads to an increase in heterogeneous complexes, mainly in the range detected by ClqSP, and masks any correlation between immune complexes and InsAb.

When Kg-AgAb results are evaluated, there is a trend for the presence of Kg-AgAb to go with medium high levels of InsAb (especially when microangiopathy is absent). An association between Kg-AgAb and InsAb has been reported also in diabetics with less than 1 year's diabetes (Chapter B2.5). Likewise, the prevalence of Kg-AgAb is higher in type I diabetics irrespective of the presence of microangiopathy (Chapter B3.2c). It appears that KgBt detects immune complexes which are somehow related to the presence of InsAb rather than to the occurrence of diabetic microangiopathy. The reason for this correlation is not obvious. Kg-AgAb are usually large complexes that fix complement and therefore are very different from most insulin-anti-insulin complexes. It cannot be excluded that KgBt reacts with some monomeric immunoglobulins, when present in very high concentrations. The levels of InsAb in these patients were not associated with the presence of microangiopathy. This is in keeping with the lack of association between Kg-AgAb and microangiopathy.

In this study there is no clear evidence of the influence of heterologous insulin treatment on diabetic microangiopathy. To some extent this result might have been predicted. If insulin treatment has any influence on the course of complications through immune mechanisms (i.e. the formation of insulin-anti-insulin complexes),

the insulin binding capacity is not the best tool for investigating this problem. Insulin antibodies vary widely in avidity and may form complexes with different pathogenetic significance. The methods for detecting InsAb usually available, and particularly those used in this investigation, give information on the amount, and not the avidity, of the antibody and measure mostly free antibodies. It is therefore not surprising that the sole immunological phenomenon found in association with the presence of severe diabetic microangiopathy is related to two other questions: does insulin treatment give rise to complexes of pathogenic significance, and do these complexes and the Clq-AgAb - chronically accumulated and deposited in the small blood vessels - play a role in aggravating local tissue damage?

B3.4 IMMUNE COMPLEXES, INSULIN ANTIBODIES, INSULIN-ANTI-INSULIN COMPLEXES RELATED TO PLATELET AND COAGULATION FACTORS IN PATIENTS WITH PROLIFERATIVE RETINOPATHY.

B3.4a Aims of the study

In this study the relationship of soluble immune complexes, insulin antibodies and insulin-anti-insulin complexes to platelet function and coagulation has been investigated in two groups of Type I diabetics chosen to represent the extremes of the retinopathy spectrum, namely those with proliferative retinopathy and those without retinopathy, and in non-diabetic controls.

B3.4b Patients and methods

Patients

20 Type I diabetics with proliferative retinopathy (mean age  $35 \pm 11$  years, mean duration of diabetes  $19 \pm 7$  years), 20 Type I diabetics without ophthalmoscopic evidence of retinopathy (mean age  $34 \pm 9$  years, mean duration of diabetes  $21 \pm 7$  years) and 20 healthy

non-diabetic controls (mean age  $35 \pm$  years), were studied. Subjects in each group were individually matched for age, sex (12 male, 8 female) and, where applicable, duration of diabetes. All subjects had a normal serum creatinine and absence of proteinuria on Albustix testing. Two of the diabetics with retinopathy had signs and/or symptoms of macrovascular disease.

A 2 hr post-prandial venous blood sample was withdrawn with minimal venous stasis from resting subjects and analysed as follows:

#### Immune complexes

Soluble immune complexes (AgAb) were evaluated by a modification of the conglutinin radioimmunoassay method (Casali et al,1977). The technical aspects, limit of positivity and expression of results have been described in chapter A3.1.

#### Insulin antibodies

Insulin antibodies (InsAb) were assayed according to the method of Andersen et al,1972, as modified by Mustaffa et al,1977 (Chapter A3.3).

#### Insulin-anti-insulin complexes

Insulin-anti-insulin complexes (Ins/iAb) were detected using a modification (Chapter A3.4) of the techniques described by Jayarao et al, 1973, and Virella et al,1980.

### Platelet and coagulation factors

Beta-thromboglobulin (beta-TG), platelet factor 4 (PF4), fibrinopeptide A (FpA), fibrinogen (Factor 1), antithrombin III activity (AT-III), and Factor VIII related antigen (VIII R:Ag) were measured routinely by the Edinburgh Blood Transfusion Centre (Borsey et al, 1984)

### Statistical analysis

Parametric and non parametric tests were used in normally and asymmetrically distributed data respectively. Student's t test, Fisher's exact test and Wilcoxon's test were used when appropriate.



B3.4      R e s u l t s

## Immune complexes

Immune complexes were found in 12 out of the 20 patients with retinopathy ( $p < 0.05$  vs the control group) and in 8 out of the 20 without retinopathy. There was no significant difference in immune complex levels between non-retinopathic patients and controls or patients with retinopathy.

## Insulin antibodies

All the diabetics had detectable insulin antibodies but there was no significant difference in insulin antibody levels between patients with retinopathy (median 54  $\mu\text{u/ml}$ , interquartile range 0 IR 20.5-135) and without retinopathy (median 117.5  $\mu\text{u/ml}$ , IR 31.5-161.5) (Figure B3.4/1).

## Insulin-anti-insulin complexes

Ins/iAb were found in 7 out the 20 patients with retinopathy (mean of the positive values  $22.5\% \pm 11.1$ ) and in 10 (mean 21.8%, 8.3) out the 20 without retinopathy (Figure B3.4/2).

## Immune complexes vs insulin-anti-insulin complexes

5 of the 7 retinopathic patients and 4 of the 10 non-retinopathic patients with circulating Ins/iAb also had AgAb in their serum.

## Immune complexes vs insulin antibodies

There was no significant correlation between immune complexes and insulin antibodies.

### Insulin antibodies vs insulin-anti-insulin complexes

There was no constant parallelism between the levels of detectable Ins/iAb and levels of insulin antibodies. However patients with retinopathy and detectable Ins/iAb in their serum showed levels of insulin antibodies (median 137 mU/ml, IR 45-430) higher than in those with Ins/iAb within the normal limits (median 42 mU/ml, IR 17-97). When the non-retinopathic diabetics were likewise divided into those with and without detectable Ins/iAb similar findings were observed (median 161 mU/ml, IR 134-260 and median 31.5 mU/ml, IR 13-96 respectively).

### Platelet and Coagulation Factors

Beta-TG and  $PF_4$  (Table B3.4/1) were higher in patients with retinopathy than in the controls ( $p < 0.01$ ) and  $PF_4$  was also elevated in the non-retinopathic patients compared to the controls ( $p < 0.05$ ). A highly significant correlation between beta-TG and  $PF_4$  was seen in each of the three groups (controls and retinopathic patients  $p < 0.01$ , non-retinopathic patients  $p < 0.01$ ).

Fibrinogen was elevated in non-retinopathic patients vs. controls ( $p < 0.01$ ), retinopathic diabetics vs. controls ( $p < 0.001$ ) and retinopathic vs. non-retinopathic patients ( $p < 0.05$ ).

There was no significant difference in FpA levels between groups, but FpA correlated with both beta-TG ( $p < 0.05$ ) and  $PF_4$  (controls and non-retinopathic patients  $p < 0.05$ ; retinopathic patients  $p < 0.01$ ) within each of the groups.

AT-III was increased in retinopathic patients vs. controls  $p < 0.001$ ; and in retinopathic vs. non-retinopathic patients  $p < 0.01$ .

Factor VIII R:Ag was higher in both the diabetic groups than in the controls ( $p < 0.001$ ).

#### Immune factors vs Haemostatic Factors

In diabetics with proliferative retinopathy there was a significant correlation between positive values of AgAb and increased levels of  $PF_4$  and beta-TG (Figure B3.4/3). In fact  $PF_4$  and beta-TG values were higher in retinopathic patients with detectable complexes ( $PF_4$  mean 32.7, SD 16.7; beta-TG mean 72, SD 30.8) than in those without complexes ( $PF_4$  mean 15.1, SD 11.4; beta-TG mean 45, SD 17.1) ( $p < 0.05$  and  $p < 0.05$  respectively).

Although there was no parallelism between the levels of insulin antibodies and any of the haemostatic factors, the presence of insulin-anti-insulin complexes was significantly correlated with increased levels of beta thromboglobulin in the diabetics with retinopathy ( $80.1 \pm 26.1$  vs.  $50.6 \pm 25.3$  in Ins/iAb negative patients,  $p < 0.05$ ).

When retinopathic patients showing the presence of both complexes and insulin-anti-insulin complexes were compared to retinopathic patients without any kind of complexes, a positive correlation between complexes and abnormalities of platelet factors was apparent in the former group ( $PF_4$   $38.7 \pm 14.7$  vs.  $13.1 \pm 10.1$ ,  $p < 0.02$ ; beta-TG  $78.4 \pm 27.4$  vs.  $39.6 \pm 16$ ,  $p < 0.02$ ) and also a correlation between complexes and fibrinopeptide A became apparent (FpA  $7 \pm 5.6$  vs.  $1.2 \pm 1$   $p < 0.05$ ). Significant correlations between immune and haemostatic factors were not found in non-retinopathic patients and in controls.

TABLE B3.4/1 Platelet and coagulation factors (median and range) in the three study groups

|            |       | Diabetics with<br>pr. retinopathy | Diabetics without<br>retinopathy | Normal controls  |
|------------|-------|-----------------------------------|----------------------------------|------------------|
|            | units |                                   |                                  |                  |
| beta-TG    | ng/ml | 51.5 (24.1-127.5)                 | 44.9 (22.2-96.3)                 | 31.5 (19.1-68.5) |
| PF4        | ng/ml | 19.6 (6.7-63.7)                   | 17.5 (8.5-72.6)                  | 10.4 (10- 52)    |
| Fibrinogen | g/l   | 2.93 (1.85-5.70)                  | 2.46 (1.56-3.46)                 | 1.70 (1.04-2.93) |
| FpA        | ng/ml | 2.80 (2.00-17.8)                  | 2.82 (2-16.6)                    | 1.64 (1.6-19.2)  |
| AT-III     | %     | 116 (92-132)                      | 104 (92-122)                     | 103 (82-119)     |
| VIII R:Ag  | %     | 267 (142-400)                     | 252 (126-460)                    | 98 (72-140)      |

FIGURE B3.4/1

Insulin antibody levels in patients with proliferative retinopathy (D1) and in those without any clinical signs of retinopathy (D2).

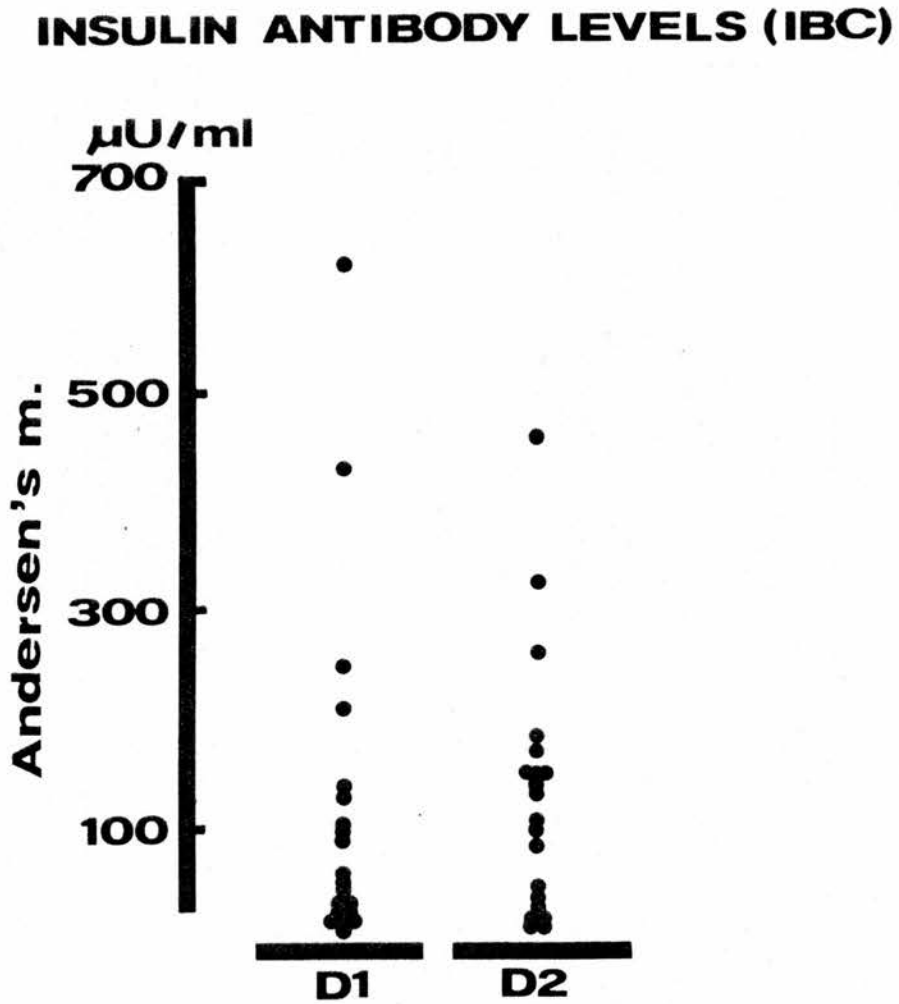


FIGURE B3.4/2

Insulin-anti-insulin complexes in patients with proliferative retinopathy (D1) and in those without any clinical signs of retinopathy (D2).

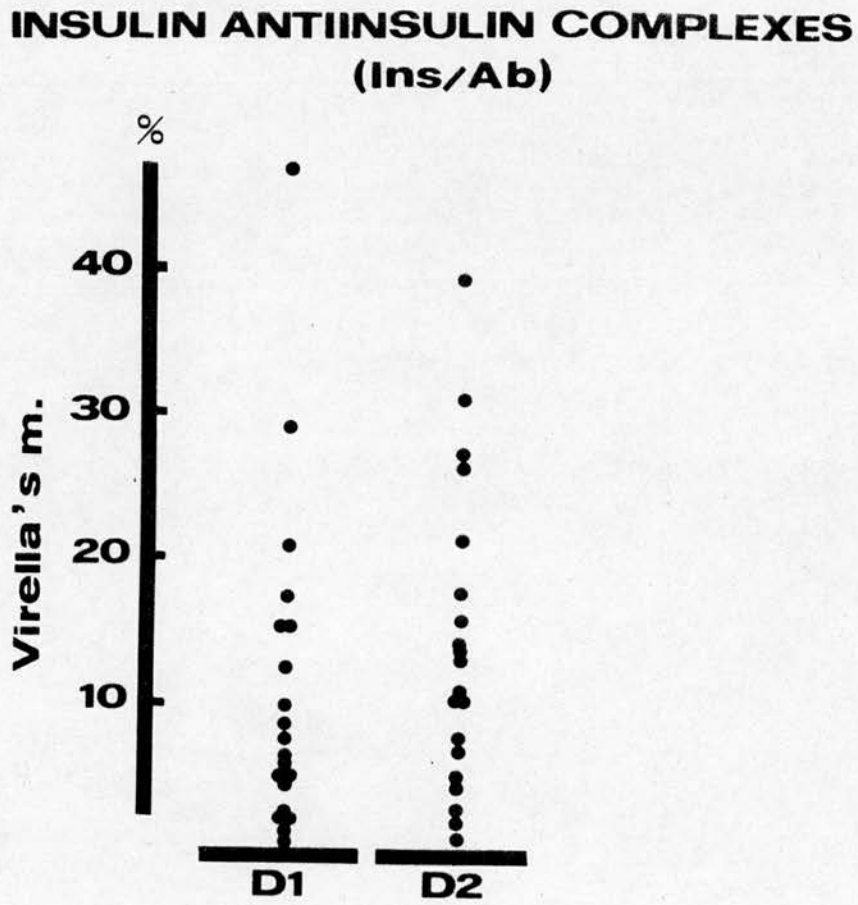


FIGURE B3.4/3

The relationship between AgAb and platelet specific proteins (PF4 and BTG) in diabetics with proliferative retinopathy.

**Ag/Ab and platelet specific proteins  
in diabetics with proliferative retinopathy**



B3.4d      D i s c u s s i o n

In diabetic retinopathy the causes and the sequence of events are still unclear. Metabolic abnormalities are thought to play a major role in initiating the vascular lesions (Cudworth et al,1982). Nevertheless, the metabolic derangement cannot explain many aspects and steps in the development of microangiopathy. It is known that the progression of retinopathy does not parallel the average degree of the metabolic control in many patients: malignant retinopathy may occur soon after the clinical onset of the disease in patients with acceptable metabolic control and with a mild form of the disease (Andreani,1980). At least in microangiopathic lesions of the kidney , the metabolic control seems to have importance only in the first phases of the disease (Cudworth et al,1982). Epidemiological studies in diabetic twins and other genetic studies support the concept that inherited constitutional factors influence the progression and the severity of retinopathy (Leslie et al,1982;Bodansky et al,1982). Furthermore a number of abnormalities of the immunological function (Jayarao et al,1974; Virella et al,1980;Chapters B3.2c, B3.2d) and of haemostasis (Jones et al,1981; Borseley et al,1984,Cudworth et al,1982) have been already reported in patients with proliferative retinopathy.

In this study the presence of both immune and haemostatic abnormalities in diabetics with retinopathy have been confirmed. Attention has been focussed on the role of factors, other than the metabolic control, and in particular on the possible reciprocal influence and modulation of immunological and haemostatic factors,



trying to understand their significance in the general multifactorial context.

In keeping with previous studies in which immune complexes were evaluated by the conglutinin method (Chapter B3.2c) immune complexes were significantly increased in diabetics with proliferative retinopathy when compared with normal subjects. The finding of a similar prevalence of insulin-anti-insulin complexes in diabetics with and without retinopathy was not unexpected since it has been reported that the frequency of insulin antibodies in these two groups does not differ significantly (Chapter B3.3). Beta-TG,  $PF_4$ , AT-III, VIII R:Ag and fibrinogen were increased in the diabetics with retinopathy when compared with the controls whilst fibrinogen and AT-III were also significantly higher in the retinopathic than in the non-retinopathic patients. Thus, platelet function and coagulation are deranged in Type I diabetics and some of the abnormalities are more apparent when retinopathy is present.

The finding of a correlation between humoral immune phenomena and abnormalities of haemostasis in diabetics with retinopathy has not been previously reported and raises many intriguing possibilities as to the pathogenesis of microangiopathy. In these patients the presence of immune complexes was significantly correlated with higher levels of Beta-TG and  $PF_4$  and in immune complex- and insulin complex-positive patients a significantly greater number of platelet and coagulation factors were elevated. The platelet specific proteins Beta-TG and  $PF_4$  are released together from alfa granules during platelet aggregation and elevated plasma levels may reflect enhanced in vivo platelet activation (Kaplan & Owen, 1981). Immune complexes

are removed from the circulation in different ways according to their size: small complexes are eliminated by the kidney whereas the medium and large complexes, such as those detected by the conglutinin method, are cleared by reticuloendothelial cells (Mannik, 1974). When phagocytic clearance is inefficient or when other factors, such as the presence of vasoactive substances, intervene, complexes may be deposited in the blood vessel walls. Platelets are the most likely source of vasoactive amines since they are rich in both histamine and serotonin. Previous work has shown that the deposition of microaggregates is directly correlated with factors which favour increased vascular permeability (Figure B3.4/4) (Williams, 1980). Local immune complex deposition is markedly diminished by agents which block the effect of vasoactive amines and by platelet depletion. On the other hand, immune complexes are among the factors favouring platelet adhesion and aggregation and thereby may induce the release of vasoactive substances and contribute to the vascular damage (Figure B3.4/5). In fact *in vitro* studies have shown that Ins/Ab may activate platelet aggregation in the blood of diabetic subjects (Virella et al, 1983).

This study shows a possibly interesting correlation between immunological and haemostatic factors. Since the pathogenesis of diabetic microangiopathy appears to be multifactorial, with an individual variability on a genetic basis, our findings suggest that an interaction between immune complexes or insulin-anti-insulin complexes and platelets may aggravate the pathological events in the development of diabetic retinopathy at least in some individuals or at certain stages of the pathogenetic sequence.

FIGURE B3.4/4

Schematic representation of the interaction between immune complexes, platelets and endothelial cells.

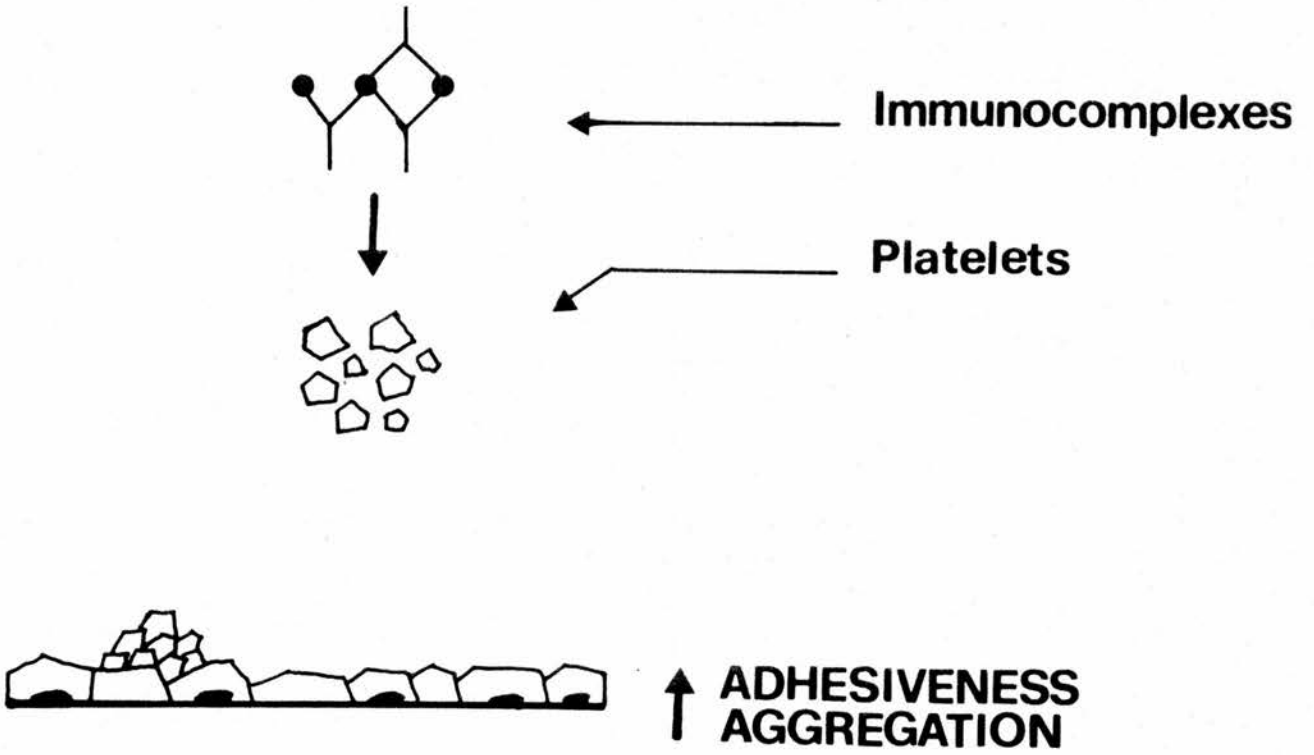
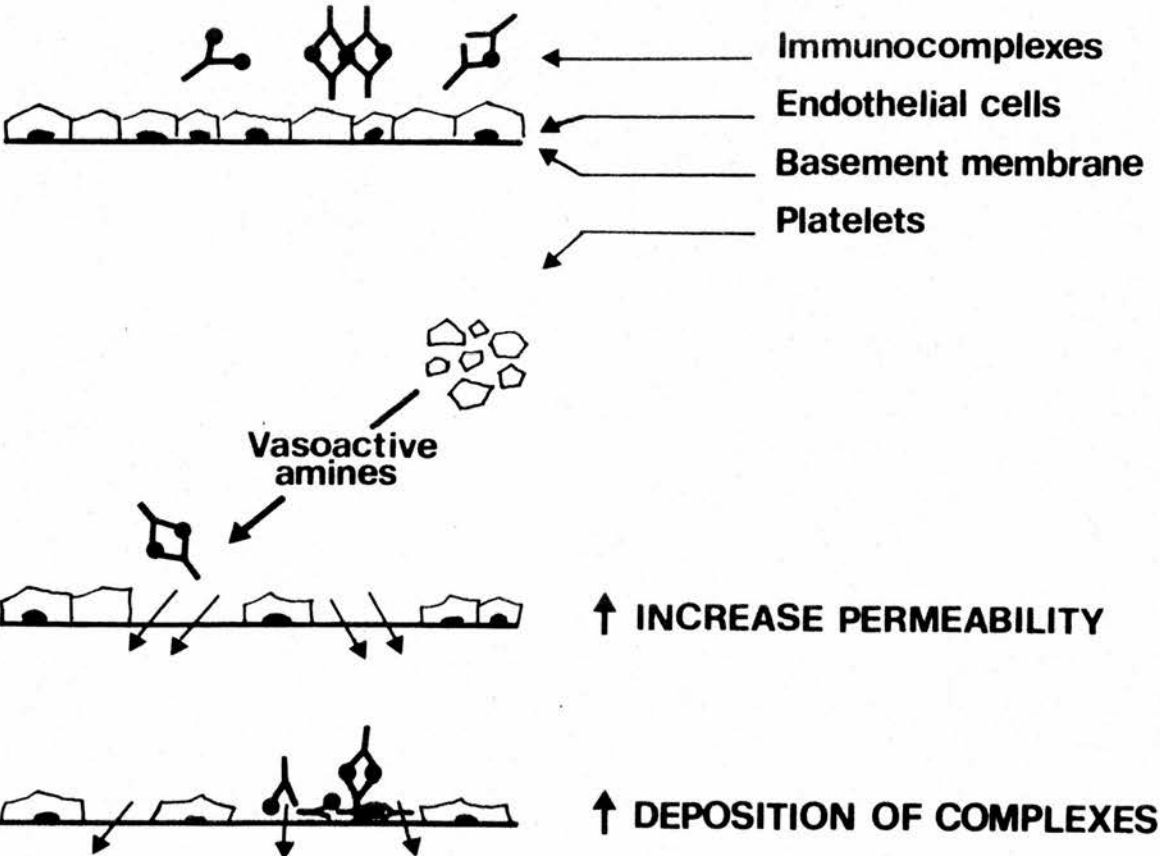


FIGURE B3.4/5

Schematic representation of the possible influence of platelet vasoactive substances on vascular permeability and on the deposition of complexes.



B3.5 IMMUNE COMPLEXES AND LYMPHOCYTE SUBSETS IN PATIENTS WITH  
MICROANGIOPATHY

B3.5a Aim of the study

The percentage of peripheral lymphocyte subsets and the occurrence of AgAb in diabetic patients of long duration has been evaluated in order to investigate a possible relationship, independent of the type of diabetes, between these two factors and the presence of microangiopathy.

B3.5b Materials and Methods

P a t i e n t s

The following subjects, attending the Rome University's Diabetic Clinic, were randomly selected and included in the study:

- 19 Type 1 diabetic patients (11 males, 8 females, mean age: 45 yr, age range 27-63 yr, mean duration of disease: 16 yr, range: 12-45 yr);
- 17 Type 2 diabetic patients (10 males, 7 females, mean age: 57

yr, age range: 42-70 yr, mean duration of disease: 16 yr, range: 10-30 yr);

- 22 normal subjects (10 males, 12 females, mean age: 40 yr, age range 24-62 yr) were included as a control group for lymphocyte studies.

One-hundred and fifteen sera from normal volunteers were used as controls in AgAb studies. In all patients, the presence of microangiopathy was evaluated by retinal fluoroangiography, albuminuria and creatinine clearance.

#### T cell subsets

Peripheral lymphocytes were obtained by gradient centrifugation of venous blood on Ficoll-Hypaque. The following monoclonal antibodies defining antigens expressed on the surface of peripheral lymphocytes were used: UCHT1 which reacts with 90% of E-rosetting cells (T cells) (Beverley and Callard, 1981); Leu 3a, which phenotypes the helper/inducer T cells subset ( $T_4$ ) (Ledbetter et al, 1981); UCHT4, which identifies the suppressor/cytotoxic T cells ( $T_8$ ) (Beverley, 1982). The UCHT4 antibody is analogous to OKT8 or Leu 2a monoclonal antibodies which phenotype the  $T_8^+$  cells (see chapter A3.2).

As total T cells have been reported previously to be normal in patients with Type 2 diabetes (Cattaneo et al, 1976), it was decided in these patients to use only monoclonal antibodies phenotyping the immunoregulatory lymphocytes (Leu 3a and UCHT4).

The indirect immunofluorescence technique using fluorescinated rabbit anti-mouse antiserum was utilised for detecting positive lymphocytes for each monoclonal antibody. The percentage of positive cells was based on a minimum count of 300 cells.

### Immune complexes

AgAb were measured by two different techniques, the Clq solid phase assay and the conglutinin binding method (Lambert et al,1978). The limit of positivity of AgAb assays was evaluated as reported in chapter A3.1.

### Statistical analysis

The Mann-Whitney U-test and the Fisher exact probability test were used.

B3.5c      R e s u l t s

## T cell subsets

In type 1 diabetic patients a significant decrease of total T cells ( $P < 0.01$ ),  $T4^+$  cells ( $P < 0.01$ ) and a significant increase of  $T8^+$  cells ( $P < 0.05$ ), were observed (Fig.B3.5/1). In patients with type 2 diabetes, the percentages of  $T4^+$  and  $T8^+$  cells were not statistically different from those of normal subjects (Figure B3.5/2). It can also be assumed that in patients with type 2 diabetes, the percentage of total T cells was not different from controls since the sum of  $T4^+$  and  $T8^+$  cells gave a result that was similar to that detected in normal subjects. However, 5 out 18 patients with type 2 diabetes showed a percentage of  $T8^+$  cells which was at least 2 SD above the mean percentage found in normal subjects.

## Immune complexes

When the Clq assay was used, AgAb were found in 41% of type 1 diabetic patients and in 25% of type 2 diabetic patients ( $P < 0.02$ ) whereas with the conglutinin binding method, the percentages were 63% and 23%, respectively ( $P < 0.01$ ).

## Relationship between

## T cell subsets and immune complexes

There was no relationship between the presence of AgAb, as measured by the Clq assay, and values for any of the lymphocyte subsets. However, when AgAb were evaluated by the conglutinin binding method, a relationship with the  $T8^+$  cells was observed (Table



A3.5/1). Thus, in 11 patients with very high  $T8^+$  cells (2 SD above the normal mean, i.e. >26%), AgAb were present only in one patient (9%). By contrast, in 25 patients with  $T8^+$  cells within the normal range, AgAb were present in 15 patients (60%) (Table B3.5/1).

#### Relationship between

##### T cell subsets, immune complexes and microangiopathy

Twenty-four patients (12 Type 1 and 12 Type 2) showed varying degrees of retinopathy (background retinopathy n=16, including two patients with nephropathy; proliferative retinopathy n=8, including one patient with nephropathy). Twelve patients (7 Type 1 and 5 Type 2) had no signs of microangiopathy. Interestingly, the incidence of microangiopathy was correlated with the level of  $T8^+$  cells and AgAb as measured by the conglutinin method. Thus, 7 out of 11 patients with raised  $T8^+$  cells (>26%) had no signs of retinopathy or nephropathy and only one was AgAb positive. By contrast, 20 out of 25 patients with  $T8^+$  cells within the normal range showed retinopathy and 11 were also AgAb positive (Table A3.5/1).

TABLE B3.5/1

The relationship between levels of T8<sup>+</sup> cells (UCTH4 positive) and circulating immune complexes and retinopathy

|                                | Immune complexes |          | Retinopathy               |        |
|--------------------------------|------------------|----------|---------------------------|--------|
|                                | Positive         | Negative | Present                   | Absent |
| UCTH4 (>26%) (n=11)            | 1                | 10       | 4 (3A, 1B) <sup>a</sup>   | 7      |
| UCTH4 (<26%) (n=25)            | 15               | 10       | 20 (13A, 7B) <sup>a</sup> | 5      |
| Fisher exact probability test: | P=0.005          |          | P=0.014                   |        |

<sup>a</sup> A = background retinopathy; B = proliferative retinopathy. In patients without retinopathy, nephropathy was also absent. Raised levels of UCTH4 positive lymphocytes are considered those exceeding 2 SD mean observed in normal subjects.

FIGURE B3.5/1

The percentages of total T cells (UCHT1 positive), T4 cells (Leu 3a positive) and T8 cells (UCHT4 positive) in 19 patients with Type 1 diabetes of long duration and in 22 normal subjects.

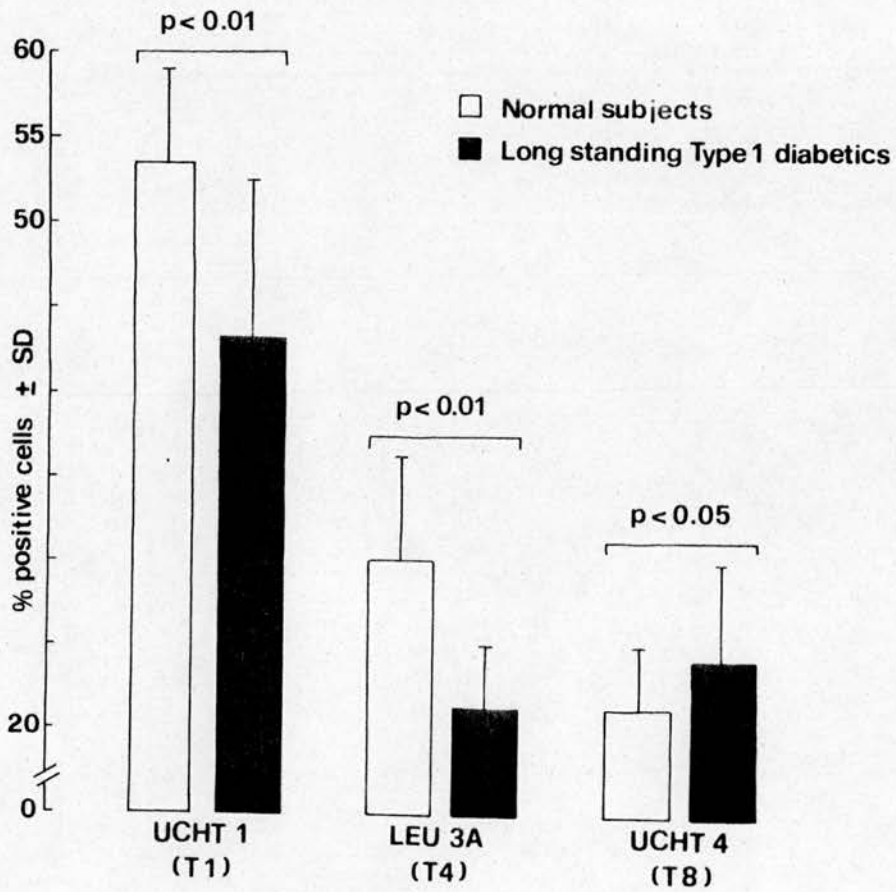
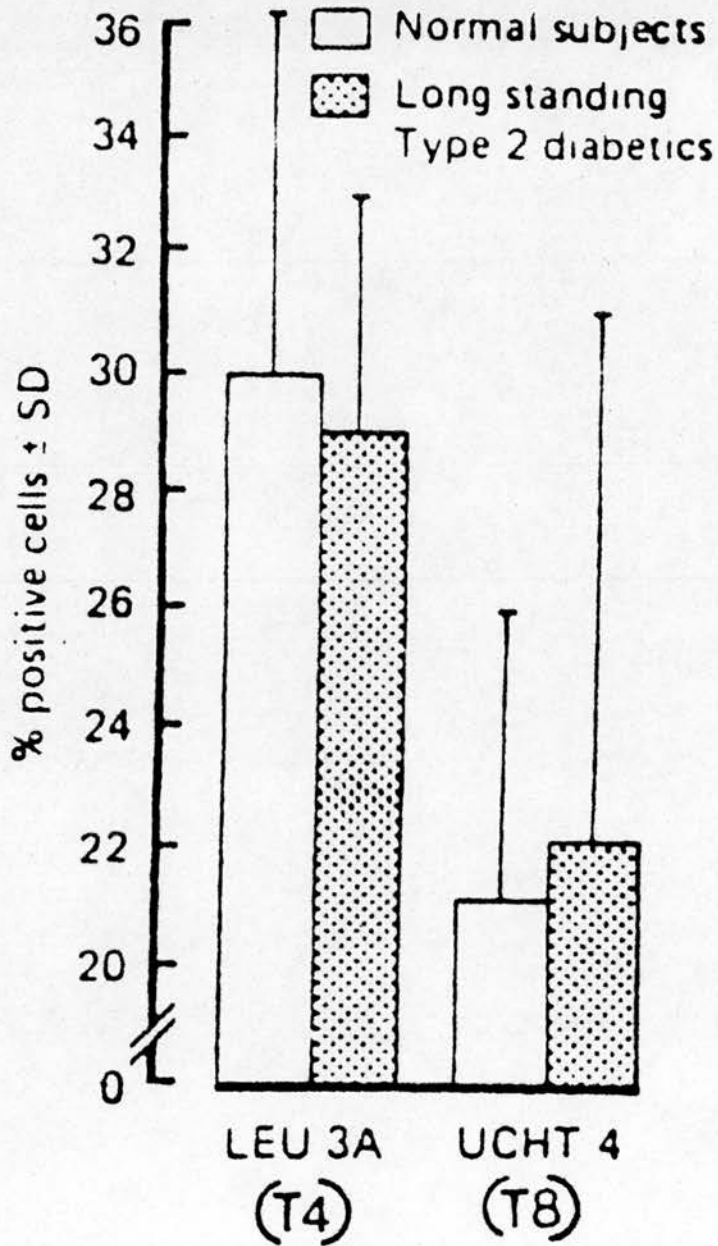


FIGURE B3.5/2

The percentages of T4 cells (Leu 3a positive) and T8 cells (UCHT4 positive) in 17 patients with Type 2 diabetes of long duration and in 22 normal subjects.



B3.5d Discussion

The present findings suggest an inter-relationship among lymphocyte subsets, immune complexes and the presence of microangiopathy. The main feature consists of an increase of  $T8^+$  cells in diabetic patients without immune complexes and no evidence of microangiopathy, despite the long duration of disease. This concept is reinforced by the opposite finding in patients with microangiopathy.

The other findings comprise a decrease of  $T4^+$  cells in type 1 diabetics with long duration of disease. As has been described herein (chapter B3.2b), the presence of AgAb is closely related with the presence of microangiopathic changes rather than with the duration of the disease. Data are now accumulating to suggest that the presence and persistence of AgAb are strictly dependent not only upon impaired function of fixed phagocytes, but also upon the number of mononuclear cells (Moretta et al, 1978; Fridman et al, 1981) which phenotypically have the characteristics of  $T8^+$  cells and functionally could be suppressor/cytotoxic cells (Beverley and Callard, 1981). The latter aspect, however, is still unclear and needs further elucidation.

The majority of cells with  $T8^+$  phenotype (defined by the UCHT4 antibody) possess receptors for the Fc portion of IgG (Fc $\gamma$ R) (Beverley and Callard, 1981). Others have demonstrated that AgAb have an affinity for the Fc $\gamma$ R even greater than for native IgG (Dickler, 1976). This phenomenon is likely to be a consequence of the multiple binding which occurs between Fc $\gamma$ R and the Fc regions of Ig when the latter are aggregated in an immune complex (Moretta et al, 1978). Therefore, as most of  $T8^+$  cells have Fc $\gamma$ R, it is likely

that these cells are capable of binding AgAb. On this basis, the relative increase of  $T8^+$  cell subset could explain the absence of detectable levels of circulating AgAb in some diabetic patients. These cells may have a role in controlling the levels of at least some types of AgAb by removing them from circulation and thus preventing their deposition over the vessel walls.

An alternative hypothesis for a favourable role played by  $T8^+$  cells is based on their function as suppressor cells. Their increase may contribute to a reduction in antibody formation by specific suppressor factors (Fridman et al,1977), and thereby limiting the formation of AgAb.

In the present study we have confirmed but also extended, using different monoclonal antibodies, previous data indicating a decrease of  $T4^+$  cells in patients with Type 1 diabetes of long duration have been confirmed and extended (Mascart-Lemone et al,1982). As  $T4^+$  cells represent the majority of the overall T lymphocyte population, the significant decrease of these cells in type 1 diabetic patients with long duration of disease may account for the reduction of total T cells reported previously (Cattaneo et al,1976;Pozzilli et al,1979). This suggests that the known susceptibility to infections in poorly controlled diabetic patients could be partly due to a reduction of  $T4^+$  cells.

A prospective study is now needed of lymphocyte subsets and of circulating serum factors such as immune complexes or other macromolecules, in particular alpha-2-macroglobulin. The latter protein is increased in patients with microangiopathy (Almer & Pandolfi,1976) and it has been recently shown that alpha-2-macroglobulin can affect lymphocyte responses in vitro (Miyayaga et al,1982). Future work

should then highlight the role of these immune parameters in influencing the appearance and evolution of diabetic microangiopathy.

### B3.6 CONCLUSIONS

When selected groups of diabetics with microangiopathy have been studied using a technique based on Clq properties, a correlation appears between the presence of immune complexes and the occurrence of severe microangiopathy. Immune complexes are increased in long standing diabetics with severe microangiopathy and in those with a precocious onset of retinopathy. Immune complexes found in patients with severe microangiopathy do not seem to have any correlation with the type of treatment and this finding is in keeping with the clinical observation that microangiopathy occurs irrespective of the type of treatment. This enhancement of immune complex levels seems to be due to a decreased rate of clearance by an impaired reticuloendothelial system in patients with severe microangiopathy.

Immune complexes detected by Clq correlated with the presence of microangiopathy but not with insulin antibody levels. On the other hand, insulin antibodies have been found to correlate with complexes detected by conglutinin but neither of these two correlate with the presence of microangiopathy.

A significant correlation between positive values of immune complexes and increased levels of platelet specific proteins in diabetics with proliferative retinopathy was found. This suggests



that an interaction between platelets and complexes may have pathological relevance in the development of diabetic retinopathy.

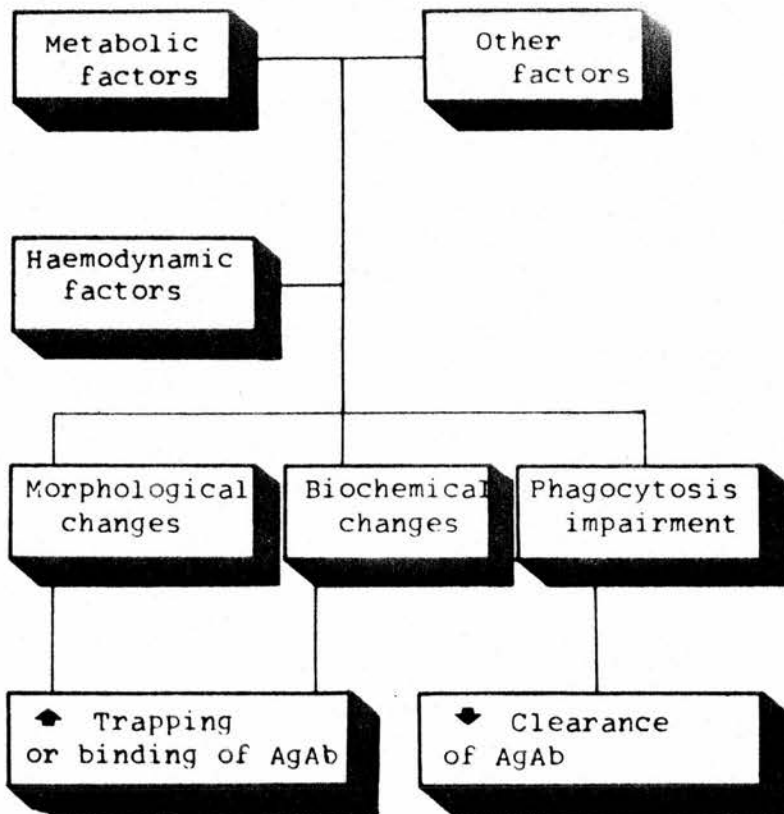
Patients with very high levels of T8 positive cells did not have detectable immune complexes and had no evidence of microangiopathy. By contrast, patients with normal levels of these cells were found to have raised immune complexes and showed retinopathy of varying degree. This relationship between cells with the T8 phenotype, some immune complexes and the presence of microangiopathy suggests that the presence and persistence of complexes and their deposition or prevention may be partly dependent upon the number of circulating mononuclear cells with Fc or C receptors.

The immunological phenomena occurring in diabetic microangiopathy are probably a limited aspect of the complicated multifactorial events leading to vessel damage. Nevertheless, evidence accumulated so far confirms that these phenomena form an important step in the building up of microangiopathy (Figure B3.6/1). The deposits of immunoglobulins, complement and complexes in microangiopathic vessels are frequent and striking. Even though experienced only in animals, immune mechanisms may well induce microangiopathic-like lesions. Circulating, potentially lesive complexes are increased in diabetics with microangiopathy and they are capable of interacting with the complement, platelets and lymphocytes. An impairment of macromolecule clearance is present in patients with severe complications. Biochemical changes of the basement membrane present in diabetes with microangiopathy may well favour the binding or trapping of macromolecules. The pathogenetic relevance of these phenomena is

still to be definitely determined. It is conceivable that immune complexes of different types, chronically accumulated in small amounts over a prolonged period of time secondarily to other events, are likely to activate the complement and the coagulation cascade and aggravate the local damage of blood vessel walls.

FIGURE B3.6/1

Possible pathogenetic involvement of circulating immune complexes in the development of microangiopathy



CHAPTER B 4

IMMUNOLOGY OF DIABETIC PREGNANCY

Chapter B4      IMMUNOLOGY OF DIABETIC PREGNANCY

- B4.1    Experimental design of the studies on diabetic pregnancy
- B4.2    Humoral immunity in diabetic pregnancy :  
Islet cell antibodies, organ specific auto antibodies,  
insulin antibodies and immune complexes.
  - B4.2a    Aim of the study
  - B4.2b    Materials and methods
  - B4.2c    Results
  - B4.2d    Discussion
- B4.3    Humoral immunity in diabetic pregnancy : insulin antibodies and insulin-anti-insulin complexes
  - B4.3a    Aim of the study
  - B4.3b    Materials and methods
  - B4.3c    Results
  - B4.3d    Discussion
- B4.4    Cellular immunity in diabetic pregnancy : T cell subsets
  - B4.4a    Aim of the study
  - B4.4b    Materials and methods
  - B4.4c    Results
  - B4.4d    Discussion
- B4.5    Cellular immunity in diabetic pregnancy : activated T cells

B4.5a Aim of the study

B4.5b Materials and methods

B4.5c Results

B4.5d Discussion

B4.6 Conclusions

## B4.1      EXPERIMENTAL DESIGN OF THE STUDIES ON DIABETIC PREGNANCY

Diabetic pregnancy is a useful model for the study of several immune phenomena. The immune system in normal pregnancy is thought to be in a state of "activation" rather than in a state of "depression". In diabetes, several immunological abnormalities may be present and these may be related to pathogenic events, metabolic derangement and insulin therapy. In diabetic pregnancy, several immune phenomena may be present temporarily as a consequence of both diabetes and pregnancy. Moreover, some abnormal immune factors can cross the placenta and interfere with fetal metabolism and development (for a larger discussion and references see chapter A1.6).

The author has planned to investigate humoral and cellular immunological aspects in diabetic pregnant women and in some of their neonates. Islet cell antibodies, organ specific autoantibodies, immune complexes, insulin antibodies, insulin-anti-insulin complexes, T cell subpopulations and activated T cells have been assayed.

The author had worked on the following hypotheses :

- a) Humoral factors, in addition to metabolic events, might influence the clinical course of pregnancy and neonatal complications.
- b) Maternal insulin antibodies may easily cross the placenta and reach the neonatal circulation. The fetal insulin that they face is in several aspects different from the therapeutic insulin in

the mother. Therefore the insulin-anti-insulin complexes formed are likely to be different in the mother and in the foetus.

- c) As the maternal immune system is "actively depressed", it is conceivable that the T cell subpopulations are somehow different in the diabetic pregnant woman in comparison with the normal pregnant or the normal woman.
- d) As the maternal immune system is informed of the presence of the antigenically foreign fetus but does not reject, it is possible that the activation of T lymphocyte either does not occur or is somewhere blocked.

Hypothesis "a" has been verified in the first study (Chapter B4.2)

The study in a large group of insulin treated diabetic pregnant women (118) has been in part cross sectional and in part prospective. The immunological factors studied have been islet cell antibodies, organ specific autoantibodies, insulin antibodies and immune complexes. The maternal metabolic control, the type of diabetes and the type of insulin preparation have been taken into account. Both the maternal metabolic control and the presence of immunological factors have been correlated with the clinical complications in the mother and in the neonate.

Hypothesis "b" was studied in the second research work (Chapter B4.3)

The study was performed in 47 pregnant diabetic patients and in 23 neonates. Insulin antibodies and insulin-anti-insulin complexes



were studied both in the mother during pregnancy and in the cord blood.

Hypothesis "c" was investigated in the 3rd study (Chapter B4.4)

Monoclonal antibodies against an antigen common to all the T cells, against cytotoxic/suppressor cells and to helper/inducer T cells were used to define T cell subpopulations in type 1, type 2 and gestational pregnant diabetic women, in normal pregnant subjects and in normal women.

Hypothesis "d" was analyzed in the 4th study (Chapter B4.5)

Monoclonal antibodies to T cell surface antigens present on activated T cells which are related both to MHC class II antigens and to other antigens, were used in T cell studies in type 1, type 2 and gestational pregnant diabetic women, in normal pregnant subjects and in normal women.

The monoclonal antibodies used in these studies were produced in the MRC Clinical & Population Cytogenetics Unit of Edinburgh.

The clinical part of these studies was done in collaboration with the "Cattedra di Puericultura Prenatale" and with "Servizio Stati Disendocrini e Dismetabolici", University of Rome, Italy.

B4.2 HUMORAL IMMUNITY IN DIABETIC PREGNANCY : ISLET CELL  
ANTIBODIES, ORGAN SPECIFIC AUTOANTIBODIES, INSULIN ANTI-  
BODIES AND IMMUNE COMPLEXES.

B4.2a Aim of the study

This study was planned to examine the presence of islet cell antibodies (ICA), complement fixing islet cell antibodies (CF-ICA), autoantibodies to thyroid, stomach, adrenal and ovary, insulin antibodies, and two different types of immune complexes in pregnant diabetic patients, at the end of pregnancy and in some of the patients regularly during pregnancy, taking into account the type of insulin therapy and the maternal metabolic control. Furthermore, both the presence of these immunological factors and the degree of metabolic control were correlated with the clinical characteristics of patients, and with maternal or neonatal complications.

## B4.2b Materials and methods

### Patients

One hundred and eighteen diabetic pregnant attending the same clinic were included in the study. All of them were treated with insulin. Since the main aim of the work was the investigation of immunological phenomena, patients were grouped according to the type of diabetes and not to the White Classes.

Group 1 included 56 insulin dependent diabetic patients, already treated with insulin before pregnancy.

Twenty-three patients were included in Group 2; they were non-insulin dependent, formerly treated with diet or oral hypoglycaemic agents (OHA) and shifted to insulin at their first clinical control in our clinic.

Group 3 was formed by 39 patients with gestational diabetes, treated with insulin from their first control in pregnancy when previously established clinical criteria (age of the patient >25 years, poor metabolic control, ketonuria) had been met.

In the first phase of the study (phase A) ninety-four patients (42 in Group 1, 17 in Group 2, 35 in Group 3) were followed clinically during pregnancy and blood was collected at the end of pregnancy for a cross-sectional study (phase A).

In the second phase of the study (phase B), 24 patients were studied prospectively (14 in Group 1, 6 in Group 2, 4 in Group 3) and were sampled at regular intervals during pregnancy (phase B).

For the immunological study forty-two normal pregnant were included as controls (mean age was  $30 \pm 3$ ; all subjects delivered spontaneously at term).

The personal and clinical data of the normal and diabetic subjects are shown in table B4.2/1.

For maternal and neonatal complications, 2000 consecutive non diabetic deliveries were considered as a control group (table B4.2/2).

The patients in phase A of the study were treated with standard preparations of insulin and some of them, selected at random, were transferred to purified mixed bovine and porcine insulin from the first control in pregnancy. All patients in phase B were treated with purified mixed bovine and porcine insulins. Rapid and long-acting insulins were used, with the aim of maintaining blood glucose levels within the most satisfactory levels without acetonuria and hypoglycaemic crisis.

#### Maternal metabolic control

Maternal metabolic control (MMC) was evaluated on the basis of three times monthly blood glucose levels, four times daily glycosuria, the occurrence of hypoglycaemia and/or acetonuria. In planning the study these criteria to evaluate the degree of maternal metabolic control were chosen: A) absence of hypoglycaemia and acetonuria, blood glucose levels never  $>7.7$  mmol/l; glycosuria as an exceptional event. B) Absence of hypoglycaemia and acetonuria, blood glucose levels never  $>8.8$  mmol/l; glycosuria as a rare event. C) Rare hypoglycaemia and/or acetonuria; blood glucose levels often  $>8.8$  mmol/l; glycosuria as habitual. D) Frequent hypoglycaemia and

acetonuria, blood glucose levels often  $>10$  mmol/l. Due to numerous correlations, in the evaluation of results we considered only two groups: good (A+B) and poor (C+D) maternal metabolic control. In patients of phase B the metabolic control was also evaluated by the glycosylated haemoglobin percentage (Bio-Rad Haemoglobin A1 column test).

#### Clinical complications

The possible presence of diabetic complications and the occurrence of other associated diseases were fully explored; the duration and obstetric aspects of pregnancy and labour were carefully examined in all the patients. In particular the occurrence in the mother of hypertension, oedema and albuminuria in the last trimester of pregnancy were evaluated. The presence at least of two of these signs was the diagnostic criterion for gestosis or toxæmia of pregnancy (table B4.2/2).

The neonatal complications were also studied. Macrosomia (above the 95th percentile in the Lubchenco charts), hypoglycaemia (blood glucose  $<1.4$  mmol/l two hours after birth), hypocalcaemia ( $\text{Ca} < 1,7$  mmol/l), hyperbilirubinaemia ( $> 171$   $\mu\text{mol/l}$ ), respiratory distress syndrome, the Apgar index and the presence of malformations were recorded in the all newborns (table B4.2/2).

#### Organ specific auto-antibodies

Organ specific autoantibodies, i.e. islet cell antibodies, thyroid microsomal antibodies, antibodies to gastric mucosa, to ovaries, to adrenal, were detected by indirect immunofluorescence. Complement fixing islet cell antibodies (CF-ICA), with fresh normal

human serum used as a source of complement, were revealed by fluoresceinated anti C3 antibodies (Chapter A3.5).

#### Insulin antibodies

Insulin antibodies, as detected by insulin binding capacity, were measured with two different methodological approaches: Christiansen's method in phase A (Christiansen,1970) and Ortved Andersen's method (Andersen et al,1972) as modified by Mustaffa et al (1977) in phase B (Chapter A3.3). The results obtained with the former method were divided into negative, low, medium and high when values were  $< 0.05$ ,  $0.05-0.5$ ,  $0.51-2$ ,  $>2$  mU/ml; whereas those with the latter when values were  $<10$ ,  $10-100$ ,  $101-300$ ,  $> 300$  uU/ml, respectively.

#### Immune complexes

Immune complexes were studied by two different approaches: small medium size complexes in antigen excess were detected by the solid phase Clq binding (ClqSP) test (Hay et al,1976); C3 binding medium-large size complexes near the equivalence point were detected by the conglutinin radioimmunoassay (KgBt) (Casali et al,1976) (Chapter A3.1). One hundred and forty three blood donor sera were used to evaluate the limit of positivity in immune complex studies. Out of these samples, aliquots of ten statistically representative sera were included in all the experiment as reference sera. The chosen limit of positivity was the mean plus 2 standard deviations (SD) of normal values. Since the methods to detect immune complexes are only semiquantitative, results were expressed as negative, low (between mean plus 2 SD and mean plus 3 SD), medium (between mean

plus 3 SD and mean plus 4 SD) and high levels (above mean plus 4 SD).

#### Statistical analysis

Fisher's exact test, chi square test with Yates' correction when applicable, Cox's test for trends in proportions and the unrelated t test were used for the statistical evaluation of results.

B4.2c      R e s u l t s

Islet cell antibodies and  
other organ specific autoantibodies

At the end of pregnancy ICA were found in 12% of patients in Group 1 and in 5% in Group 3. None of the Group 2 patients was ICA positive. None of the sera were CF-ICA positive. In patients in phase B, two out of the 14 patients in Group 1 showed autoantibodies to thyroid and gastric mucosa; 1 of the 6 in Group 2 had antithyroid antibodies and 1 out of the 4 in Group 3 presented with antibodies to gastric mucosa.

Insulin antibodies

Insulin antibodies, studied at the end of pregnancy, were found in 67% of Group 1 patients, in 73% of Group 2 and in 25% of Group 3 (Figure B4.2/1). There was no significant difference in insulin antibody levels between patients treated with standard insulins throughout pregnancy and those transferred to purified mixed insulins. Insulin antibody levels in Group 1 diabetics followed during pregnancy did not vary significantly from the first trimester (mean  $5.5 \pm 1.8$ uU/ml) to the end of pregnancy (mean  $15 \pm 5.5$ uU/ml).

Immune complexes

Immune complexes, as detected by ClqSP, were found to be increased in diabetic pregnant women in comparison with normals ( $p < 0.001$ ) (Figure B4.2/2). There was no significant difference in immune complex levels between patients in Groups 1, 2 and 3. In the



patients followed throughout pregnancy there was a significant increase in immune complex levels during the course of pregnancy (Figure B4.2/3).

On the other hand, the presence of immune complexes, as detected by KgBt, was not significantly different between diabetic and normal pregnant. The positivity was 26% in Group 1 and 21% in Group 3. None of Group 2 was positive.

#### Metabolic control

Data on the maternal metabolic control are shown in table B4.2/3. The HbA1 percentage, evaluated in the patients of phase B, was found to be  $7.5 \pm 0.3$  %. These values were significantly higher than in controls.

#### Correlation between

immunological parameters and clinical characteristics of patients  
or maternal and neonatal complications

No correlation was found between the presence of organ specific autoantibodies and clinical aspects of diabetic pregnancy. On the contrary the levels of insulin antibodies were significantly correlated with the occurrence of gestosis ( $p < 0.05$ ). There was a significant association between the levels of insulin antibodies and the occurrence of neonatal hypoglycaemia ( $p < 0.025$ ), hypocalcaemia ( $p < 0.01$ ) and respiratory distress syndrome ( $p < 0.01$ ) (Figure B4.2/4). The presence of immune complexes detected by ClqSP was positively correlated with the duration of diabetes, ( $p < 0.05$ ), whereas the immune complexes detected by KgBt were correlated with the age of the patients: the younger the patients the lower the complexes ( $p < 0.01$ ).

#### Correlation between

metabolic control and maternal and foetal complications

A higher frequency of gestosis ( $p < 0.02$ ) and neonatal complications ( $p < 0.05$ ) were found in those cases with a poor maternal metabolic control (table B4.2/3).

#### Correlations between

metabolic control, insulin antibodies and neonatal complications

In patients with comparable metabolic control, the presence of insulin antibodies was significantly correlated with the occurrence of neonatal complications (table B4.2/4).

TABLE B4.2/1

Personal and clinical data of the diabetic and normal pregnant women.

| Group                                       | 1              | 2                 | 3            | N*           |
|---|----------------|-------------------|--------------|--------------|
| Number                                      | 56             | 23                | 39           | 2000         |
| Type of diabetes                            | I              | II                | GD**         | --           |
| Age (years)                                 | 28 $\pm$ 5.8   | 34 $\pm$ 4.7      | 32 $\pm$ 6.2 | 28 $\pm$ 3   |
| Age at onset (years)                        | 17 $\pm$ 7.2   | 30 $\pm$ 4.6      | 32 $\pm$ 6.2 | --           |
| Mean gestational age<br>at delivery (weeks) | 38 $\pm$ 0.2   | 37 $\pm$ 0.2      | 39 $\pm$ 0.2 | 40 $\pm$ 0.4 |
| Duration of insulin<br>treatment (years)    | 10.5 $\pm$ 7.2 | only in pregnancy |              |              |
| White Class: A                              | --             | --                | 39           | --           |
| B   | 13             | 20                | --           | --           |
| C-F   | 43             | 3                 | --           | --           |

\* N : normal pregnant women

\*\* GD: Gestational diabetes

TABLE B4.2/2

Maternal and neonatal complications of diabetic and normal pregnant women.

| Group                  | 1   | 2   | 3   | N*   |
|------------------------|-----|-----|-----|------|
| Gestosis               | 20% | 9%  | 13% | 5%   |
| Macrosomia             | 35% | 18% | 27% | 12%  |
| Hypoglycaemia          | 45% | 48% | 13% | 0.6% |
| Hypocalcaemia          | 18% | 22% | 16% | 1.8% |
| RDS**                  | 10% | 30% | 8%  | 2.7% |
| Hyperbilirubinaemia    | 61% | 74% | 42% | 16%  |
| Apgar index (<7 at 5') | 15% | 31% | 6%  | 8%   |
| Malformations          | 4%  | 13% | 0%  | 1.4% |

\* N : normal pregnant women

\*\* RDS : respiratory distress syndrome

TABLE B4.2/3

Maternal metabolic control (MMC) and clinical data of 118 diabetic pregnant women.

| MMC   | GOOD           | POOR           | "p" value< |
|---|----------------|----------------|------------|
| Number subjects                             | 75             | 43             |            |
| Age (years)                                 | 31.2 $\pm$ 1.1 | 32.1 $\pm$ 1.2 |            |
| Mean gestational age<br>at delivery (weeks) | 38,2 $\pm$ 0.3 | 37.5 $\pm$ 0.2 |            |
| <u>Maternal complications(%)</u> :          |                |                |            |
| gestosis                                    | 11             | 28             | 0.02       |
| polyamnios                                  | 16             | 16             | --         |
| <u>Neonatal complications(%)</u> :          |                |                |            |
| total                                       | 53             | 75             | 0.05       |
| macrosomia                                  | 35             | 42             | --         |
| hypoglycaemia                               | 24             | 45             | 0.05       |
| hypocalcaemia                               | 8              | 20             | --         |
| RDS*  | 13             | 15             | --         |
| hyperbilirubinaemia                         | 25             | 52             | 0.01       |

\* RDS : respiratory distress syndrome.

TABLE B4.2/4

Maternal metabolic (MMC) and neonatal complications relation to the presence of insulin antibodies (InsAb) in 118 diabetic pregnant.

| MMC                     | GOOD   |         | "p"< | POOR   |         | "p"< |
|-------------------------|--------|---------|------|--------|---------|------|
|                         | Absent | Present |      | Absent | Present |      |
| <u>Neonatal</u>         |        |         |      |        |         |      |
| <u>complications(%)</u> | 54     | 89      | 0.01 | 88     | 93      | --   |
| macrosomia              | 33     | 38      | --   | 31     | 44      | --   |
| hypoglycaemia           | 20     | 41      | --   | 31     | 56      | --   |
| hypocalcaemia           | 3      | 15      | --   | 12     | 30      | --   |
| RDS*                    | 3      | 24      | 0.05 | 6      | 22      | --   |
| hyperbilirubinaemia     | 27     | 59      | 0.01 | 69     | 63      | --   |

\* RDS : respiratory distress syndrome

FIGURE B4.2/1

Insulin antibodies (InsAb) in all the diabetic pregnant women studied at the end of pregnancy. Patients in phase A are indicated with circles. The open circles indicate patients treated with commercial insulins; the closed circles those treated with highly purified insulins. Patients in phase B are indicated with squares. For further information on patients and methods used, see text.

## InsAb in diabetic pregnant at the end of pregnancy

**InsAb  
levels**

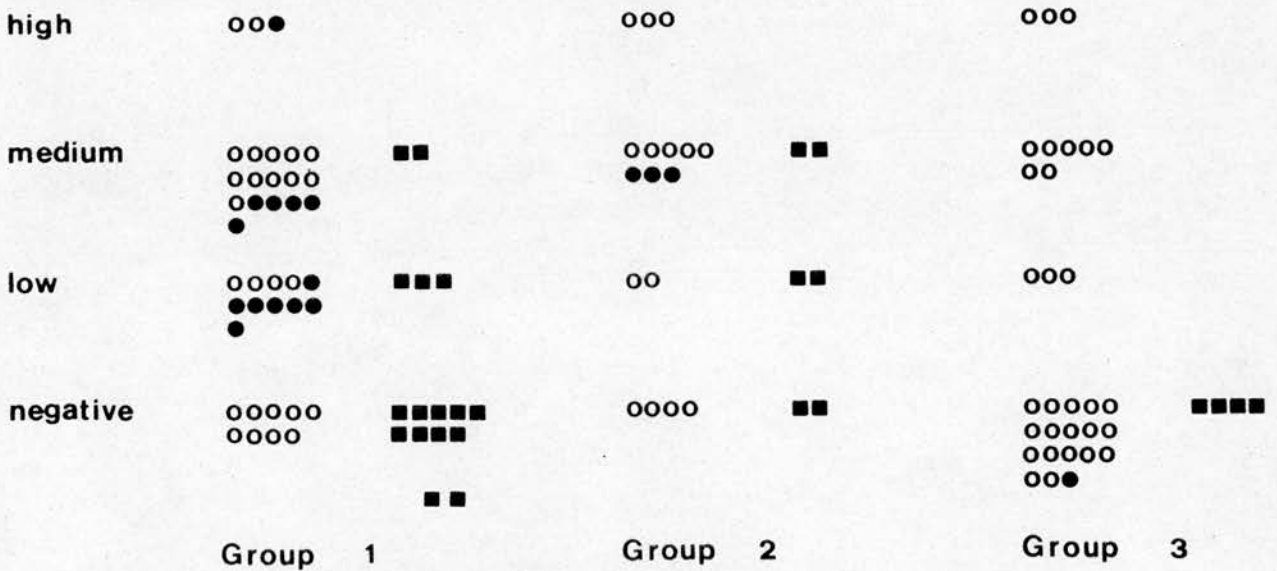


FIGURE B4.2/2

Immune complexes (AgAb), as detected by C1qSP, in diabetic pregnant women at the end of pregnancy in comparison with normals.

## AgAb(C1qSP) in diabetic pregnant studied at the end of pregnancy

AgAb  
levels

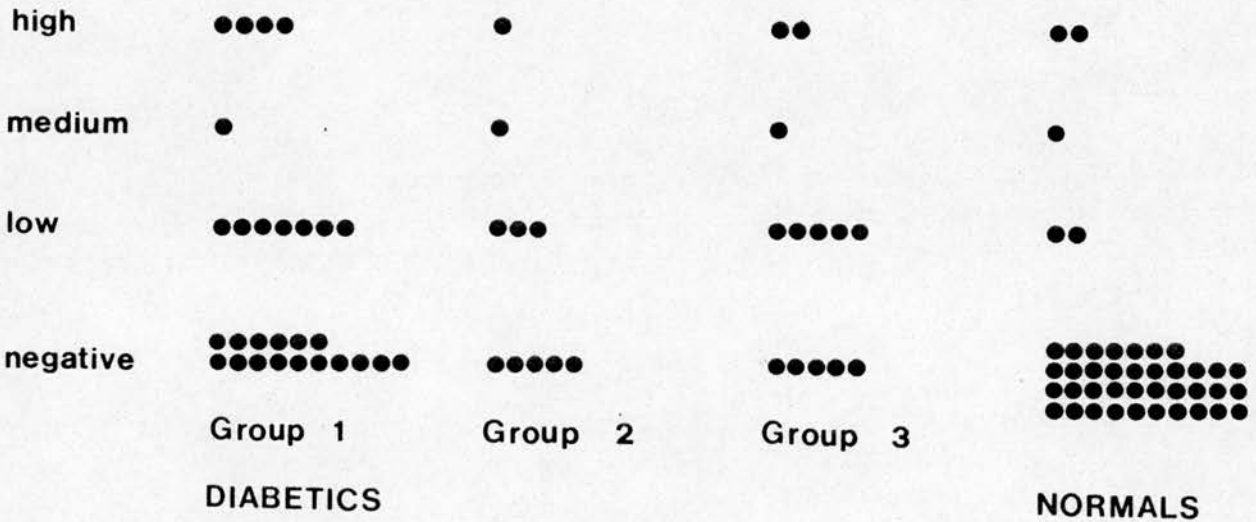
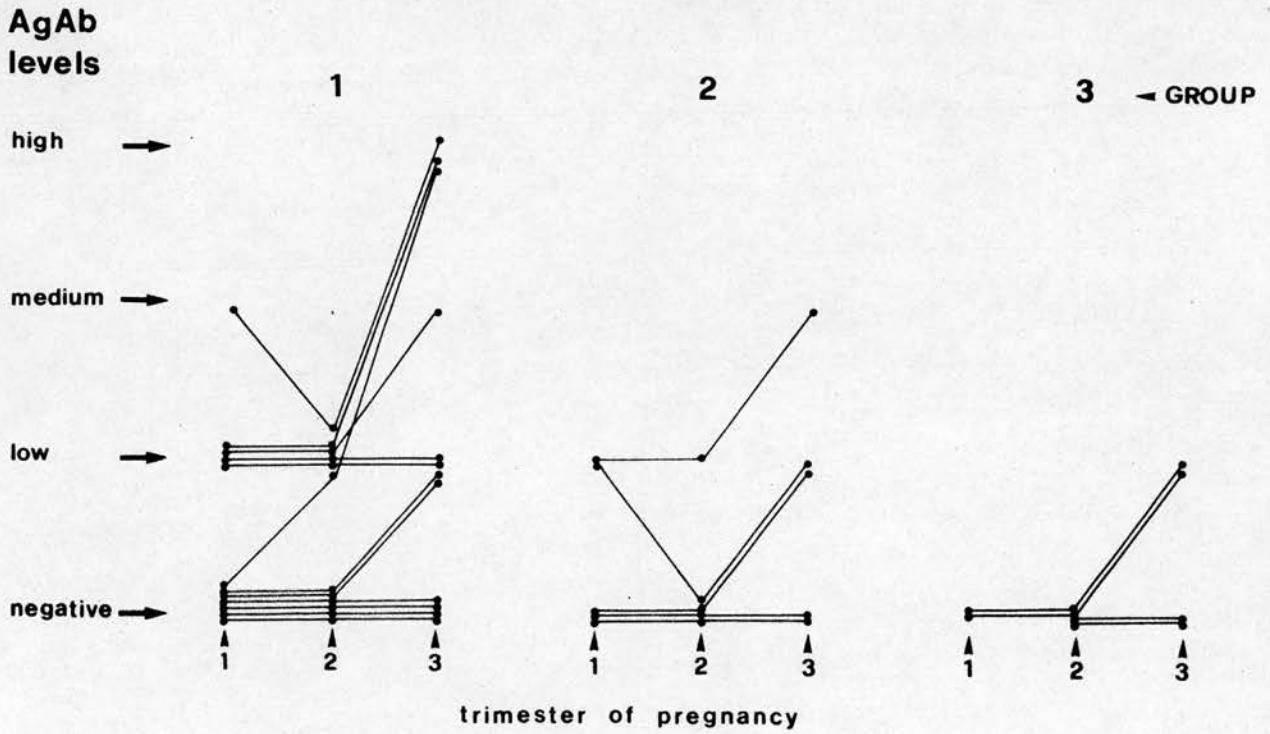




FIGURE B4.2/3

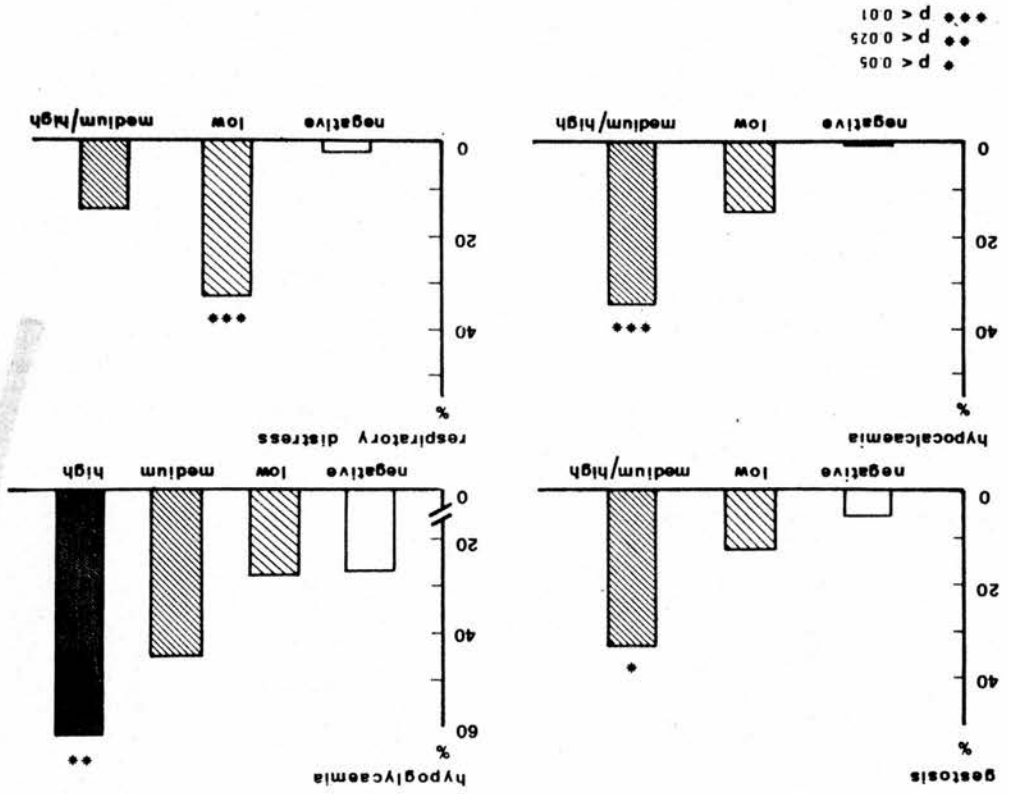
Immune complex levels (AgAb), as detected by C1qSP, during pregnancy in phase B patients.

### AgAb(C1qSP) in diabetic pregnant in the course of pregnancy



Insulin antibodies correlated with occurrence of maternal and neonatal complications.

InsAb and maternal/newborn complications



B4.2d      D i s c u s s i o n

The maternal and/or foetal pathological events in the diabetic pregnancy could be the effect of several different pathogenic agents including metabolic and immunological factors.

Immunological factors in pregnancy have not been adequately investigated in the past. Therefore, the main concern in planning this work was to reconsider the pathological events in the diabetic pregnancy from this approach: the presence and the possible influence of changes in the immunological aspect of the patients. The results of the work have, in fact, substantiated this hypothesis.

The correlation between medium-high insulin antibody levels and increased occurrence of maternal gestosis or neonatal hypoglycaemia, hypocalcaemia and respiratory distress syndrome, suggests a possible influence of these antibodies on the maternal and neonatal morbidity in the diabetic pregnancy. This hypothesis is reinforced by the finding that in patients with good metabolic control the presence of insulin antibodies was still correlated with the occurrence of neonatal complications. The presence of these antibodies may increase the deleterious effect of hyperglycaemia.

Furthermore, the increased presence of circulating immune complexes in diabetic pregnant, when compared to normal pregnant, and their tendency to increase during pregnancy, reinforces the concept of an involvement of immune factors; as it is frequently observed only one type of complexes was found to be increased: those of small-medium size, mostly in antigen excess, as detected by the solid phase Clq binding test.

The presence of islet cell antibodies in a few gestational diabetics, is in accordance with a few other similar reports (Steel et al,1980;Tingle et al,1979).The presence of islet cell auto-antibodies suggests the occurrence of some damage of the islet cell function, presumably sustained by immune factors, despite the clinical finding of only an impaired glucose tolerance. In this regard, the report of the development of overt diabetes a few years after pregnancy in islet cell antibody positive gestational diabetics is relevant (Tingle et al,1979).

Of course, the finding of a statistical correlation between the occurrence of immunological factors and pathological events does not necessarily imply a cause-effect relationship. Nevertheless,the finding herein and those of previous reports (Galbraith and Page Faulk,1979), suggest that immunological factors may play a role in the development of pathological events in diabetic pregnancies.

Although insulin does not cross the placenta in significant amounts, insulin antibodies of IgG class do. Their presence in the foetal circulation, buffering the foetal insulin, may well increase the demand for insulin from the foetal pancreas (Heding et al,1980); if this is the case, the higher the antibody levels, the more likely is the occurrence of hypoglycaemia in the newborn. In this respect a higher insulin and/or C-peptide level in the cord blood of infants of diabetic mothers having elevated levels of circulating insulin antibodies has been reported (Heding et al,-1980;Fallucca et al,1980). This effect is evident even when the influence of the maternal metabolic control has been taken into account (Fallucca et al,1980).

It is interesting that the switch from standard insulin preparations to purified mixed bovine and porcine insulins did not substantially modify the correlations found. Bovine insulins, though purified, are still immunogenic to some extent (Chance et al,1976).

Noteworthy also is the correlation between insulin antibody levels and the occurrence of infants' respiratory distress syndrome. Although the pathogenesis of infants' lung distress syndrome is not elucidated, the presence of immunological abnormalities in the alveolar basement membrane has been suggested by previous studies (Benatre et al,1974;Smith et al,1975). On the other hand, foetal hyperinsulinism, indirectly induced by high insulin antibody levels, appears to be a very important factor. Insulin has in fact an inhibitory effect on lung surfactant factor production (Smith et al,1975;Gajl-Perzalska,1964 ). More obscure is the possible relationship between high insulin antibody titers and neonatal hypocalcaemia. It is likely that other complicating conditions, such as macrosomia and respiratory distress, both related to hyperinsulinaemia, may indirectly link, through an acidotic condition, the presence of these antibodies to an impaired calcium and phosphate homeostasis (Tsang et al,1972).

Other papers have reported a correlation between the presence of circulating immune complexes and the occurrence of maternal gestosis (Scott et al, 1978;Jenkins,1977;Gleicher et al,1978). The increased presence of immune complexes in our diabetic pregnant failed to be significantly correlated with the occurrence of gestosis, but a correlation was found with the levels of insulin antibodies. It is known that the increased presence of circulating macromolecules is correlated with the presence of diabetic vascular lesions and that,

in some diabetics, a deposition of immune materials and of macromolecules in placental blood vessels has been described (Galbraith and Page Faulk, 1979). Therefore, the possibility should be considered that higher levels of insulin antibodies, and the consequent insulin anti-insulin complex formation, contribute to increased deposition of macromolecules and damage of blood vessels, leading to maternal clinical manifestations.

In conclusion, the study of immunological parameters during pregnancy in diabetics might give useful information on the probability of the development of maternal complications and even predict and somehow help in preventing the possible occurrence of neonatal complications. Moreover an appropriate choice of highly purified insulins, avoiding immunological phenomena connected with insulin therapy, is a measure which will facilitate metabolic control and may help to reduce the possibility of clinical complications.

B4.3 HUMORAL IMMUNITY IN DIABETIC PREGNANCY : INSULIN  
ANTIBODIES AND INSULIN-ANTI-INSULIN COMPLEXES

B4.3a Aim of the study

In this study attention has been focused on assaying the possible presence of insulin-anti-insulin complexes in the cord blood and on the relationships of these complexes of the newborn with the maternal insulin antibodies and insulin-anti-insulin complexes.

B4.3b Materials and methods

Patients

A total of forty-seven pregnant diabetic patients, all attending the same clinic, were included in the study. Twenty-three of these diabetic women were investigated together with their infants. All the pregnant patients were sampled at the end of pregnancy and 27 also in the first trimester of pregnancy. Twenty-six had type 1 diabetes, 14 type 2 and 7 gestational diabetes. All the patients were treated with insulin; during pregnancy monocomponent insulins or semisynthetic

human insulins were used, except in 2 cases, regardless of the type of treatment previously used. The cord blood of the infants was studied.

#### Insulin antibodies

Insulin antibodies, measured as insulin binding capacity (InsAb), were evaluated according to the technique originally described by Ortved Andersen (1972) and modified by Mustaffa et al. (1977) with a few other minor modifications (Chapter A3.3). Values above 10 uU/ml were considered indicative of the presence of antibodies.

#### Insulin-anti-insulin complexes

Insulin-anti-insulin complexes (Ins/iAb) were measured according to a modification (Chapter A3.4) of the techniques described by Jayarao et al.(1974) and Virella et al.(1980). The insulin-anti-insulin complexes are calculated as the difference between the "total" antibody and the "free" antibody. The sensitivity of this technique in assaying the sole presence of insulin antibodies is slightly different from Andersen's method (Chapter A3.4).

#### Statistical analysis

Fisher's exact test was used for statistical evaluations.



B4.3c      R e s u l t s

## Insulin antibodies

## Diabetic Mothers

InsAb were found at the end of pregnancy in 62% of the type 1 diabetic patients (median:15.5 uU/ml,interquartile range, or i.r., <10-45),in 71% of the type 2 (m.:26.5 uU/ml, i.r.<10-79) and in 43% of the gestational diabetic patients (m.:<10 uU/ml,i.r. <10-13). No significant difference was found when the levels of InsAb at the beginning of pregnancy (median: 18 uU/ml,i.r.<10-43) were compared with those at the end of pregnancy in the same type 1 diabetic patient.

## Neonates

InsAb were found in 48% of the newborn infants studied. InsAb levels were similar in the newborns (m.:<10 uU/ml,i.r.<10-28) and in the mothers at the end of pregnancy (m.:13 uU/ml, i.r.<10/30)(Table B4.2/1).

## Insulin-anti-insulin complexes

## Mothers

Quantifiable Ins/Ab were found in 37% of the type 1 diabetic patients studied at the beginning of pregnancy (m.:<7, i.r.<7-13.3) and in 27% of the patients with the same type of diabetes studied at the end of pregnancy (m.:<7, i.r. <7-7.1). Ins/iAb were also present in 21% of type 2 diabetic mothers at the end of pregnancy (positive values: 8,9.1,82.1) and in 14% of gestational diabetic patients (p.v.:7.9).

### Neonates

Ins/iAb were found in infants of diabetic mothers (38% of infants of type 1 and 43% of type 2 diabetic mothers) (Table B4.3/1). There was a significant correlation between the presence of Ins/iAb in newborn infants and the occurrence of these complexes in their relative mothers; nevertheless, this correlation was much stronger when the findings in newborns were correlated with those found in mothers in the first trimester of pregnancy ( $p < 0.0005$ ) than at the end of it ( $p < 0.01$ ).

TABLE B4.3/1

| Patient no. | Type of diabetes | Duration of diabetes (yrs) | Type of insulin |    | Mother IBC |     | Ins/iAb |      | Newborn IBC | Ins/iAb (Total) | (Complexes) |
|-------------|------------------|----------------------------|-----------------|----|------------|-----|---------|------|-------------|-----------------|-------------|
|             |                  |                            | a.              | b. | 1.         | 2.  | 1.      | 2.   |             |                 |             |
| 1           | 1                | 15                         | C               | H  | 18         | 20  | <10     | <7   | <10         | <7              | <7          |
| 2           | 1                | 10                         | C               | H  | 18         | 27  | <10     | <7   | <10         | <7              | <7          |
| 3           | 1                | 12                         | MC              | H  | 158        | 167 | 94.1    | 19.1 | 99          | 41.4            | 21.4        |
| 4           | 1                | 6                          | MC              | MC | <10        | <10 | <10     | <7   | <10         | <10             | <7          |
| 5           | 1                | 6                          | MC              | MC | <10        | <10 | <10     | <7   | <10         | <10             | <7          |
| 6           | 1                | 4                          | MC              | MC | <10        | <10 | <10     | <7   | <10         | <10             | <7          |
| 7           | 1                | 2                          | C               | C  | <10        | <10 | <10     | <7   | <10         | <10             | <7          |
| 8           | 1                | 3                          | C               | MC | 35         | <10 | 29.7    | 19.9 | 15          | 18              | 8.7         |
| 9           | 1                | 16                         | C               | MC | 14         | 14  | 17.3    | 7.5  | 15          | 10.1            | 7.3         |
| 10          | 1                | 5                          | C               | MC | <10        | <10 | <10     | <7   | <10         | <10             | <7          |
| 11          | 1                | 10                         | C               | MC | 11         | <10 | <10     | <7   | <10         | <10             | <7          |
| 12          | 1                | 16                         | C               | MC | 37         | 74  | 18.2    | 10.2 | 28          | 22.7            | 10.2        |
| 13          | 1                | 9                          | C               | MC | 44         | 11  | 33.4    | 21.2 | 35          | 26.7            | 17.1        |
| 14          | 2                | 1                          | *               | C  | 153        | 202 | 42      | <7   | 234         | 39.1            | 11.1        |
| 15          | 2                | 6                          | MC              | MC | <10        | 28  | <10     | <7   | 11          | <10             | <7          |
| 16          | 2                | 11                         | *               | MC | 207        | 236 | 104.2   | 66.8 | 121         | 50.4            | 44.3        |
| 17          | 2                | 7                          | MC              | MC | <10        | 30  | <10     | <7   | 13          | <10             | <7          |
| 18          | 2                | 7                          | H               | H  | <10        | <10 | <10     | <7   | <10         | <10             | <7          |
| 19          | 2                | 16                         | H               | H  | <10        | 18  | <10     | <7   | 14          | <10             | <7          |
| 20          | 2                | 14                         | *               | H  | 198        | 79  | 46.5    | 23.2 | 51          | 32.1            | 26.8        |
| 21          | GD               | -                          | MC              | MC | <10        | <10 | <10     | <7   | <10         | <10             | <7          |
| 22          | GD               | -                          | H               | H  | <10        | 10  | <10     | <7   | <10         | <10             | <7          |
| 23          | GD               | -                          | H               | H  | 17         | 13  | <10     | <7   | <10         | <10             | <7          |

legenda:

a. type of insulin used for most of the time before pregnancy b. type of insulin prescribed during pregnancy

1. values obtained in blood sample taken in the first trimester and 2. values obtained in samples taken at the end of pregnancy

C conventional non-highly purified insulins MC monocomponent insulins H human insulins

\* the patient was treated with conventional insulins in previous pregnancies H human insulins

B4.3d Discussion

Evidence has been attained in this study that insulin- anti-insulin complexes are present in the circulation of a number of infants of insulin-treated diabetic mothers.

The amount of complexed insulin antibody is not only related to the total amount of the antibody but may be influenced by the affinity and avidity of the maternal insulin antibody.

There is a strong correlation between the presence of insulin-anti-insulin complexes in the cord blood and their presence in the maternal circulation. Nevertheless, the presence of insulin-anti-insulin complexes in the fetus does not merely represent a passive transfer of the insulin antibodies from the mother to the fetus. The levels of insulin-anti-insulin complexes in the newborn do not reflect those of the mother at the end of pregnancy but appear to correlate with the average of the levels of insulin- anti-insulin complexes during pregnancy.

Indeed, even if insulin antibodies can easily cross the placenta whereas insulin itself is not transferred in significant proportions, when insulin antibodies reach the fetal circulation they face a different situation. These antibodies react with an antigen slightly different from the maternal one: fetal endogenous insulin and not therapeutic heterologous insulin. The antigen/antibody molar ratio is substantially different. While in the mother the antibody interacts with massive doses of insulin, of high or low immunogenicity, administered in subcutaneous boluses twice a day on average, in the fetal circulation the antibody reacts with a continuously produced

pancreatic insulin. The amount of insulin itself produced by the fetal pancreas varies tremendously from the first months until the end of pregnancy, whereas the amount of insulin antibodies transferred by the mother usually does not vary significantly during pregnancy. From a situation very likely characterized by antibody excess at the beginning of pregnancy, as the fetal pancreas produces insulin in increasing amounts, the proportion between antigen and antibody becomes more balanced. It is highly probable that both the fate and the physiopathological significance of the fetal insulin-anti-insulin complexes are different from those of the mother.

The clearance of fetal insulin-anti-insulin complexes seems, to some extent, slower than that of the mother. It is not known whether insulin antibodies in fetal circulation may easily be retransferred to the mother's circulation, but it is not probable that the complexes may be freely exchanged.

Whereas in the mother small medium-size complexes are rapidly removed by the kidney and the reticulo-endothelial system (Mannik et al, 1974), in the fetus, the complex clearance mechanisms are not so efficient, especially in infants of diabetic mothers in whom the normal duration of pregnancy is usually shortened. This could explain why in those few cases in which the insulin antibody levels are high in the mother at the beginning of pregnancy and low at the end, the insulin-anti-insulin complex levels in the fetus are comparable to those found in the mother in the first months of pregnancy.

A few research works, among which the previous chapter of the thesis, have already described a correlation between the presence of insulin antibodies and a few neonatal clinical complications (Martin et al, 1975; chapter B4.2))

On the other hand, the possible damage induced by the deposition and presence of immune complexes is well known both in diabetic patients (Jayarao et al,1974;chapter B3) and in pregnant women with clinical complications (Theophilopoulos et al,1981;Vazquez-Escobosa et al, 1983). Moreover, it is well known that the injection of insulin antibodies may induce a diabetic-like syndrome in normal animals (Wright,1961). The presence of insulin-anti-insulin complexes in detectable amounts may be theoretically associated with the occurrence of defects in the circulation of the small blood vessels of the placenta, some clinical complications in the newborn and, in particular, with the macrosomia and the neonatal hypoglycemia neutralising or abruptly releasing substantial amounts of newborn insulin.

B4.4 CELLULAR IMMUNITY IN DIABETIC PREGNANCY:  
T CELL SUBSETS

B4.4a A i m o f t h e s t u d y

The aim of this study was to investigate the T cell subpopulations in diabetic pregnant patients and to compare the findings with those in normal pregnant subjects and in normal women. The type of diabetes has been taken into consideration. The total number of T cells, the proportion of T helper and of T cytotoxic/suppressor lymphocytes and to ratio of T helper to T cytotoxic-suppressor cells have been analyzed.

B4.4b M a t e r i a l s a n d M e t h o d s

Patients

37 diabetic pregnant patients, 16 with type 1 , 10 with type II and 11 with gestational diabetes were studied together with 22 normal pregnant subjects and 20 normal women. All the pregnant women were attending the same Clinic. The medians of the age of type 1, type 2

and gestational diabetic pregnant, of normal pregnant and of normal controls were 25 (Range 22-34), 30 (R. 25-40), (R 21-31) and 26 (R 18-40) respectively. The duration of diabetes was 12 years (R 3-16) in type 1 diabetics, and 10 years (2-16) in type 2 diabetics. 13 diabetic patients were sampled at the end of pregnancy, 11 at the end of the second trimester and 14 at the end of the first trimester of pregnancy.

#### T cell subsets

The monoclonal antibody OKT3 (Ortho Diagnostic) was used to count the total T cell population and OKT4 to enumerate the helper/inducer T cells. The antibody UCHL1, was used to detect the suppressor/ cytotoxic T cells. For details see chapter A3.2

#### Statistical analysis

Unpaired t test was used to evaluate the results.



B4.4c      R e s u l t s

## Total T cell number

The total number of T cells as measured by OKT3 was essentially similar in type 1, type 2, gestational diabetic pregnant, normal pregnant subjects and normal women (Table B4.4/1).

## Helper T cells (Th)

The percentages of helper/inducer T cells, as measured by OKT4, were found in  $35.8\% \pm 8.7$  of type 1,  $42.8\% \pm 9.2$  of type 2, in  $41.2\% \pm 8.7$  of gestational diabetic pregnant, in  $38.2\% \pm 7.3$  of normal pregnant subjects and in  $41.5\% \pm 6.4$  of normal women (Table B4.4/1). The reduction in T helper cells in type 1 diabetics in comparison with normal subjects was statistically significant ( $p < 0.01$ ).

## Suppressor/cytotoxic T cells (Tc/s)

The percentage of suppressor/cytotoxic T cells, as measured by UCHT4, in type 1, type 2, gestational diabetic pregnant, normal women were  $19\% \pm 7$ ,  $13\% \pm 4.2$ ,  $18.5\% \pm 3.8$ ,  $17.7\% \pm 5$  and  $19.8\% \pm 5.7$  respectively (Table B4.4/1). The reduction in T cytotoxic-suppressor cells in type 2 diabetics in comparison with normal subjects was statistically significant ( $p < 0.05$ ).

## Th/Tc-s ratio

When the ratio between the percentages of helper T cells and of cytotoxic/suppressor T cells was calculated in type 1, type 2, gestational diabetic pregnant, normal pregnant subjects and normal

women the results obtained were  $2.1 \pm 0.9$ ,  $3.4 \pm 0.9$ ,  $2.4 \pm 0.8$ ,  $2.4 \pm 0.97$  and  $2.2 \pm 0.67$  respectively (Table B4.4/1). The ratio was significantly increased in type 2 diabetics in comparison to normal pregnant women, gestational diabetics, type 1 diabetics and normal subjects ( $p < 0.02$ ).

#### T cell subsets during pregnancy

The values of T cell subsets at the end of the 1st, 2nd and 3rd trimester of pregnancy in type 1, type 2 and gestational diabetic pregnant women were as indicated below:

|                    | OKT3 |      | OKT4 |      | UCHT4 |      |
|--------------------|------|------|------|------|-------|------|
|                    | mean | SD   | mean | SD   | mean  | SD   |
| type 1 pregnant d. |      |      |      |      |       |      |
| 1st trim.          | 55.2 | 10.3 | 37.2 | 13.2 | 18.6  | 11.2 |
| 2nd trim.          | 56.1 | 5.5  | 35.1 | 5.9  | 21.2  | 3.6  |
| 3rd trim.          | 55.0 | 2.1  | 35.3 | 3.6  | 18.3  | 3.9  |
| type 2 pregnant d. |      |      |      |      |       |      |
|                    | 48.9 | 0.7  | 33.7 | 2.7  | 8.4   | 0.6  |
|                    | 56.3 | 3.4  | 38.9 | 0.8  | 15.9  | 6.7  |
|                    | 59.2 | 4.7  | 46.6 | 9.4  | 13.5  | 2.2  |
| gestational d.     |      |      |      |      |       |      |
|                    | 57.9 | 4.6  | 42.6 | 9.1  | 18    | 2.2  |
|                    | 54.9 | 5.1  | 41.6 | 5.5  | 16.3  | 2.2  |
|                    | 51.9 | 0.7  | 36.6 | 0.9  | 27.5  | 0.8  |

#### Normal pregnancy

The percentages of total T cells, of helper/inducer T cells and of suppressor cytotoxic lymphocytes were lower in normal pregnant

women in comparison with normal controls. These differences were clearer when the values at the end of the first trimester of pregnancy were considered.

TABLE B4.4/1 T cell subsets in diabetic and normal pregnancy

|                           | OKT3 |     | OKT4 |     | UCHL1 |     |
|---------------------------|------|-----|------|-----|-------|-----|
|                           | mean | SD  | mean | SD  | mean  | SD  |
| Type 1 pregnant diabetics | 55.4 | 6.7 | 35.8 | 8.5 | 19.1  | 7.0 |
| Type 2 pregnant diabetics | 57.5 | 5.3 | 42.5 | 9.1 | 13    | 4.2 |
| Gestational diabetics     | 56.5 | 5   | 41.7 | 7.9 | 18.5  | 3.8 |
| Normal pregnant women     | 56   | 7   | 38.2 | 7.3 | 17.7  | 5   |
| Normal women              | 59.1 | 2.3 | 41.5 | 6.4 | 19.8  | 5.7 |

B4.4d      D i s c u s s i o n

Changes in the relative proportions of T cell subsets, and in particular in the balance between helper and suppressor T cells, could be important in the maternal immune tolerance towards the fetus. It is interesting to evaluate the possible changes in T cell subpopulations especially in diabetic pregnancy where immune abnormalities related to diabetes may be superimposed on the modifications related to pregnancy.

In normal pregnancy contradictory studies on the proportion of T cell subpopulations have so far been reported. Absolute T cells counts (Dodson et al 1977, Cornfield et al 1979), and the proportion of T cells (Scott et al 1978; Dodson et al 1977, Birkeland & Kristoffersen, 1977; Sumiyoshi et al 1981; Campion & Currey 1972; Garewal et al 1978) are reported to remain unchanged in a few studies whereas others report a marked fall in the first trimester (Strelkauskas et al 1978) and a minor fall in the third trimester (Baines et al 1977, Cornfield et al 1979; Bulmer & Hancock, 1977; Vanderbeeken et al 1982). A decrease in T helper cells during pregnancy was reported (Vanderbeeken et al, 1982) in contrast with others who did not find significant changes (Moore et al, 1983). These contradictory results may be explained by differences in the methodology and techniques used and also by the relatively small number of subjects investigated.

In this study a minor decrease in the percentages of total T cells and helper T cells, especially at the end of the first

trimester of pregnancy is in agreement with several of the above reported studies (Strelkauskas et al 1978; Vanderbeeken et al 1982).

Regular follow up of a large number of normal pregnant women and studies with the use of standardized and accurate techniques to evaluate the T cell populations are at present needed.

In diabetic pregnancy very few studies on cellular immunity are reported (Galluzzo & Bompiani).

In type I diabetic pregnant women a significant reduction in helper/ inducer T cells is observed. This reduction is observed also in long standing type I diabetics (chapter B 3.5). Thus the reduction in cells bearing the T4 phenotype seems to be related to the immune abnormalities linked to diabetes more than to the events occurring in pregnancy.

In type 2 diabetic pregnant women a significant increase in the ratio of helper T cells/ cytotoxic-suppressor T cells is observed. In long standing type 2 diabetes no significant modifications in the percentage of lymphocyte subsets was detected (chapter B 3.5).

This increase in the ratio of helper/suppressor lymphocytes seems to be related to events occurring in pregnancy. The explanation of this finding is not obvious. Since type 2 pregnant patients already treated with diet or oral agents are at the beginning of pregnancy and thereafter treated with heterologous insulin, this cellular modification might reflect this event.

In gestational diabetic patients no significant difference in T cell subsets in comparison with normal pregnant women has been found.

The modifications of T cell subpopulations reported above do not seem to reflect adequately the complex and fascinating sequence of immunological events occurring in pregnancy. The failure to respond

of the maternal immune system to the antigenically foreign fetoplacental unit must involve complex immunological mechanisms. More sophisticated techniques are needed to study cellular immunity in pregnancy including monoclonal antibodies defining T cells during activation and differentiation.

B4.5 CELLULAR IMMUNITY IN DIABETIC PREGNANCY :  
ACTIVATED T CELLS.

B4.5a Aim of the study

Aim of this study was to investigate the possible presence of activated T cells in diabetic pregnant patients and to compare the findings with those in normal pregnant subjects and in normal women. The type of diabetes has been taken into consideration. The expression of surface antigens such as the transferrin receptor, the T cell growth factor, a protein of 120 k molecular weight linked to the insulin receptor, and different determinants of class II MHC has been analyzed.

B4.5b Materials and Methods

Patients

See chapter B4.4b

Cells



4F2 positive cells (chapter A3.2a) were enumerated in all the patients. Since 4F2 positive cells show a peaked asymmetric distribution in the normal population, the limit of positivity was chosen as the 90th percentile of the values found in normal controls

Monoclonal antibodies 5E9 and TAC (see chapter A3.2a) have been used to define activated T cells in some subjects.

The monoclonals 231, 164 and L 243 (see chapter A3.2a) were used to reveal Class II antigens expressed on the surface of T lymphocytes. A double staining method and Medicell chambers were used to enumerate class II positive T cells (see chapter A3.2b&c)

#### Statistical analysis

Wilcoxon's test and unpaired t test were used to evaluate the results.

B4.5c      R e s u l t s

## Type 1 diabetic pregnant

4F2 positive cells, in numbers above the 90 th percentile of values found in normal controls, were found in 75% of type 1 diabetics (Figure B4.5/1).

Increased levels of 5E9 and TAC positive T lymphocytes were not found in any of the patients.

DA6.231 positive/T3 positive lymphocytes were found in 42% of this type of diabetics whereas DA6.164 positive/ T3 positive cells were in 37% and L243 positive cells in 37% of type 1 diabetic pregnant women. The positivity for the antibodies 231, 164 and 243 was defined taking as limit of positivity the mean + 2 SD of values found in normals. 50% of the patients tested showed a class II MHC T cell positivity with at least one of these 3 monoclonals. (Figure B4.5/2).

## Type 2 diabetic pregnant

4F2 positive cells were present in 50% of type 2 diabetics (Figure B4.5/1) whereas 5E9 and TAC positive cells were within the normal range.

231 +/T3+ lymphocytes were found in 25% whereas 164+/T3+ and 243+/T3+ cells were within the normal range (Figure B4.5/2).

## Gestational diabetics

4F2+ cells were present in 44% of patients (Figure B4.5/1) whereas 5E9+ and TAC+ in none of them 231+/T3+ cells were present in

20%, 164+/T3+ in 25% and 243+/T3+ cells in 30% of gestational diabetic pregnant (Figure B4.5/2).

#### Normal pregnant

4F2 positive cells were found in 70% of subjects (Figure B4.5/1).

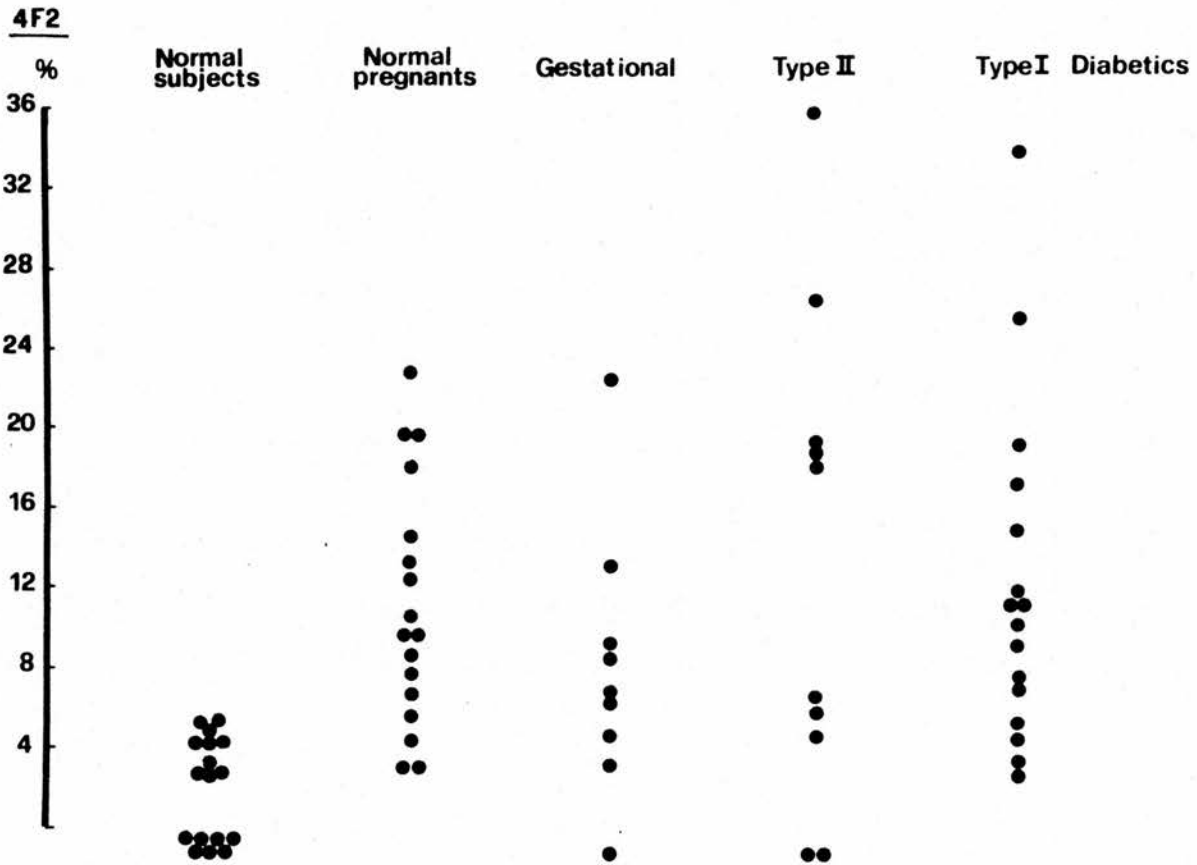
DA6.231 +/T38 cells, DA6.164+/ T3+ and L243+/T3+ lymphocytes were found in none of the normal pregnant women studied.

#### Normal women

The mean values for 4F2, 231, 164, 243 were 3.4 (2.2), 1.1 (1.1), 0.5 (0.8) and 1.3 (1).

FIGURE B4.5/1

Percentages of 4F2 positive mononuclear cells in normal subjects, normal pregnant women, in gestational, type 2 and type 1 pregnant diabetics.





B4.5d      D i s c u s s i o n

In diabetic pregnancy, the immune phenomena occurring in diabetes must be viewed against a background of immune events that occur in normal pregnancy. On the other hand the sum of the immunological events related to diabetes and to pregnancy may generate a particular immunological situation with possible effects on the disease itself, on the course of pregnancy, on the placenta and on the fetus.

In pregnancy there is an apparent failure to recognise fetal antigenic components or an inability to generate an immune response to the histoincompatible fetoplacental unit. Modifications in cellular immunological processes are mainly responsible for this immune adaptation to pregnancy. The proportions of T regulator and T effector cells can be evaluated with accuracy by the use of monoclonal antibodies (Chapter B4.4). More recently attention has been focussed on the evaluation and presence of the significance of T lymphocytes expressing antigens on their surface not present in the resting state. The mitogenic or allogeneic stimulation of T lymphocytes results in the appearance of activated T lymphocytes, i.e. cells expressing surface determinants not present on resting cells. According to some authors these antigens are expressed sequentially in the process of activation: the 4F2 antigen being among the earliest, the Tac among the last ones to be expressed whereas the class II MHC molecules are among the best defined antigens expressed on activated T lymphocytes (Cotner et al, 1983)

To study activated T cells in diabetic pregnancy is of interest for several reasons. Since the presence of activated T cells is a marker for allogeneic stimulation, T cell activation would be expected to be present in pregnancy but blocked or somehow modified, so as to avoid an immune rejection of the fetus. Activated T cells have been reported to be increased in type 1 diabetes ( Rowley & Eisenbarth, 1982; Pozzilli et al, 1983). When diabetes and pregnancy are concomitant the effects on T cell activation is not obvious.

In this study activated T cells have been studied in diabetic pregnant women and in normal pregnant subject by the use of a panel of monoclonal antibodies.

A clear increase in 4F2 positive cells is present both in normal and in diabetic pregnant women. There is an increase in MHC class II positive lymphocyte in diabetic pregnant women but not in normal pregnant subjects. The increase in class II positive T cells is higher in type 1 pregnant diabetics. In those patients in whom Tac and 5E9 antigen expression was examined, no increase of these antigens on T cells was found.

In normal pregnancy functionally mature and fully activated T lymphocytes, namely those expressing class II, Tac and 5E9 antigens, are not present. On the other hand the increase in 4F2 positive cells is an interesting finding. It is conceivable that resting T cells in normal pregnancy are activated or induced to enter the first phase of the cell cycle (G0 → G1 transition) and that this coincides with the expression of the 4F2 antigen at the cell surface . Further progression into the cell cycle , leading to mitosis and expression of the Tac antigen, class II antigens and functional maturity,

appears to be blocked. This is in keeping with the modern concept that pregnancy represents a state of immunoactivation and not of immunodepression (Gleicher & Siegel, 1983). In other words pregnancy seems to be a state in which immunosuppressive components are activated. It cannot be excluded that the 4F2 positive mononuclear cells in pregnancy are immature cells or fetal cells (Gleicher & Siegel, 1983).

In diabetic pregnancy, in addition to the increase in 4F2 antigen on mononuclear cells, the increase in class II positive T cells in a number of type 1 diabetics, and in a few type 2 or gestational diabetics is likely to reflect the immune abnormalities preexistent to pregnancy. Fully functionally mature and activated T cells, namely those expressing Tac and 5E9 antigens have not been found. This is in keeping with other reports in type 1 diabetes where 5E9 positive cells were not found to be increased (Pozzilli et al, 1983). As expected in diabetic pregnancy the cellular immune factors are simultaneously influenced by phenomena related to diabetes and to pregnancy. It remains to be shown whether the superimposition of diabetes upon normal pregnancy may represent an immunological "final straw" with consequences for the fetus (Page Faulk et al, 1980; Galbraith & Page Faulk, 1981).



## B4.6 CONCLUSIONS

The hypotheses formulated in planning the work have been investigated. The results reported in this chapter show that :

a) Humoral factors, in addition to metabolic events, seem to influence the clinical course of pregnancy and neonatal complications.

Islet cell antibodies were found not to be correlated with maternal or neonatal clinical complications. In particular it is confirmed that neonates of islet cell antibody positive mothers apparently do not suffer from any specific metabolic disorder. Nevertheless the presence of islet cell antibodies in some gestational diabetic pregnant women is evidence of a likely immunological response damaging the islets, as confirmed by the reports of others of an early insulin failure in a similar group of patients.

Insulin antibodies were found to be increased in a significant proportion of pregnant diabetic patients who presented with gestosis. The presence of maternal insulin antibodies was correlated with a number of clinical complications in the neonates. Indeed, theoretically, insulin antibodies may interfere, either directly or indirectly, in a number of ways with the fetal metabolism and development. Some types of immune complexes were increased in pregnant diabetics but their correlation with gestosis failed to reach statistical significance.

b) Insulin anti-insulin complexes are here proved to be present also in neonatal circulation and they may have physiopathological effects. Insulin-anti-insulin complexes in the neonate do not reflect simply a passive passage from the maternal circulation. The clearance of fetal insulin-anti-insulin complexes seems slower than that of the mother.

Insulin antibodies in the mother and in cord blood show similar levels. The differences in the antigens encountered may explain in part the different levels of insulin complexes.

c) The absolute number of total T cells is not significantly different in diabetic pregnant women and in normal pregnant.

Nevertheless T helper cells are significantly decreased in type I diabetic pregnant women.

An increase in the ratio T helper/T suppressor-cytotoxic cells has been observed in pregnant diabetics in comparison with normal women.

The expected difference in cellular immunological status in diabetic pregnant, seems to be only marginally reflected in variations of the absolute T cell subpopulation number.

d) Mononuclear cells expressing surface antigens not usually present in non-activated lymphocytes are increased in diabetic pregnant women.

A clear increase in 4F2 positive cells is present both in normal and in diabetic pregnant women.

There is an increase in MMC class II antigen positive lymphocytes in type 1 diabetic pregnant women but not in normal pregnant subjects.

Antigens usually found in mature and activated lymphocytes were not found in the subjects studied.

A population of aberted but not fully activated immunocomponent cells seems to be increased in pregnancy.

In diabetic pregnancy T cell subsets are simultaneously influenced by phenomena related to diabetes and to pregnancy.

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A P P E N D I X

P U B L I C A T I O N S

by the author  
submitted in support of the thesis

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## **The correlation between insulin antibodies and circulating immune complexes in diabetics with and without microangiopathy**

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### SUMMARY

The relationship between immune complexes and insulin antibodies was evaluated in 237 insulin treated subjects with a duration of diabetes of more than 1 year. Ninety-seven diabetics were selected at random (group 1) whereas 140 according to the presence of diabetic microangiopathy (group 2). Immune complexes were evaluated by the solid phase C1q binding test in all patients and by conglutinin radioimmune assay in most of them. Insulin antibodies were determined by Christiansen's and Anderson's methods. Immune complexes as detected by the solid phase C1q method were found increased in group 1 and there was an inverse correlation between these complexes and insulin antibody levels. In group 2 patients with microangiopathy the amount of this kind of complex was significantly greater than in those without microvascular lesions and there was no correlation with insulin antibodies. Immune complexes as detected by conglutinin were found increased in group 2 patients and these were significantly correlated with the level of insulin antibodies. No increase in these immune complexes was found in patients with microangiopathy when compared with patients without microangiopathy. Insulin antibodies were not correlated with the presence of complications. Overall, immune complexes detected by C1q binding were significantly correlated with the presence of microangiopathy. In patients with high insulin antibody levels the complexes formed were not detected by C1q binding. The immune complexes detected by conglutinin are correlated with insulin antibodies, but not with the presence of microangiopathy.

### INTRODUCTION

Since insulin antibodies (InsAb) were first described, there have been numerous investigations of the possible influence of prolonged heterologous insulin treatment on the course of diabetic microangiopathy. This question is still unanswered.

So far there have been a few conflicting studies that have tried to correlate the level of insulin antibodies with the severity of microangiopathy (Ortved Andersen, 1976; Page Faulk, Karam & Fudenberg, 1971; Iavicoli *et al.*, 1980). There have also been several contributions in animals showing a direct pathogenic role of exogenous insulin in microangiopathic lesions (Andreev, Ditzov & Dashev, 1970; Zampa & Mancini, 1965; Mohos, Heningar & Fogelman, 1963). On the other hand, it is well known that the lesions of small blood vessels are present in patients treated with both insulin and oral agents, although differences have been described in the severity and frequency of the lesions.

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If insulin treatment has a role in aggravating blood vessel damage through immune mechanisms, the formation and deposition of insulin-anti-insulin complexes must be considered. This is supported by the finding that in animals microangiopathy is induced only when insulin is injected with Freund's adjuvant and insulin antibodies are formed (Wehner, Huber & Kronenberg, 1973).

The study of insulin-anti-insulin complexes is complicated by a few factors (Folling, 1976). Insulin is a weak antigen and is antigenically bivalent. Insulin complexes are usually small and soluble both in antigen and in antibody excess. Only a small part of these complexes, near the equivalence point, are large enough to be capable of triggering a sequence of events that can result in tissue damage. So far there are no routine methods available to measure these different kinds of complexes. When a few years ago circulating immune complexes (IC) were described in some diabetic conditions (Di Mario, Iavicoli & Andreani, 1980) and when more recently these complexes were reported to be significantly increased in diabetics with severe microangiopathy compared with those without complications (Irvine *et al.*, 1978a; Andreani *et al.*, 1982), it was natural to suppose that part of the complexes found in patients with microangiopathy would be comprised of insulin.

This study was planned to investigate the correlation between the presence of insulin antibodies and the occurrence of immune complexes in patients with and without microangiopathy. Since there are substantial differences in the results of different techniques aimed at determining circulating immune complexes, two methods were employed, one detecting medium sized complexes in antigen excess through the Fc portion of complexed immunoglobulins—the solid phase C1q binding test (C1qSP)—and the other detecting C3 binding complexes near the equivalence point—the conglutinin radioimmune assay (KgBt).

Patients were selected using two different approaches: one group was composed of randomly selected insulin treated diabetics and another group was selected so as to have patients in opposite conditions, i.e. with severe microangiopathy and without any clinical signs of microangiopathy.

#### PATIENTS AND METHODS

*Patients.* Two hundred and thirty-seven diabetics treated with insulin for more than 1 year were selected for the study. Ninety-seven were randomly selected consecutive patients (group 1) and 140 patients were selected according to the presence and degree of microangiopathy: 58 showed severe microangiopathy, 82 had no sign of vascular lesions (group 2). The clinical and personal data of the patients are shown in Table 1. One hundred and eighty-nine blood donors were used as the control population in IC studies. The evaluation of the presence and degree of microangiopathy was performed as follows: retinopathy was assessed by retinal fluorangiography—patients with

Table 1. Clinical characteristics of the patients

|                                | No. | Sex |    | Age<br>(mean $\pm$ s.d.) | Type<br>of<br>diabetes |    | Complications* |    |     |
|--------------------------------|-----|-----|----|--------------------------|------------------------|----|----------------|----|-----|
|                                |     | M   | F  |                          | I                      | II | R              | N  | R+N |
| Group 1                        | 97  | 51  | 46 | 33.8 $\pm$ 17.4          |                        |    |                |    |     |
| Group 2                        |     |     |    |                          |                        |    |                |    |     |
| with severe<br>microangiopathy | 58  | 25  | 33 | 49.5 $\pm$ 16.1          | 38                     | 20 | 36             | 12 | 10  |
| without<br>microangiopathy     | 82  | 44  | 38 | 37.8 $\pm$ 16.7          | 67                     | 15 |                |    |     |

\* R = proliferative retinopathy; N = advanced nephropathy; R + N = both complications.

proliferative retinopathy or recidivant vitreous haemorrhages were considered to have severe retinopathy and patients with the absence of any sign of retinopathy were considered free of ocular complications. Patients with intermediate forms of retinal involvement were excluded from the study. Blood urea  $> 16$  mmol/l, serum creatinine levels  $> 2$  mg/dl and/or proteinuria  $> 2$  g/l were considered the index of kidney involvement.

**Methods.** C1qSP in group 1 and 2 patients as described by Hay, Nineham & Roitt (1976) and KgBt in group 2 patients according to Casali *et al.* (1977) with some personal modifications (Irvine *et al.*, 1978b) were used to detect immune complexes. Andersen's radioimmune assay (Ortved Anderson, Brunfeldt & Albigard, 1972) as modified by Mustafa, Dagget & Nabarro (1978) and Christiansen's electrophoretic method (Christiansen, 1970) were used to detect InsAb respectively in group 1 and 2 patients. The results were expressed in  $\mu$  ml of insulin binding capacity in the first method and  $\mu$  ml in the second one. The characteristics of the methods, the limit of positivity, the expression and analysis of the results for IC tests have been extensively discussed elsewhere (Andreani *et al.*, 1982; Di Mario *et al.*, 1980). As in most studies on circulating IC, the limit of positivity of IC was chosen as the 90th percentile of the values found in the normal population, because of their peaked asymmetric distribution. In Fig. 2 IC values were also expressed in percentage of radioactivity ( $^{125}$ I-protein A) bound radioactivity added (Casali *et al.*, 1977; Irvine *et al.*, 1978a, 1978b; Iavicoli *et al.*, 1982). Fisher's exact test, the  $\chi^2$  test, Cox's test, the *t*-unpaired test and Kendall's test were used for the statistical evaluation of the results.

## RESULTS

### Group 1

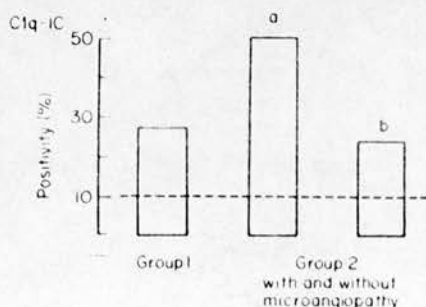
**Immune complexes.** In randomly selected diabetics, C1q-IC were found in 26 out of 97 patients (27%,  $P < 0.01$  vs normal population; Fig. 1).

**Insulin antibodies.** In the same patients, InsAb, as evaluated by Andersen's method, showed a mean value of  $116.4 \pm 113.2$   $\mu$ u/ml.

**Immune complexes vs insulin antibodies.** When InsAb were correlated with C1q-IC values, an inverse correlation was found between InsAb levels and C1q-IC values ( $P < 0.02$ ) and patients with high levels of InsAb tended to be C1q-IC negative (Fig. 2).

### Group 2

**Immune complexes.** C1q-IC were found in 29 out of 58 patients with severe microangiopathy (50%,  $P < 0.0001$  vs normals) and in 20 out of 82 of those without microangiopathy (24%,  $P < 0.01$  vs normals,  $P < 0.001$  vs complicated diabetics; Fig. 1). Kg-IC were found in 21 out of 58 patients without vascular lesions (36%,  $P < 0.0001$  vs normals) and in 20 out of 53 diabetics with severe microangiopathy (38%,  $P < 0.0001$  vs normals, N.S. vs uncomplicated diabetics).



**Fig. 1.** Immune complex positivity, as detected by C1q-SP (C1q-IC), in group 1 and 2 patients. The positivity (%) of C1q-IC is indicated in the Y axis. The dotted line indicates the positivity in the control population (10%). Group 2 patients are divided into those with (column a) and without (column b) microangiopathy.

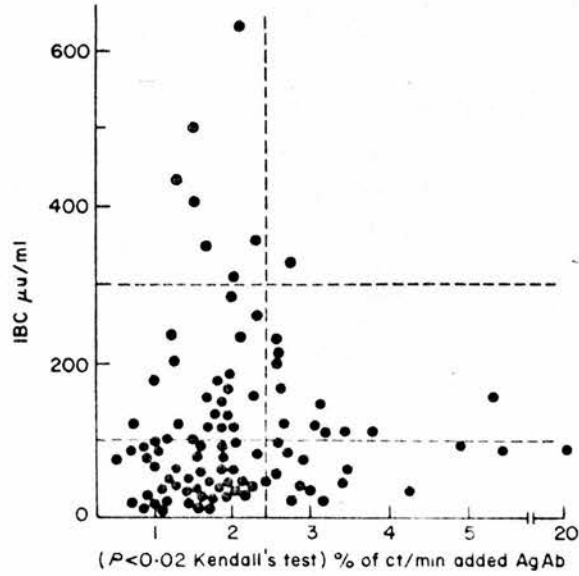


Fig. 2. Immune complexes (AgAb), as detected by C1q-SP, and insulin antibodies (IBC), as assayed by Andersen's method, in group 1 diabetics. The horizontal dotted lines divide insulin antibody levels into negative, low, medium and high levels. The vertical dotted line divides immune complex values into negative and positive.

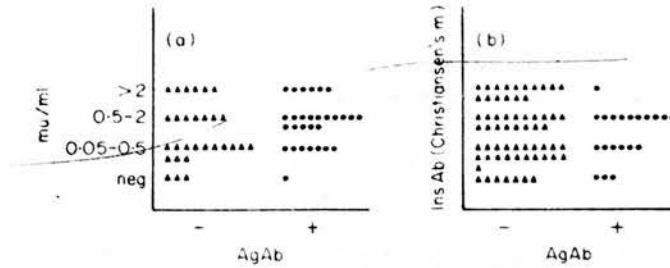


Fig. 3. The association between C1q-IC (AgAb) and insulin antibodies (InsAb) in group 2 patients with (a) and without (b) complications. InsAb are indicated in the Y axis and divided into negative, low (0.05-0.5  $\mu\text{u/ml}$ ), medium (0.5-2  $\mu\text{u/ml}$ ) and high (>2  $\mu\text{u/ml}$ ) levels.

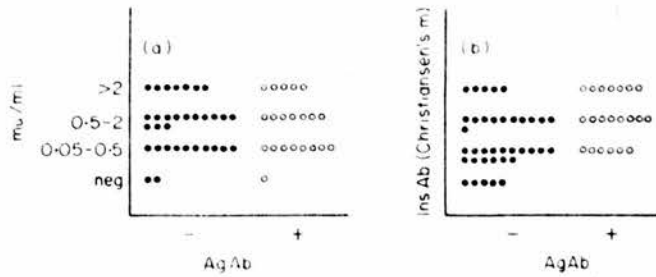


Fig. 4. The association between immune complexes (AgAb), as detected by conglutinin (Kq-IC), and insulin antibodies (InsAb) in group 2 patients with (a) and without (b) complications. InsAb as in Fig. 3.

*Insulin antibodies.* Mean InsAb levels, as evaluated by Christiansen's method, were 1.33 mu/ml in group 2 patients. There was no significant difference in InsAb levels between those with severe microangiopathy ( $1.27 \pm 1.47$  mu/ml) and those without complications ( $1.38 \pm 1.88$  mu/ml).

*Immune complexes vs insulin antibodies.* The correlation between C1q-IC and InsAb was significantly different in patients with and without microangiopathy (Fig. 3). In microangiopathic patients the positivity of C1q-IC did not vary significantly at increasing levels of InsAb, whereas in patients without microangiopathy, when InsAb levels were high, C1q-IC positivity was significantly reduced in comparison with that found in those with lower InsAb levels ( $P < 0.05$ ). Diabetics without microangiopathy having medium/high levels of InsAb ( $> 0.5$  mu/ml) showed a significantly reduced IC positivity in comparison with diabetics with microangiopathy showing similar InsAb levels. A different correlation was found between Kg-IC and InsAb (Fig. 4). In group 2 there was a positive trend for Kg-IC positivity to go with increasing levels of InsAb ( $P < 0.05$ ). This trend was even more evident when only patients without microangiopathy were considered ( $P < 0.01$ ; Fig. 4b).

## DISCUSSION

As expected the correlation between InsAb and immune complexes is rather different when C1q-IC or Kg-IC are studied.

In insulin treated diabetics selected at random, when InsAb are present in high amounts, C1q-IC are not usually detectable. Since C1qSP lacks complexes in antibody excess (Scullion, Balint & Whaley, 1979), in patients with high levels of InsAb, insulin antibody complexes would not be expected to be detectable. It is conceivable that in patients with lower InsAb levels some immune complexes may be related to insulin treatment. In fact, in another study by our group, C1q-IC were found to be higher in randomly selected insulin treated patients than in comparable diabetics not treated with insulin (Andreani *et al.*, 1982). Similar results were found in group 2 patients without microangiopathy. It is interesting to note that when microangiopathy is present, C1q-IC levels are much higher and not correlated with the levels of InsAb. This study confirms the recently reported (Irvine *et al.*, 1978a; Andreani *et al.*, 1982) increase in C1q-IC in diabetics with microangiopathy. When microangiopathy is present, some factors seem to modify the dynamics of formation and clearance of medium large sized complexes, leading to an increase in circulation of these macromolecules. These factors do not seem to be directly related to insulin treatment since C1q-IC were increased both in type I and in type II diabetics with microangiopathy (Andreani *et al.*, 1982). It has been suggested that impairment of phagocytosis, described in patients with microangiopathy (Drivas & Wardle, 1978; Bagdade, 1976), might result in an increase of circulating macromolecules. Indeed it was recently reported that diabetic patients with an impaired phagocytic clearance also have an increase in circulating C1q-IC (Iavicoli *et al.*, 1982). It can be accepted that in diabetics with microangiopathy an impairment of the phagocytic system leads to an increase in heterogeneous complexes, mainly in the range detected by C1qSP, and masks any correlation between C1q-IC and InsAb.

When Kg-IC results are evaluated, there is a trend for the presence of Kg-IC to go with medium high levels of InsAb (especially when microangiopathy is absent). An association between Kg-IC and InsAb has been recently reported in diabetics with less than 1 year's diabetes (Iavicoli *et al.*, 1983). Likewise, Kg-IC are higher in type I diabetics irrespective of the presence of microangiopathy (Andreani *et al.*, 1982). It appears that KgBt detects immune complexes which are somehow related to the presence of InsAb rather than to the occurrence of diabetic microangiopathy. The explanation of this correlation is not obvious. Kg-IC are usually large complexes that fix the complement and therefore very different from most insulin anti-insulin complexes. It cannot be excluded that KgBt reacts with some monomeric immunoglobulins, when present in a very high concentration, as we have seen in experimental conditions (unpublished personal observations). The levels of InsAb in these patients were not associated with the presence of microangiopathy. This is quite in keeping with the similar lack of association between Kg-IC and microangiopathy.

In this study there is no clear evidence of the influence of heterologous insulin treatment on

diabetic microangiopathy. To some extent this result was expected. If insulin treatment has any influence on the course of complications through immune mechanisms (i.e. the formation of insulin-anti-insulin complexes), the insulin binding capacity is not the best tool for investigating this problem. Insulin antibodies vary widely in avidity and form complexes with different pathogenetic significance. The methods for detecting InsAb usually available, and particularly those used in this investigation, give information on the amount, and not the avidity, of the antibody and measure mostly free antibodies. It is not therefore surprising that the sole immunological phenomenon found in association with the presence of severe diabetic microangiopathy is the increase of immune complexes detected by the solid phase C1q radioimmunoassay. The question as to whether heterologous insulin treatment might influence microangiopathy is related to two other questions: does insulin treatment give rise to complexes of pathogenic significance, and do these complexes and the C1q-IC—chronically accumulated and deposited in the small blood vessels—play a role in aggravating local tissue damage?

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## CIRCULATING IMMUNE COMPLEXES IN DIABETICS: THE INFLUENCE OF SEX, AGE, DURATION OF DISEASE AND TYPE OF TREATMENT

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**SUMMARY** The influence of sex, age, duration of diabetes and type of antidiabetic treatment on soluble immune complexes levels was investigated in the sera of 276 randomly selected diabetics. Immune complexes were detected by the solid phase C1q binding test.

The prevalence of immune complexes was significantly higher in insulin treated than in non-insulin treated diabetics. Within the insulin treated group, the prevalence in diabetics of 11-20 yr duration was significantly higher than in the remainder. No difference in immune complexes levels was found between males and females. The age of the patients did not have any correlation with the levels of immune complexes.

These findings suggest that some of the immune complexes detected in randomly selected diabetics are related to insulin treatment, reflecting either differences in the type of diabetes or the effects of heterologous insulin.

### INTRODUCTION

CIRCULATING immune complexes (AgAb) were reported to be increased in diabetics, especially in those who are type I insulin dependent near diagnosis (1) and patients with severe microangiopathy (2). The role of immune complexes in the disease and especially in relation to the vascular and renal complications is still controversial and further studies are necessary. The techniques presently available for the detection of AgAb do not give information on the antigen(s) involved and do not differentiate between immune complexes involved in a specific disease process and those present also in clinically normal subjects. Thus it is relevant to study the influence of the many variables and factors that could influence the levels of AgAb and it is necessary to compare the results with those of a large group of clinically normal subjects (3).

In this study we have investigated the influence of sex, age, duration of disease and type of antidiabetic treatment (insulin or oral agents) on AgAb levels.

### MATERIALS AND METHODS

Two hundred and seventy-six diabetics attending the Diabetic Clinics of Rome and of Edinburgh were selected at random. One hundred and forty-seven were males and 129 were females. Twenty-five diabetics

were less than 20 years old, 42 were in the third decade, 29 in the fourth, 32 in the fifth, 53 in the sixth and 95 were more than 60 years old. In 67 patients the duration of diabetes was between 1 and 5 yr, whereas in 61, 62, 42 and 44 patients the duration of diabetes was between 6 and 10, 11-15, 16-20 and over 20 yr, respectively. One hundred and forty-eight were treated with insulin at the time of the study and 128 with diet or oral hypoglycaemic agents. Insulin treated patients received ordinary commercial insulins (Lente, NPH, crystalline). None of those was treated with purified insulins.

One hundred and thirty blood donors were included in the study as normal controls.

Circulating AgAb were assayed by the solid phase C1q binding test according to Hay *et al.* (4) with minor modifications (5). The limit of positivity was chosen as the 90th percentile of blood donor values (6).

The Chi square test was used for the statistical analysis of the results.

### RESULTS

AgAb levels were above the limit of positivity in 65 out of the total 276 diabetics studied and in 13 out of the 130 blood donors. Therefore AgAb were significantly increased in the diabetics ( $p < 0.002$ ).

Among the diabetics, AgAb were present in 24.5% of males and in 22.5% of females.

When the diabetics were divided according to age, there was no significant difference in AgAb levels (table 1(A)).

When the duration of diabetes was taken into account considering all the patients together, there was no significant correlation between AgAb and duration of disease (table 2(A)).

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Table 1 The influence of age on AgAb

|                           | Age of patients (years) |       |       |       |       |     |
|---------------------------|-------------------------|-------|-------|-------|-------|-----|
|                           | <20                     | 21-30 | 31-40 | 41-50 | 51-60 | >60 |
| (A)                       |                         |       |       |       |       |     |
| No. of patients           | 25                      | 42    | 29    | 32    | 53    | 95  |
| AgAb positive             | 8                       | 12    | 7     | 11    | 11    | 16  |
| (B)                       |                         |       |       |       |       |     |
| Insulin treated (no.)     | 25                      | 37    | 26    | 16    | 24    | 20  |
| AgAb positive             | 8                       | 12    | 6     | 7     | 5     | 7   |
| Non-insulin treated (no.) | 0                       | 5     | 3     | 16    | 29    | 75  |
| AgAb positive             | 0                       | 0     | 1     | 4     | 6     | 9   |

Table 2 The influence of the duration of diabetes on AgAb

|                           | Duration of diabetes (years) |      |       |       |     |
|---------------------------|------------------------------|------|-------|-------|-----|
|                           | 1-5                          | 6-10 | 11-15 | 16-20 | >20 |
| (A)                       |                              |      |       |       |     |
| No. of patients           | 67                           | 61   | 62    | 42    | 44  |
| AgAb positive             | 15                           | 13   | 16    | 13    | 8   |
| (B)                       |                              |      |       |       |     |
| Insulin treated (no.)     | 36                           | 32   | 24    | 26    | 30  |
| AgAb positive             | 9                            | 7    | 11*   | 12*   | 6   |
| Non-insulin treated (no.) | 31                           | 29   | 38    | 16    | 14  |
| AgAb positive             | 6                            | 6    | 5     | 1     | 2   |

\*  $p < 0.001$  vs the remainder.

However, when patients were divided according to the type of treatment, insulin treated diabetics showed a significantly higher prevalence of AgAb compared to the non-insulin treated diabetics (30% vs 16%,  $p < 0.005$ ). The difference in AgAb positivity between insulin treated diabetics and normal controls was highly significant ( $p < 0.0001$ ), whereas the difference between non-insulin treated diabetics and normals failed to reach statistical significance ( $\chi^2 1.9$ ).

Within the insulin treated group there is a statistically significant peak in the prevalence of AgAb in diabetics of 11-20 years duration ( $p < 0.001$ ) (table 2(B), fig. 1(B)), but there was no correlation with the age of patients.

#### DISCUSSION

In this study on a randomly selected population of diabetics, AgAb were found to be increased in insulin

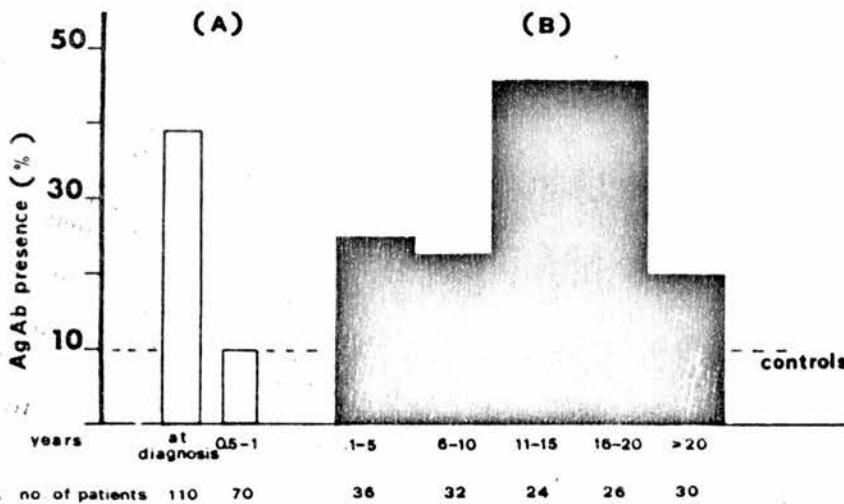


FIG. 1. The prevalence of AgAb in diabetes. (A) The percentage positivity for AgAb in recently diagnosed patients (see text). (B) The percentage positivity in diabetics according to the duration of clinical diabetes.

treated diabetics in comparison with non-insulin treated diabetics or normal controls. As the nature of AgAb has not been analyzed in the present study we can only surmise as to what the antigen components of these complexes might be. This correlation between insulin treatment and AgAb can be explained either as an intrinsic difference between type I and type II diabetes or as the effect of heterologous insulin administration. In some type I diabetics the increase in AgAb positivity may be explained as a consequence of a genetically determined increased immunoresponsiveness to exogenous agents present in some insulin dependent diabetics (7, 8). On the other hand the administration of exogenous insulin induces the formation of insulin-anti-insulin complexes and several clinical and experimental studies have already shown the presence of these complexes in insulin treated subjects (9, 10).

It is however by no means certain that insulin-anti-insulin complexes are detected by the C1q technique because of the immunological characteristics of insulin. Considering the bivalency of insulin, most insulin-anti-insulin complexes tend to be of very small size and so are not detectable by the techniques which use complement to reveal circulating AgAb. Only a small proportion of insulin-anti-insulin complexes form polymers and aggregates of detectable size. Nevertheless, in a randomly selected diabetic population it is possible that some of the antigen-unknown complexes found to be increased may be a consequence of an increased formation of such complexes induced by exogenous insulin.

This seems to be true especially when insulin-anti-insulin complexes are formed in antigen excess, whereas in the presence of antibody excess these complexes are not detected by the method used. Indeed an inverse correlation has been found between increasing levels of insulin antibodies and higher titres of AgAb in randomly selected diabetics (11).

Within the insulin treated diabetics there is a pronounced peak in the prevalence of AgAb in diabetics of 11-20 yr duration. Thus in insulin treated diabetics it is possible so far to describe two peaks of AgAb positivity according to the duration of diabetes. The first peak in AgAb levels is found within a few days of diagnosis with a sharp decline in positivity thereafter (fig. 1(A)), as already reported in Edinburgh insulin dependent diabetics (5). Since the techniques used to detect AgAb are antigen non-specific it is likely that the two peaks in AgAb positivity are due to different types of AgAb. AgAb in the first peak seem to be part of the immunopathological phenomena occurring at diagnosis and are statistically correlated to the presence of islet cell antibodies (ICAb). In the second peak AgAb may be induced by other factors even although in that minority of long standing insulin treated diabetics with persistently positive ICAb, AgAb were reported to be increased (12). While the highest titres of insulin antibodies tend to

occur at 9-12 months of insulin treatment, the highest titres of AgAb in the second peak (as detected by C1qSP) occurs at 11-20 yr. It is therefore likely that antigens other than insulin are involved in the second peak of AgAb.

This conclusion is also supported by the fact that, when severe microangiopathy is present, AgAb are increased in insulin as well as in non-insulin treated diabetics. This suggests that insulin-anti-insulin AgAb may not be the main component of these complexes, but that there may be a decreased clearance of miscellaneous types of complexes resulting from an impaired phagocytic system (2, 13).

The decline in AgAb positivity in diabetics with more than 20 yr of diabetes may be explained by the presence of milder complications in this group of patients or by the presence of some insulin treated type II diabetics. This study shows that the age of patients cannot explain this decline.

These results, together with our earlier findings, suggest that a heterologous population of immune complexes is present in diabetics. A careful selection of patients and the identification of antigens involved in complexes are necessary to understand their significance in diabetes.

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## HUMORAL IMMUNITY IN TYPE 1 DIABETES MELLITUS: A PROSPECTIVE STUDY

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**SUMMARY** Cytoplasmic islet cell antibodies, as detected by anti-IgG (ICAb), and circulating immune complexes (AgAb), detected by the solid phase C1q test (C1qSP), were evaluated in 153 insulin dependent diabetics (IDD) at diagnosis and subsequently in 88 of these patients who were studied prospectively at regular intervals for up to 3 yr. AgAb detected by the conglutinin (KgBt) and Raji cell (RAJI) techniques were also studied at diagnosis in 34 and 50 diabetics respectively. Normal controls were included in the AgAb studies. Complement fixing islet cell antibodies (CF-ICAb) were evaluated in 30 randomly selected diabetics both at diagnosis and after 6 months. Viral antibodies (VAb) were measured in 30 IDD at diagnosis and in 30 matched controls. Insulin antibodies (IBC) were measured 9 months after diagnosis in 35 diabetics and HLA studies (B8 and B15) performed in 115 patients.

In the prospective study the ICAb positivity declined from 50% at diagnosis to 45, 38, 36, 31, 26, 19 and 17% at 1, 3, 6, 9, 12, 24 and 36 months after diagnosis respectively. CF-ICAb were found in 30% of the diabetics at diagnosis and in 23% at 6 months. All patients with CF-ICAb at diagnosis were ICAb positive whilst only 47% of patients with ICAb also had CF-ICAb in the serum. AgAb were found at diagnosis in 35% of patients by C1qSP ( $p < 0.001$  vs. normals), in 35% by KgBt ( $p < 0.001$ ) and in 54% by RAJI ( $p < 0.002$ ). Eighty-four patients were studied at diagnosis by more than one AgAb method and of these 57% had at least one positive AgAb result. AgAb by C1qSP declined to less than 20% within 6 months of diagnosis. AgAb, as measured by C1qSP and RAJI techniques, correlated with ICAb at diagnosis whereas there was no correlation with VAb levels, IBC values, nor with the HLA antigens. There was no correlation between AgAb (C1q) and CF-ICAb. HLA B15 positive patients tended to form higher IBC levels than B15 negative patients. Thus, AgAb presence seems to parallel that of ICAb in the early stages of diabetes and both phenomena may be primarily or secondarily involved in the development of the disease.

### INTRODUCTION

DURING the last decade a great deal of evidence has accumulated to support the role of immunological phenomena in the pathogenesis of insulin dependent (Type 1) diabetes mellitus (IDDM) (1-4). Islet cell damage may be produced by cytotoxic antibodies, harmful immune complexes, T cells or by K cells. We have been particularly interested in circulating immune complexes (AgAb) since Irvine *et al.* reported, in 1977, their increased presence in some IDD's at diagnosis (5). Several sensitive techniques for the assay of AgAb are presently available including those which utilise C1q, Raji cells and conglutinin to bind complexes (6, 7). These methods reveal different types of complexes: C1q detects

mainly medium sized AgAb in antigen excess through the Fc portion of immunoglobulin aggregates, whilst conglutinin and Raji cells detect large size AgAb near the equivalence point, mostly through the third component of the complement. The concordance between these techniques is low (6). The above methods are antigen non-specific and consequently do not differentiate between complexes related to specific disease processes and those found in commoner conditions, such as viral infections. The significance of AgAb in a particular disease must be assessed following a comparison of the results with those found in the normal population and the selection of an appropriate limit of positivity (8). Several workers have confirmed the increase of AgAb in different diabetic states (9-12). The cross-sectional nature of these studies and methodological differences in AgAb assay may be responsible for some apparent discrepancies.

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In this study our aims have been to examine the humoral immune response in IDDM to endogenous and exogenous antigens such as islet cells, heterologous insulin and viruses, and to investigate the presence of antigen-antibody complexes. The relationship of these factors with the HLA antigens was also considered. We have therefore evaluated AgAb and cytoplasmic islet cell antibodies as detected by anti-IgG (ICAb) in a large group of IDD's at diagnosis and prospectively at intervals up to 3 yr following diagnosis. In view of the possible heterogeneity of AgAb in diabetes, immune complexes were detected by three different techniques: the solid phase C1q binding test (C1qSP), the Raji cell radio-immune assay (RAJI) and the conglutinin binding assay (KgBt). Complement fixing islet cell antibodies (CF-ICAb) at diagnosis and after 6 months, viral antibodies (VAb) at diagnosis, insulin antibodies (IBC) at 9 months after diagnosis and HLA antigens B8 and B15 were also evaluated in a number of these patients.

## MATERIALS AND METHODS

One hundred and fifty-three newly diagnosed, insulin dependent diabetics attending an adult clinic were studied within 2 days of diagnosis and of these 88 consecutive patients were studied prospectively for up to 3 yr following diagnosis. Details of the patients are shown in Table 1.

ICAb were evaluated in all diabetics at diagnosis and in 88, 79, 76, 68, 57, 31 and 6 patients at 1, 3, 6, 9, 12, 24 and 36 months after diagnosis respectively. AgAb (C1q) were also measured in all patients at diagnosis but only in 52, 58, 51, 58, 53, 29 and 5 patients at the above intervals. CF-ICAb were evaluated in 30 randomly selected diabetics both at diagnosis and after 6 months. AgAb (Kg) and (RAJI) were studied at diagnosis in 34 and 50 insulin dependent diabetics, respectively. A group of normal controls were included in the AgAb studies for the detection of the limit of positivity as described below.

Viral antibodies were assayed in 30 insulin dependent diabetics at diagnosis and in 30 age and sex matched normal controls residing in the same geographical region. Samples from the matched pairs of subjects were obtained within 7 days of each other.

Insulin antibodies were measured 9 months after diagnosis in 35 diabetics treated with highly purified ("monocomponent") insulins from diagnosis. HLA B8 and B15, representing the two HLA diabetogenic series, were evaluated in 115 insulin dependent diabetics.

### Detection of Islet Cell Antibodies

ICAb were detected as previously described by indirect immunofluorescence on human pancreatic cryostat sections using antihuman

IgG conjugated with fluorescein isothiocyanate (13). CF-ICAb were detected by an indirect immunofluorescence test using fresh complement and anti C3 conjugated with fluorescein isothiocyanate (14).

### Detection of Immune Complexes

**Solid phase C1q radioimmunoassay (C1qSP).** C1q was purified according to Yonemasu and Stroud (15). The assay was carried out as described by Hay (16) with minor modifications. <sup>125</sup>I labelled Staphylococcal protein A was used to detect AgAb fixed to C1q coated tubes. Protein A was labelled according to Dorval *et al.* (17). One hundred and eighty-five blood donors were included in the study for the detection of the limit of positivity as described elsewhere (8, 18).

**Conglutinin binding test (KgBt).** Conglutinin was purified and the test performed according to Casali *et al.* (19). <sup>125</sup>I labelled protein A was used to reveal conglutinin bound complexes. One hundred and five blood donors were tested for the evaluation of the limit of positivity (8, 18).

### Raji Cell Radioimmunoassay (RAJI)

Raji cells were used to detect AgAb by a minor modification of the technique of Theophilopoulos *et al.* (20). Raji cell bound AgAb were detected by the addition of <sup>125</sup>I labelled Staphylococcal protein A. The limit of positivity was calculated to be 20 µg AHG (aggregated human immunoglobulin G) eq/ml on the basis of the results of 68 blood donors (8).

### Detection of Virus Antibodies

Inactivated sera (56°C for 30 min) were tested in a microtitre assay for complement fixing antibodies to chlamydia and to a number of viruses and viral antigens, namely coxsackie B1-6, influenza A and B, adenovirus, respiratory syncytial, measles, mumps S and V, herpes simplex and varicella zoster. Sera were screened at a dilution of 1:32 with 3 HD<sub>50</sub> guinea pig complement. Sera positive at 1:32 were titrated at further two-fold dilutions to 1:512. Antibodies to coxsackie viruses B1-6 were estimated in a quantal neutralisation test. 100 TCID<sub>50</sub> of each virus was reacted with serum dilutions and inoculated into virus cell cultures in tubes. End points were determined by low power microscopy of the viral cytopathic effect. All sera were negative for hepatitis B surface antigen by a reverse passive haemagglutination test.

### Detection of Insulin Antibodies

IBC was evaluated according to the method of Ortvad Andersen (21) as modified by Mustafa (22).

### Statistical Analysis

The Chi-squared (2 × 2) test ("X") with Yates's correction, Fisher's test ("F") and Cox's test for trend ("C") were used in the analysis of the results.

Table 1 Details of the 153 insulin dependent diabetics studied

| Study                   | ICAb status at diagnosis | No. of patients | Sex (% male) | Age at diagnosis (mean ± S.D.) years | Associated autoimmune disease* |
|-------------------------|--------------------------|-----------------|--------------|--------------------------------------|--------------------------------|
| Diagnosis and follow up | Positive                 | 44              | 63.6         | 25.6 ± 15.4                          | 0                              |
|                         | Negative                 | 44              | 75.0         | 24.8 ± 14.5                          | 2                              |
| Diagnosis alone         | Positive                 | 24              | 41.7         | 19.4 ± 8                             | 0                              |
|                         | Negative                 | 41              | 63.4         | 23.7 ± 12.0                          | 0                              |

\* Thyroid disease in all cases.

**Table 2** Islet cell antibody status of the 88 insulin dependent diabetics studied prospectively from diagnosis

| Time after Diagnosis | No. of patients | No. ICAb positive (%) |
|----------------------|-----------------|-----------------------|
| Diagnosis            | 88              | 44 (50%)              |
| 1 month              | 88              | 40 (45%)              |
| 3 months             | 79              | 30 (38%)              |
| 6 months             | 76              | 27 (36%)              |
| 9 months             | 68              | 21 (31%)              |
| 1 year               | 57              | 15 (26%)              |
| 2 years              | 31              | 6 (19%)               |
| 3 years              | 6               | 1 (17%)               |

**RESULTS**

**ICAb**

ICAb were found in 68 of the 153 insulin dependent diabetics studied at diagnosis (table 1). Forty-four (50%) of the insulin dependent diabetics studied prospectively were ICAb positive at diagnosis, the presence of ICAb declining to 45, 38, 36, 31, 26, 19 and 17% at 1, 3, 6, 9, 12, 24 and 36 months after diagnosis respectively (table 2).

**CF-ICAb**

CF-ICAb were found in 30% of the diabetics at diagnosis and in 23% at 6 months. All patients with CF-ICAb at diagnosis were ICAb positive whilst only 47% of patients with ICAb also had CF-ICAb in the serum.

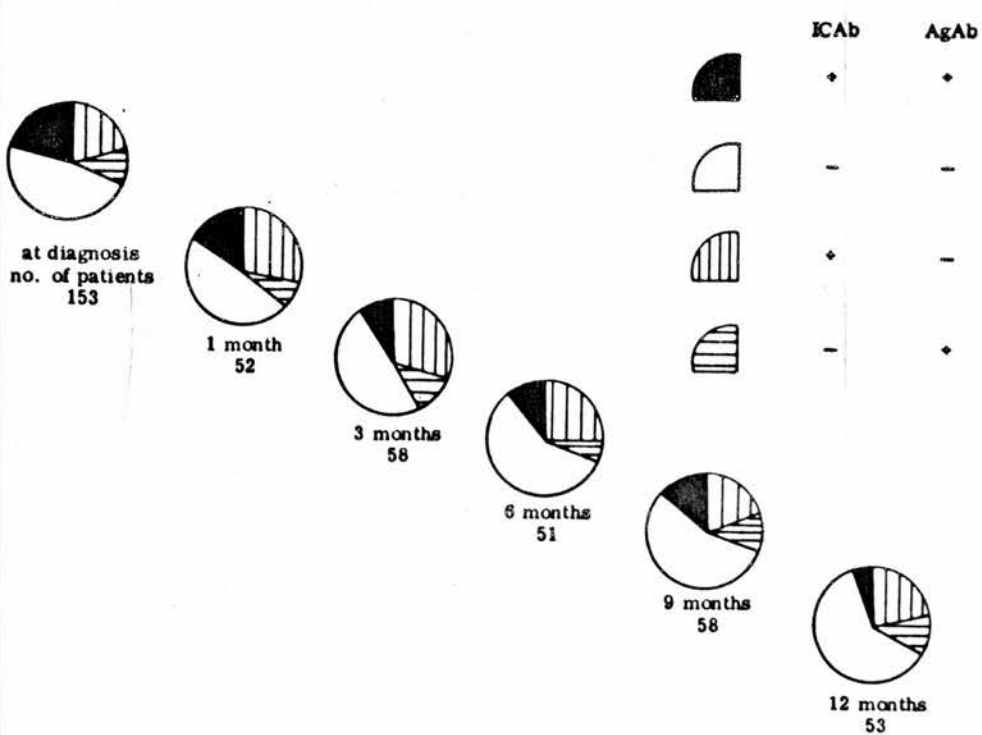
**AgAb**

**C1qSP.** AgAb (C1q) were found in 54 of the 153 insulin dependent diabetics studied at diagnosis ( $p < 0.001$  vs. normals, "X"). Twenty-nine of the 65 insulin-dependent diabetics studied at diagnosis alone and 25 (28%) of the 88 studied prospectively had AgAb (C1q) in their serum at diagnosis. In the prospective study the AgAb (C1q) positivity declined to 25% ( $p < 0.01$  vs. normals, "X"), 22% ( $p < 0.02$ , "X"), 17% (n.s., "X"), 19%, 17%, 17% and 0% after 1, 3, 6, 9, 12, 24 and 36 months from diagnosis respectively.

**KgBt.** AgAb (Kg) were found in 12 of the 34 diabetics studied at diagnosis ( $p < 0.001$  vs. normals, "X").

**RAJI.** AgAb (RAJI) were found in 27 of 50 diabetics studied at diagnosis ( $p < 0.002$  vs. normals, "X"). Eighty-four patients were studied at diagnosis by more than one AgAb method and of these 57% had at least one positive AgAb result.

**ICAb vs. AgAb.** A significant correlation between the presence of AgAb, as detected by C1qSP, and the occurrence of ICAb was found in IDDM at diagnosis ( $p < 0.001$ , "X") (fig. 1). Fifty-four percent of the ICAb positive insulin dependent subjects and 20% of those without ICAb showed AgAb in their serum at diagnosis. AgAb (RAJI) were also significantly increased in ICAb positive diabetics ( $p < 0.02$ , "F") but there was no significant correlation between AgAb (Kg) and ICAb.



**FIG. 1.** A comparison of the results of ICAb and AgAb (C1q) studies in insulin dependent diabetics at diagnosis and during the 12 months following diagnosis.

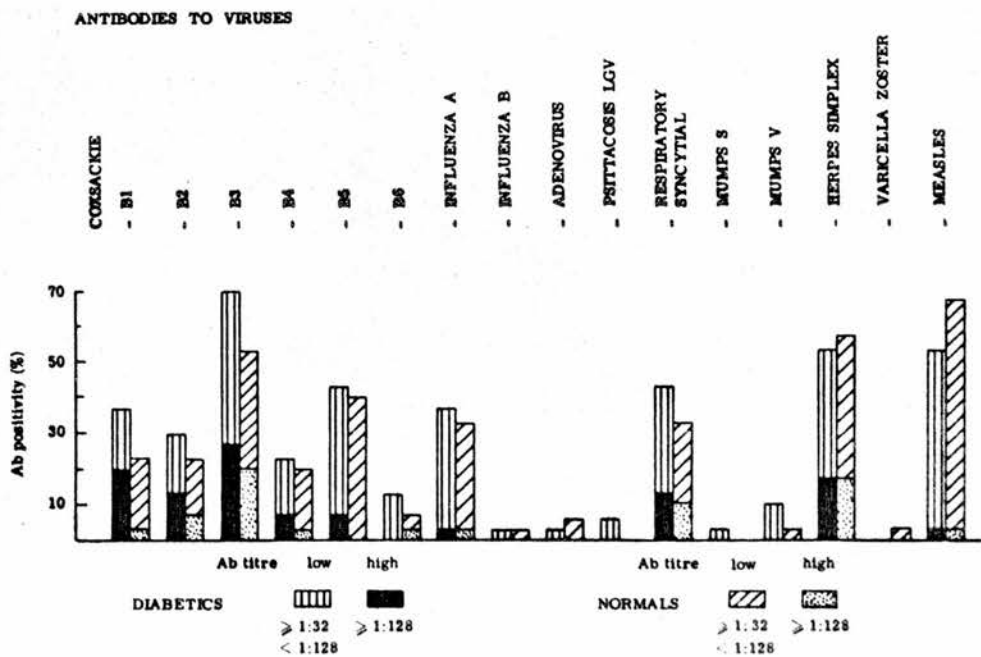


FIG. 2. A comparison of the serum viral antibody titres in 30 insulin dependent diabetics at diagnosis and in 30 matched controls.

*CF-ICAb vs. AgAb (CIq).* There was no significant correlation between CF-ICAb and AgAb (CIq) either at diagnosis or after 6 months ("X").

#### Viral Antibodies

There was no significant difference ("F") in the pre-

Table 3 Antibodies to Coxsackie B(1-6) correlated with AgAb (CIq) and ICAb in the serum of 30 newly diagnosed insulin dependent diabetics

| Coxsackie B(1-6) antibodies |                 | AgAb (CIq)       | ICAb             |
|-----------------------------|-----------------|------------------|------------------|
| Titres                      | No. of patients | No. of positives | No. of positives |
| < 32*                       | 6               | 0                | 5                |
| 32-64†                      | 14              | 4                | 3                |
| ≥ 128‡                      | 10              | 2                | 6                |

\* Coxsackie B 1-6 titres all < 32.

† One or more of the Coxsackie B 1-6 titres = 32-64.

‡ One or more of the Coxsackie B 1-6 titres ≥ 128.

sence or titre of VAb between the diabetics and the controls studied (fig. 2). No correlation was found between VAb presence and AgAb or ICAb positivity at diagnosis (table 3).

#### Insulin Antibodies

The mean ( $\pm$  S.D.) IBC value was 33.6 ( $\pm$  46.4)  $\mu$ U/ml. No correlation was found between the IBC levels at 9 months after diagnosis and the presence, both at diagnosis and after 9 months, of ICAb or AgAb (table 4).

#### HLA Antigens

Forty-six percent of the insulin dependent diabetics were HLA B8 positive and 15% were HLA B15 positive. No correlation was found between these two diabetogenic series and the presence of ICAb, AgAb or VAb at diagnosis. HLA B15 positive patients tend to form significantly higher IBC levels than B15 negative patients ( $p < 0.05$ , "C") (table 5).

Table 4 Insulin antibodies, measured 9 months after diagnosis, correlated with the presence of AgAb (CIq) and ICAb in the serum at diagnosis and at 9 months after diagnosis

| IBC                  |                 | AgAb(CIq)    |             | ICAb         |             |
|----------------------|-----------------|--------------|-------------|--------------|-------------|
| Values ( $\mu$ U/ml) | No. of patients | At diagnosis | At 9 months | At diagnosis | At 9 months |
| < 10                 | 17              | 7            | 3           | 7            | 6           |
| 10-50                | 13              | 7            | 4           | 8            | 6           |
| > 50                 | 5               | 3            | 1           | 2            | 1           |

Table 5 HLA B8 and B15 correlated with serum levels of ICAb, AgAb, VAb and IBC

|                                 |       | No. of patients | HLA B8 positive patients | HLA B15 positive patients |
|---------------------------------|-------|-----------------|--------------------------|---------------------------|
| <b>HLA vs. ICAb</b>             |       |                 |                          |                           |
| ICAb at diagnosis               | +     | 52              | 23 (44%)                 | 6 (12%)                   |
|                                 | -     | 63              | 31 (49%)                 | 11 (17%)                  |
| After 12 months                 | +     | 12              | 6 (50%)                  | 3 (25%)                   |
|                                 | -     | 36              | 21 (58%)                 | 6 (17%)                   |
| <b>HLA vs. AgAb</b>             |       |                 |                          |                           |
| AgAb (C1q) at diagnosis         | +     | 39              | 18 (46%)                 | 7 (18%)                   |
|                                 | -     | 76              | 35 (46%)                 | 10 (13%)                  |
| AgAb (C1q) after 12 months      | +     | 8               | 4 (50%)                  | 1 (12.5%)                 |
|                                 | -     | 40              | 22 (50%)                 | 7 (17.5%)                 |
| AgAb (Kg) at diagnosis          | +     | 7               | 3 (43%)                  | 0 (0)                     |
|                                 | -     | 18              | 8 (44%)                  | 2 (14%)                   |
| AgAb (RAJI) at diagnosis        | +     | 19              | 11 (58%)                 | 2 (10%)                   |
|                                 | -     | 14              | 8 (57%)                  | 2 (14%)                   |
| <b>HLA vs. Coxsackie Ab</b>     |       |                 |                          |                           |
| Coxsackie B titres at diagnosis |       |                 |                          |                           |
|                                 | < 32  | 4               | 2 (50%)                  | 0 (0)                     |
|                                 | 32-64 | 12              | 4 (33%)                  | 2 (17%)                   |
|                                 | ≥ 128 | 9               | 4 (44%)                  | 1 (11%)                   |
| <b>HLA vs. IBC</b>              |       |                 |                          |                           |
| IBC (μU/ml)                     |       |                 |                          |                           |
|                                 | < 10  | 16              | 9 (56%)                  | 2 (12%)                   |
|                                 | 10-50 | 10              | 7 (70%)                  | 3 (30%)                   |
|                                 | > 50  | 5               | 0 (0)                    | 3 (60%)                   |

## DISCUSSION

The increased presence of circulating immune complexes in Type 1 diabetics at diagnosis is confirmed by this large study. Immune complex positivity tends to decline rapidly following diagnosis and within only a few months their prevalence is near to that found in the normal population. The methods used to assay AgAb differ in principle and detect different types of complexes. However, with all techniques employed, the increased prevalence of complexed immunoglobulins is similar and complexes are detected that can lead to tissue damage by activating the complement system and by binding to cells with Fc or C<sub>3</sub> receptors on their surface. Thus, we have shown that a heterogeneous group of potentially harmful complexes is present at the time of diagnosis of Type 1 diabetes.

ICAb were found in approximately half of our newly diagnosed diabetics. This prevalence is similar to that which we have reported previously (13, 23) but lower than that reported by other workers (24, 25). The older age of our patients and our decision to define as negatives all doubtful results may account for this apparent discrepancy. There is a need to standardize the detection of ICAb since differences in the substrate and in the

methodology have sometimes resulted in poorly reproducible findings.

It has been reported that antibodies to certain viruses are increased in Type 1 diabetics at diagnosis (26). However, in epidemiological studies, where the parameter being investigated is also present in the normal population, there is often difficulty in selecting an appropriate control group. In our viral studies, we matched each diabetic with a normal subject of the same age and sex who resided in the same geographical area. The diabetic and the control subjects were sampled within the same 7-day period to exclude differences resulting from the seasonal variation of viral infections in the community. Although the antibody positivity to certain viruses appeared high in the diabetic patients there was no significant difference from the results obtained in the control group (fig. 2) and the findings simply reflect the viruses prevalent at the time of sampling.

This finding is not unexpected since it is likely that only in a very few cases does an overwhelming viral infection produce rapid beta cell necrosis. In other cases, a viral infection of the islets may trigger an autoimmune reaction against the islets which in turn produces beta cell damage and leads to the development of diabetes



months, or even years, after the initial infection. However, viral antibodies, which represent a positive reaction by the body against an aggressive agent, may not be the most appropriate phenomena to study since our aim is to determine why the host fails to cope adequately with a viral attack and thereby sustains beta cell damage.

Insulin antibody levels generally increase slowly from the time insulin therapy is commenced and assume a steady level after several months. IBC levels were normal or only modestly elevated in our patients despite their estimation 9 months after diagnosis. This finding can be explained by the use of purified insulins alone in the patients studied. Furthermore, a correlation between AgAb (C1q) and IBC was not expected since AgAb presence tends to decline after diagnosis in contrast to IBC levels. AgAb seem to reflect other phenomena occurring at diagnosis more than the presence of circulating antibodies to insulin and viruses.

This study has demonstrated a tendency for HLA B15 positive patients to form significantly higher IBC levels than B15 negative patients. This genetic predisposition for some patients to produce higher amounts of insulin antibodies is in accordance with previous work (27-29). It is well recognised, at least in animals, that the major histocompatibility genes are very close to genes that regulate the immune response and, in particular, the T-cell mediated response. In our patients, this genetic influence was apparent only in the response towards therapeutic insulin since there was no correlation between AgAb, ICab and VAb measured at diagnosis and HLA types. The lack of correlation between ICab at diagnosis and HLA types confirms our previous findings (30).

Immune complexes (C1q, RAJI) were correlated with the occurrence of ICab at diagnosis but not with the other antibodies studied. Thus, it would appear that AgAb and ICab are related and that the presence of AgAb at diagnosis does not simply reflect a recent viral infection. This is supported by previous reports of a strong correlation between ICab and immune complexes in insulin-dependent diabetics with persistent ICab (31) and in newly diagnosed non-insulin dependent diabetics (32). In the present context, the CF-ICab studies did not provide any additional information regarding the immunological mechanisms which are operative at, and soon after, the onset of IDDM.

It is possible that AgAb and ICab have separate roles in the early stages of Type 1 diabetes or that they may be involved together, either primarily or secondarily. Complexes may reflect damage to the islets and be present near the clinical onset of the disease together with other immune phenomena such as ICab. Medium or large size antigen-antibody complexes, that fix complement and react with cells having surface receptors for Fc or C<sub>3</sub>, may produce cell damage either directly or by interacting with specific T-cell subpopulations. AgAb, if present in antibody excess, may arm K cells against a specific antigen(s)

in or on the pancreatic islets and thereby result in the development of diabetes mellitus. The correlation between the presence of AgAb and ICab in the serum, at the time IDDM is diagnosed, suggests that some of the AgAb may be comprised of I.C. Antibody/I.C. Antigen.

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## Aspects of Humoral Immunity in a Prospective Study of Type I (Insulin-Dependent) Diabetic Subjects Treated with Insulins of Different Purity

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**Summary.** In 41 Type I (insulin-dependent) diabetic patients, islet cell antibodies, anti-insulin antibodies, and immune complexes measured by two different methods (the C1q solid phase assay and the conglutinin binding test) were studied at diagnosis, and the influence of treatment with insulins of different purity was investigated during the first year of treatment. Twenty subjects were treated with conventional insulins (group 1) while 21 were treated with monocomponent porcine insulins (group 2). The prevalence of islet cell antibodies significantly decreased during the 12-month study period in the 41 patients. From the first month anti-insulin antibodies were always significantly higher in group 1 than in group 2. At diagnosis the prevalence of both types of immune complexes in the 41 patients was higher than in normal subjects. The im-

mune complexes measured by the C1q solid phase method showed a significant and progressive reduction during the follow-up period, whereas the immune complexes assayed by conglutinin showed no significant variation in the same period. The presence of C1q immune complexes was found to correlate with the occurrence of islet cell antibodies both at diagnosis and during the follow-up period. The presence of conglutinin immune complexes, on the other hand, tended to parallel the increase of anti-insulin antibody levels.

**Key words:** Type 1 diabetes, anti-insulin antibodies, islet cell antibodies, immune complexes, C1q solid phase assay, conglutinin binding test, monocomponent insulins.

Islet cell antibodies, anti-insulin antibodies and immune complexes have been demonstrated in the sera of diabetic subjects. Islet cell antibodies have been found in Type I (insulin-dependent) diabetic patients at diagnosis and in non-diabetic patients with other autoimmune diseases. The role of these antibodies in the pathogenesis of Type 1 diabetes remains to be elucidated [1-3]. Anti-insulin antibodies are present in most insulin-treated diabetic patients. It has been established that anti-insulin antibodies may play a role in the transient complications of insulin treatment (i.e. allergic reactions, insulin resistance, etc.), whereas no influence on late diabetic complications has yet been demonstrated [4-9].

It has been suggested that the presence of islet cell and anti-insulin antibodies in the circulation may reflect the presence of circulating immune complexes [10, 11]. Circulating immune complexes have been described in a large percentage of Type I diabetic patients at the time of diagnosis [12] and in some cases with severe complications [13]. Detection methods based upon different principles have also revealed the presence of heterogeneous populations of immune complexes [14,

15]. A correlation was recognized in some of these studies between immune complexes detected by the solid phase C1q binding test and the presence of islet cell antibodies [16], whilst in others a correlation appeared to exist between immune complexes detected by conglutinin binding assay and insulin treatment [17].

The present investigation was carried out in a group of patients at the time of clinical diagnosis of Type I diabetes. These patients were treated with either conventional insulin or monocomponent insulin and were observed at regular intervals for one year.

The aim of the present study was to attempt to correlate circulating immune complexes (detected by C1q and conglutinin) and the presence of islet cell and anti-insulin antibodies, bearing in mind the type and duration of the diabetes and antigenicity of the insulin used in the treatment.

### Patients and Methods

Forty-one newly diagnosed Type I diabetic patients, with age at onset < 30 years, were included in the study before insulin treatment was started. Twenty of these patients (12 males and eight females, mean

**Table 1.** Anti-insulin antibodies in Type 1 diabetic patients at diagnosis and during 1 year's treatment

|         | Duration of treatment (months) |                        |                        |                        |
|---------|--------------------------------|------------------------|------------------------|------------------------|
|         | 1                              | 3                      | 6                      | 12                     |
| Group 1 | 0.8 ± 0.8 <sup>a</sup>         | 1.7 ± 1.5 <sup>b</sup> | 1.8 ± 1.9 <sup>b</sup> | 1.9 ± 1.3 <sup>b</sup> |
| Group 2 | 0.08 ± 0.11                    | 0.3 ± 0.4              | 0.3 ± 0.5              | 0.2 ± 0.3              |

Results are presented as mean ± SD; <sup>a</sup>  $p < 0.02$ ; <sup>b</sup>  $p < 0.001$

**Table 2.** Immune complexes assayed with C1q solid phase method and with conglutinin binding test in Type 1 diabetic patients at diagnosis and during 1 year's treatment

|                                 | Duration of treatment (months) |       |            |       |       |
|---------------------------------|--------------------------------|-------|------------|-------|-------|
|                                 | 0                              | 1     | 3          | 6     | 12    |
| <i>C1q solid phase method</i>   |                                |       |            |       |       |
| Group 1                         | 9/20                           | 5/17  | 4/16       | 2/18  | 3/20  |
| Group 2                         | 10/21                          | 6/19  | 3/15       | 3/19  | 2/20  |
| Total                           | 19/41                          | 11/36 | 7/31       | 5/37  | 5/40  |
|                                 |                                |       | $p < 0.01$ |       |       |
| <i>Conglutinin binding test</i> |                                |       |            |       |       |
| Group 1                         | 6/20                           | 6/17  | 6/17       | 7/19  | 8/20  |
| Group 2                         | 6/21                           | 4/18  | 5/18       | 7/18  | 4/20  |
| Total                           | 12/41                          | 10/35 | 11/35      | 14/37 | 12/40 |

Results are presented as positive/total subjects

age 9 years, range 5–11 years) were treated with conventional insulins (group 1), whereas 21 (15 males and six females, mean age 13 years, range 8–22 years) were treated with monocomponent porcine insulins (group 2). Patients were randomly assigned to each group. Blood samples were collected at diagnosis and 1, 3, 6 and 12 months later in all patients.

A series of 189 blood donors was also studied to determine the normal range of the immune complex assays. Islet cell antibodies were assayed by indirect immunofluorescence on cryostat sections of blood group 0 human pancreas. The presence of islet cell antibodies was evaluated by two independent observers using a Leitz Dialux microscope.

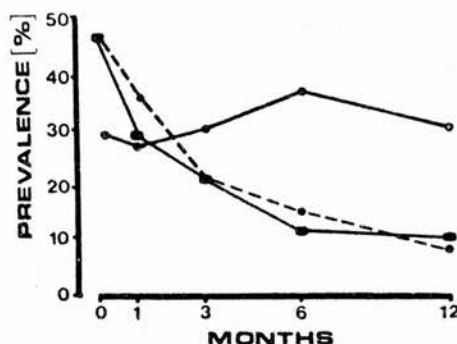
Anti-insulin antibodies, evaluated as insulin binding capacity, were measured by an immunoelectrophoretic method [18]; the sensitivity of the method is 0.05 mU/ml, the interassay variation 3%.

Immune complexes were determined by the C1q solid phase method according to Hay et al. [19] and by the conglutinin binding test according to Casali et al. [20]. The characteristics of the methods are described elsewhere [21].

The  $\chi^2$  test, Fisher's exact test, Cox's test, Student's t-test for independent variables and the binomial test were used for statistical evaluation of the results.

## Results

Islet cell antibodies were detected in 19 of the 41 diabetic patients at diagnosis (46%). During the follow-up period their prevalence decreased progressively to 10% after 12 months. This decrease was statistically significant ( $p < 0.001$ ). No difference in occurrence of islet cell antibody was found between the two groups of patients. Anti-insulin antibodies were detectable after the first month of treatment, being significantly higher in

**Fig. 1.** Prevalence of immune complexes assayed with C1q solid phase method (■—■) and with conglutinin binding test (●—●) and of islet cell antibodies (●—●) in 41 Type 1 diabetic patients at diagnosis and during 1 year follow-up

group 1 than in group 2 at each stage of follow-up (Table 1).

C1q immune complexes were present in 19 of the 41 diabetic patients at diagnosis (46%) and progressively decreased thereafter (Table 2). Prevalence at 12 months' follow-up was 12%. This decrease was statistically significant ( $p < 0.01$ ). No difference was found between groups 1 and 2. Conglutinin immune complexes were present in 12 of the 41 diabetic patients at diagnosis (29%), prevalence varying during follow-up from a minimum of 26% to a maximum of 38% (NS). Although at 12 months the prevalence was 40% in group 1 and 20% in group 2, the differences between the two groups were not significant.

Islet cell antibodies and C1q immune complexes showed similar decreases in frequency from diagnosis to 12 months in all patients together and in the two groups examined separately. C1q immune complexes were detected in 58% of the 19 islet-cell-antibody-positive subjects at diagnosis. The agreement between the two tests (both positive and negative) was significant (1 month  $p < 0.05$ , 6 months  $p < 0.001$ , 12 months  $p < 0.001$ ).

Islet cell antibodies and conglutinin immune complexes showed different profiles during follow-up (Fig. 1). Analysis of the relationship between these two immunological factors demonstrated a difference in prevalence at diagnosis (46% versus 29%), and only four out of the 19 islet-cell-antibody-positive subjects also showed conglutinin immune complexes. No significant agreement was found between the two tests.

No significant correlation was found between C1q immune complexes and anti-insulin antibodies, either in the 41 diabetic patients or in groups 1 and 2 considered separately. The presence of conglutinin immune complexes, on the other hand, showed a tendency to parallel the increase in anti-insulin antibody levels in the 41 diabetic patients ( $p < 0.001$ ). This trend was still present when the two groups were examined separately ( $p < 0.001$ ). However, whereas in group 1 maximum

prevalence of conglutinin immune complexes (85%) was reached when anti-insulin antibody levels were above 2 mU/ml ( $p < 0.002$ ), the maximum (75%) in group 2 was reached at lower anti-insulin antibody levels (0.5–2 mU/ml) ( $p < 0.002$ ).

## Discussion

These results demonstrate a correlation between C1q immune complexes and islet cell antibodies, and between conglutinin immune complexes and anti-insulin antibodies, whereas no correlation was found between C1q immune complexes and anti-insulin antibodies or between conglutinin immune complexes and islet cell antibodies. Furthermore, C1q and conglutinin probably detect different populations of immune complexes.

It is possible that islet cell antibodies and C1q immune complexes are present at the time of diagnosis independently of each other and are different expressions of the immune response to the same aetiological agent; on the other hand, the correlation between C1q immune complexes and islet cell antibodies observed in the present study suggests that the islet cell antibodies might circulate in the form of soluble complexes bound to antigens of islet origin. In this case immune complexes detected by C1q assay at diagnosis may be a reflection of such a phenomenon.

Of interest in the present study is the relationship observed between conglutinin-immune complexes and insulin binding capacity. We have demonstrated previously a relationship between this type of immune complex and insulin treatment [17]; these complexes were significantly more frequent in patients treated with insulin than in others [21]. The present data, showing the existence of a significant trend in the prevalence of circulating conglutinin-immune complexes with increasing levels of insulin binding capacity, appear to suggest that the conglutinin method detects insulin-anti-insulin complexes present in diabetic patients following treatment. On the other hand, assay of immune complexes using conglutinin could be affected by the anti-insulin antibodies and by the aggregates of these antibodies.

It is more difficult to offer an explanation for the observation that the relationship between conglutinin-immune complexes and the levels of insulin binding capacity in patients treated with conventional insulins (group 1) differs from that in patients treated with monocomponent insulins (group 2). It would, in fact, appear that while the former tend to produce immune complexes at high levels of insulin binding capacity, the latter form these complexes at low or medium levels of insulin binding capacity.

This finding may be due to the difference in immunogenicity of the two types of insulin. Even though because of their purity, monocomponent insulins produce low overall levels of insulin-binding capacity, the antibodies induced have a relatively high affinity

for insulin. In contrast, conventional insulins, which contain extractive polypeptides with a greater molecular weight and immunogenic capacity than pure insulin, tend to produce larger families of antibodies only some of which display marked affinity for insulin, even though all may be detectable as insulin binding capacity by Christiansen's method [4, 8].

It is thus possible that for the same insulin-binding capacity level, patients treated with monocomponent insulin produce a larger quantity of specific complexes than those using conventional insulins.

In conclusion, the present findings confirm the presence of circulating immune complexes in patients with Type 1 diabetes mellitus, some of which may be related to factors present at diagnosis, others to anti-insulin antibodies.

On the other hand conglutinin-immune complexes might provide a useful method with which to follow the pathological events associated with circulating anti-insulin antibodies.

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## Immune Abnormalities in Diabetic Patients Not Requiring Insulin at Diagnosis

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**Summary.** Islet cell antibodies (ICA), complement fixing islet cell antibodies, immune complexes and thyro-gastric autoantibodies were studied in newly diagnosed diabetic patients not requiring insulin at diagnosis. Particular attention was focussed on that minority of patients who are initially treated with diet or oral agents but show ICA in their serum. One hundred and six non-insulin-requiring patients were studied at clinical diagnosis. Seventeen who had ICA in their serum were compared with a control group of 89 who did not. The 17 ICA-positive diabetic patients were followed serologically for approximately 1 year from diagnosis. Patients were followed clinically for 3 years. Forty-seven percent of ICA-positive and 19% of ICA-negative patients had immune complexes in their serum. Eleven of the 17 ICA-positive patients also had serum complement fixing islet cell antibodies. Thyro-gastric anti-

bodies were found in 29% of ICA-positive and 18% of ICA-negative diabetic patients. ICA, complement fixing antibody and immune complex positivity declined with time. Ten of the 17 ICA-positive and two of the 89 ICA-negative patients required insulin within 3 years of diagnosis. There was a positive trend for the presence of complement fixing islet cell antibodies at diagnosis to be associated with the early development of insulin dependency. The type of diabetes in ICA-positive patients not requiring insulin at diagnosis has strong immunological and clinical similarities to classical Type 1 (insulin-dependent) diabetes.

**Key words:** Type 1 diabetes, Type 2 diabetes, islet cell antibodies, complement fixing islet cell antibodies, immune complexes, thyro-gastric autoantibodies.

Circulating immune complexes (AgAb) have been reported to be present in higher levels in newly diagnosed Type 1 (insulin-dependent) diabetic patients and to be correlated with islet cell antibodies (ICA) at and shortly after diagnosis [1-3]. These circulating complexes may have a pathogenic role or simply reflect the presence of ketosis and metabolic derangement in the early phases of clinical diabetes, which could modify the dynamics of formation and clearance of AgAb [4].

In this study, we investigated the presence of soluble AgAb in diabetic patients who did not require insulin at the time of clinical diagnosis. Particular attention was focussed on the minority who did not have ketonuria but showed ICA in the serum at diagnosis. In this relatively uncommon group of patients, ICA, complement fixing islet cell antibodies (CF-ICA) and AgAb were tested at diagnosis and at regular intervals thereafter. Clinical and subclinical associations with other autoimmune phenomena were studied also.

Our aims were to highlight the presence of AgAb in patients who were ICA-positive but not ketotic, the modifications with time of ICA, CF-ICA and AgAb and the correlation between the presence of ICA, CF-

ICA or AgAb at diagnosis and in the following months with the tendency to progress to insulin dependency.

### Subjects and Methods

#### Subjects

Over a period of a year most patients newly diagnosed at a few Edinburgh Diabetic Clinics as Type 2 (non-insulin dependent) diabetic subjects, according to the National Diabetes Data Group Classification [5], were tested for ICA at their first attendance. Two hundred and sixty patients were tested of whom 106 were included in this study: all those who were ICA-positive (17), together with 89 who were selected randomly from the newly diagnosed ICA-negative diabetic patients.

In the ICA-positive group, there were five males and 12 females with a mean age at diagnosis of  $55.4 \pm 16.1$  years. In the ICA-negative group, 42 were male and 47 were female and the mean age at diagnosis was  $61.6 \pm 12$  years. Two of the 17 ICA-positive and two of the 89 ICA-negative patients had other associated autoimmune diseases (Hashimoto's thyroiditis, pernicious anaemia and primary hypothyroidism). Almost all the 17 ICA-positive patients could be considered to have Type 2a diabetes [5], the median of the ideal body weight being 98.5%. No other substantial difference in the symptoms preceding diagnosis or in the clinical and metabolic signs at the onset of overt diabetes was noticed between ICA-positive and ICA-negative patients.

Since there are no reports of ICA-negative diabetic patients later becoming positive, we concentrated our study on the 17 initially ICA-positive patients; they were followed serologically at regular intervals for approximately 1 year from diagnosis and serum antibodies were tested twice, once between 2 and 4 months and again 10 or more months after diagnosis.

This research was part of a more general study, so that the clinicians did not know the result of the ICA test, at least in the first months, and in any case ICA status was not among the parameters taken into account when the decision to put patients on insulin was taken.

## Methods

AgAb were evaluated by the solid phase Clq radioimmunoassay according to Hay et al. [6], with minor modifications. Details of the technique used, the limit of positivity, the expression of results and the reproducibility of the test have been published previously [1, 7]. ICA, thyroid cytoplasmic and gastric parietal cell antibodies were determined by indirect immunofluorescence using fresh frozen cryostat sections [8, 9]. CF-ICA were evaluated with the addition of fresh normal serum as the source of complement and fluorescein conjugated anti-C3 antibody [10].

Statistical evaluation was done by  $\chi^2$  test, with Yates' correction when appropriate, and by the two tailed Cox's test for trends in proportions when indicated.

## Results

### Islet Cell Antibodies

In the ICA-positive patients, ICA were still present in 14 out of 17 between 2 and 4 months after diagnosis and in 10 out of 16 after 1 year or more of clinical diabetes (Fig. 1).

### Immune Complexes

AgAb were found in eight of the 17 ICA-positive diabetic patients ( $p < 0.01$  versus normal subjects) and in 16 of the 89 ICA-negative patients (NS versus normal subjects,  $p < 0.02$  versus ICA-positive patients), both groups being studied at diagnosis.

### Complement Fixing Islet Cell Antibodies

CF-ICA were found in 11 of the 17 ICA-positive diabetic patients. At the end of the first 3 months, ten patients were positive; five were still positive 10 months or more from diagnosis (Fig. 1). None of the patients who were CF-ICA-negative at diagnosis became CF-ICA-positive with time.

### Thyro-gastric Antibodies

Autoantibodies to thyroid or to gastric parietal cells, studied in all the patients at diagnosis, were found in five of the 17 ICA-positive and in 16 of the 89 ICA-negative diabetic patients (NS).

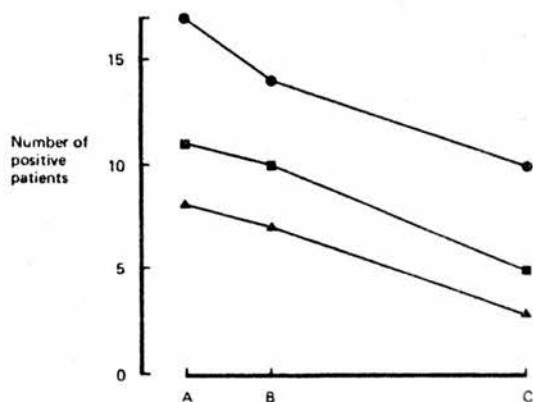


Fig. 1. ICA (●—●), CF-ICA (■—■) and AgAb (▲—▲) in the 17 ICA-positive diabetic patients initially treated without insulin, at diagnosis (A), between the second and the fourth month of the disease (B), and after 10 months or more of clinical diabetes (C)

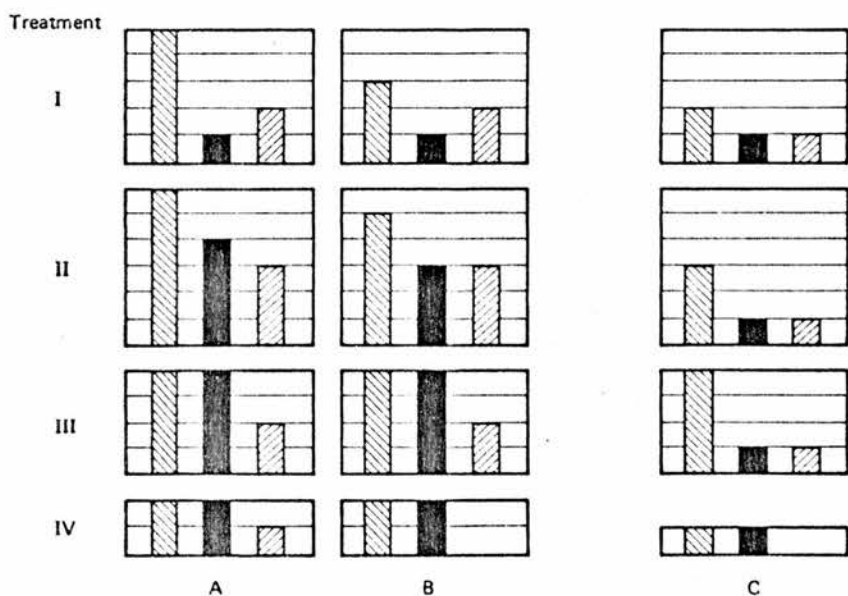
### ICA, CF-ICA, AgAb and Clinical Tendency to Progress to Insulin Dependency

Ten of the 17 ICA-positive and two of the 89 ICA-negative diabetic patients required insulin within 3 years of diagnosis ( $p < 0.0001$ ), either because of the presence of ketonuria and/or because of a metabolic derangement (glycaemia constantly  $> 12$  mmol/l) non-controllable with other therapy. When the patients were divided according to tendency to progress to insulin dependency (Fig. 2), five were still on oral hypoglycaemic agents more than 3 years from diagnosis, six went on to insulin after 20 months of clinical diabetes, four between 6 and 10 months and two died from vascular complications after 5 and 21 months, respectively. There was a significant trend for the presence of CF-ICA at diagnosis to be associated with earlier development of insulin dependency ( $p < 0.05$ ). The occurrence of AgAb and of thyro-gastric antibodies were not correlated with the tendency to progress to insulin dependency.

## Discussion

While an increased prevalence of immune complexes has been shown in the serum at diagnosis of Type 1 diabetes [1-3], this is not the case here where a substantial number of randomly selected Type 2 diabetic patients have been studied at diagnosis and compared with control subjects. However, the present study demonstrates that the prevalence of AgAb is increased in the minority of diabetic patients who have ICA in the serum but do not require insulin at diagnosis or during the first months of the disease. A correlation between serum AgAb and ICA has been demonstrated also in Type 1 diabetic patients close to diagnosis [1-3] and in those Type 1 diabetic patients in whom ICA persists for some years [11]. The correlation between serum ICA and AgAb suggests that islet cell antigen-antibody complexes may be part of the AgAb detected in the subjects.





**Fig. 2.** ICA (▨), CF-ICA (■), AgAb (□) and tendency to progress to insulin dependency. The horizontal lines within the rectangular frames indicate the number of patients. Group 1 patients ( $n=5$ ) were still on oral hypoglycaemic agents 3 years from clinical diagnosis. Group 2 ( $n=6$ ) required insulin between 20 and 36 months from diagnosis, Group 3 ( $n=4$ ) needed insulin within 10 months and the two Group 4 patients died during the follow-up study. A, B and C as in Figure 1

Whether AgAb are involved in islet cell damage in these patients has not yet been established, but they could be involved in serum cytotoxic activity or they could interact in several ways with cells which have receptors for the Fc portion of immunoglobulins or for the third component of complement [12]. As our ICA-positive diabetic patients not requiring insulin at diagnosis were not ketotic, severe metabolic derangement would not seem to be a major factor in the development of serum AgAb in these subjects. Furthermore, hyperglycaemia is unlikely to be responsible as an increased presence of AgAb was seen in ICA-positive non-insulin diabetic patients but not in the majority of patients with Type 2 diabetes.

The present study demonstrates that a small proportion of apparent Type 2 diabetic patients show the same immunopathological phenomena that occur in classical Type 1 diabetes at diagnosis; i.e. ICA, CF-ICA and AgAb. It has been demonstrated previously that the presence of serum ICA in diabetic patients initially treated with diet and/or oral hypoglycaemic agents is a useful marker for predicting subsequent insulin dependency [13]. The findings of this study suggest that when serum CF-ICA are present, the development of insulin dependency tends to occur earlier. A similar predictive value for CF-ICA was recently shown by other workers in different situations, i.e. Type 1 diabetes, relatives of patients with Type 1 diabetes or patients with autoimmune disorders [10, 14, 15].

ICA-positive diabetic patients, not requiring insulin at diagnosis, who may also have CF-ICA and AgAb at diagnosis, clearly do not fit logically into the broad clinical subdivision of the majority of idiopathic diabetic patients according to the presence or absence of insulin dependency [14]. At clinical diagnosis, Type 1 diabetic patients are ketosis prone and have islet cell autoimmunity. Type 2 diabetic patients classically have neither of

these characteristics. The small group of subjects included in the present study showed no ketonuria and did not require insulin at diagnosis but had evidence of islet cell autoimmunity and tended to progress to insulin dependency. As argued previously [16], the type of diabetes in ICA-positive non-insulin requiring diabetic patients should be regarded as a subgroup of Type 1 diabetes, probably representing a stage earlier in the same disease process (or group of disease processes) that tends to culminate in classical Type 1 diabetes.

*Acknowledgements.* The authors wish to thank Mr. C. Tiberti for his skilful technical work and Miss F. Craik and Miss P. Henley for their excellent secretarial assistance.

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## METHODS

# A METHOD TO FACILITATE THE READING OF UP TO 60 SAMPLES OF MONOCLONAL ANTIBODY-FLUORESCENCE STAINED CELLS USING MEDICELL HLA-DR PLATES

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(Received 16 November 1983)

### INTRODUCTION

An important development in the characterization of cell surface functional antigens has been the development of monoclonal antibodies. To date more and more specificities are described in the scientific literature and no doubt even more will be found in the future. As an example, for accurate characterization of T and B lymphocyte subclasses, K/NK cells and activated T cells, at least 10 monoclonal antibodies per blood sample are required. If a fluorescence activated cell sorter (FACS) is available, the number of samples poses no problem. At present however not many researchers can use this facility routinely and therefore they find themselves in the unenviable position of spending a great deal of time preparing just a single slide for each monoclonal antibody.

In this report we describe an adaptation of existing equipment to facilitate the load of work.

### MATERIALS AND METHODS

The equipment consists of Medicell glass-bottomed plates (see fig. 1) in conjunction with an inverted fluorescence microscope. The plates, which are readily available (MEDICELL INTERNATIONAL LTD, 239 Liverpool Road, London N1 1LX, UK) since they are routinely used for HLA-DR tissue typing, are made of a thin sheet of glass, subdivided into 60 flat circular areas by raised rings, attached to a plastic frame with an inner ridge. The plates can be covered with a transparent plastic cover which fits over the inner ridge of the frame thus effectively reducing the rate of evaporation of the cell samples. The latter are placed within the circular areas where diffusion is prevented by the raised rings. The microscope stage can be easily adapted to take the plates; alternatively the plates can be moved along by hand.

The method described is for blood mononuclear cells although it can be applied to any other cell preparation.

1. Isolate mononuclear cells by density gradient centrifugation on Ficoll-Hypaque.

2. Wash cells three times in Phosphate Buffer Saline (PBS), pH 7.3, and suspend them at a concentration of  $2.0 \times 10^6$  cell/ml in RPMI 1640 medium containing 10% heat inactivated foetal calf serum (complete medium). 1.0 ml of this suspension is enough for a panel of up to 10 monoclonal antibodies.
3. Divide the cell suspension in 0.1-0.2 ml aliquots into small plastic centrifuge tubes (10 x 60 mm).
4. Spin tubes at 1,700 g for 1.5 min, preferably in a centrifuge equipped with a fixed-angle rotor. Remove supernatant by tipping tubes then

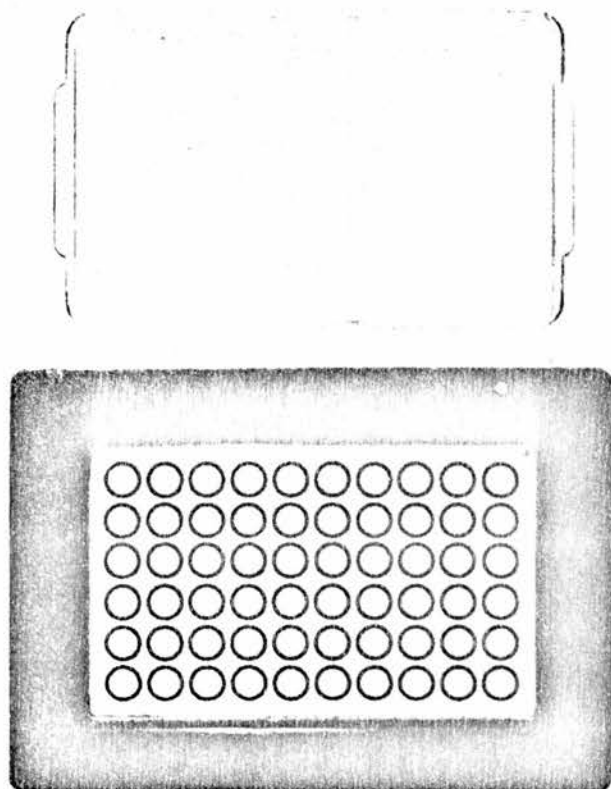


FIG. 1

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- add 50  $\mu$ l of the appropriate monoclonal antibody. Quickly vortex tubes to suspend cells and incubate for 30 min in an ice-bath.
5. Wash cells three times with PBS at 1,700 g for 1.5 min then add 50  $\mu$ l of the appropriate dilution of fluorescein conjugate antimouse IgG antibody. Vortex tubes and incubate for 30 min in an ice-bath.
  6. Wash cells three times in PBS and finally suspend them in 50  $\mu$ l of complete medium.
  7. Place 5  $\mu$ l of each cell sample in a circular area of the Medicell plate and leave for 3–5 min to allow cells to sink to the bottom.
  8. Count the proportion of rim fluorescence positive cells in the inverted fluorescence microscope.

#### COMMENTS

The problem of the length of time required to complete the characterization of mononuclear cells from a single subject was very acute in our laboratory where a family

follow-up study involved 3–5 subjects at a time with 8–10 monoclonal antibodies. The method described has now been used for over one year and it has been found extremely useful. Many of the problems connected with eye adaptation to dark room conditions are avoided since the microscope reader does not need to leave the machine to prepare single slides.

In addition to increasing the number of cell samples that can be read at any given time, there is the added bonus that the number of cells required for each monoclonal antibody can be greatly reduced (0.2–0.5  $\times 10^5$  cells). This can be of considerable importance when phenotyping mononuclear cells from small blood samples, in particular those from small children and difficult patients.



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## METHODS

# EVALUATION OF CLASS II ANTIGEN POSITIVE T CELLS: A SIMPLE DOUBLE STAINING METHOD USING MONOCLONAL ANTIBODIES

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### INTRODUCTION

ATTENTION of investigators has been recently focused on that subset of lymphocytes which expresses the class II (or DR) antigen on their surface. This class II antigen is a marker of T cell activation and is normally absent on non-activated or otherwise committed T cells. On the other hand, this antigen is normally present on the surface of B cells. The fact that this surface antigen is present in both B cells and in a few T cells creates some technical difficulties.

Since the percentage of activated T cells is usually very low, a method to easily and clearly identify this T cell subset is needed. Most investigators identify and isolate T cells on the basis of their ability to form spontaneous rosettes when incubated with sheep red blood cells (1-3). This widely used technique is time consuming and not accurate enough for the evaluation of activated T cells: some T cells are lost—and a particular subpopulation of T cells may be preferentially removed—and on the other hand some B cells still remain in the rosetting-enriched T population making it difficult to discriminate the activated T cells from the contaminating B cells. Other investigators use a double immunofluorescence staining—using both fluoresceine and rhodamine—to label respectively the antibody defining the DR antigen and that defining another antigen either of T or B cells. This technique is rather tedious and elaborate; the immunofluorescence pattern is usually faint, the different cells need to be read with different fluorescence microscope filters and the results obtained are usually poorer than expected.

We have devised a straightforward double staining technique to overcome these difficulties using commercially available monoclonal antibodies.

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### METHOD

#### *Immunological or Other Specific Reagents Needed*

Anti-class II antigen monoclonal antibody (this antibody should not be cytotoxic), anti-total T cell monoclonal antibody (for instance Leu 1a, BECTON DICKINSON or OKT3, ORTHO DIAGN.), ethidium bromide, fresh rabbit complement, rabbit anti-mouse antibody conjugated with fluorescein. All the monoclonal antibodies are diluted in medium (RPMI 1640) plus 10% foetal calf serum.

#### *Procedure*

Purify lymphoid cells by density gradient centrifugation; check viability of cells and count them;

- add anti-class II antigen monoclonal antibody (0.05 ml appropriate dilution to  $5 \times 10^5$  cells); incubate 30 min at 4°C and wash twice in medium;
- add rabbit anti-mouse antibody conjugated with fluorescein; incubate 30 min at 4°C and wash twice in medium;
- add a cytotoxic anti-T cell monoclonal antibody (0.05 ml appropriate dilution) and fresh complement (200 µl rabbit or guinea pig complement after checking for spontaneous cytotoxicity); incubate 30 min at 37°C;
- add ethidium bromide at a concentration of 50 µg/ml and read with a fluorescence microscope, fitted with a filter for fluorescein.

The B cells will appear green on the surface, the class II negative T cells will appear red in the nuclei whereas the class II positive T cells will show a red nucleus and a green surface.

### COMMENTS

With the technique described, the difficulties found with the other methodologies seem to be overcome. Under the fluorescence microscope it is possible to see clearly the bright red of the nuclei of the T cells, the green continuous linear fluorescence of the surface of the B cells and a few cells with a red nucleus and a fluorescent green surface, i.e. the class II antigen positive T cells. Both colours are read with the same filter, i.e. that for fluorescein. The technique is fast: 3 or 4 hr are needed to complete it. No significant amount of cells are lost with this procedure

which implies only washings but not separation of cells. Furthermore the total number of B and T lymphocytes is easily obtained.

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# Semisynthetic Human Insulin: Biologic and Immunologic Activity in Newly Treated Diabetic Subjects During a Six-month Follow-up

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Biologic and immunogenic activities of semisynthetic human monocomponent insulins were examined in insulin-dependent diabetic patients (group 1). Patients treated with porcine monocomponent (group 2) and conventional (group 3) insulins were studied for control purposes. The patients were examined before the beginning of insulin treatment and for a 6-mo follow-up period. The data collected during the study show that insulin antibody levels were significantly lower in group 1 than in groups 2 and 3. Furthermore, the prevalence of immune complexes assays with the C1q solid phase technique failed to reveal any differences between the three groups. When the conglutinin binding test was used, the prevalence of immune complexes showed a slight but not significant reduction in group 1 and a significant increase in group 3. The metabolic control was similar in the three groups during follow-up and the insulin requirement was lower, but not significantly, in group 1 than in groups 2 and 3. These data suggest that with human monocomponent insulins equivalent glycemic control may be achieved at similar doses than those required with porcine monocomponent insulins. Furthermore, human insulin is the least immunogenic of the present available insulins. *DIABETES CARE* 7: 128-131, MARCH-APRIL 1984.

The main problems arising in insulin treatment today are the mode of administration and the type of insulin to be used. Numerous studies have shown that purified insulins considerably reduce the complications occurring with insulin treatment, such as allergies, lipodystrophy, and resistance.<sup>1-5</sup> This improvement has been attributed to a significant decrease in the amount of antibodies produced after the injection of the purified hormone.<sup>6,7</sup>

The benefits of purified insulin on late diabetic complications, particularly those of a microangiopathic nature, are not yet known. Marked prevalence of circulating immune complexes in diabetic subjects with severe microangiopathy<sup>8</sup> suggests, however, that immune factors may play a role in the pathogenesis and/or in the evolution of microangiopathy.

The recent production of human insulins, the biosynthetic human insulins<sup>9</sup> and the semisynthetic monocomponent human insulins,<sup>10</sup> has made available new tools that should lead to further insight into insulin treatment,<sup>11</sup> especially concerning the rise in insulin antibodies.

The aim of the present investigation was to study human monocomponent insulin and its biologic and immunologic activity, and to draw a comparison with porcine monocomponent insulins and other conventional types of insulin preparations.

## MATERIALS AND METHODS

Sixty-eight diabetic patients receiving insulin for the first time were examined. None of these patients had evidence of other diseases likely to involve the immune system, and none exceeded 10% of mean body weight values. No pregnant women were included in the series.

Patients comparable for sex, age, and duration of disease were divided into the following three groups according to the type of insulin received.

**Group 1 (15 subjects).** Eight men and seven women comprised group 1 (mean age,  $28.2 \pm 6.3$  yr; mean duration of diabetes,  $4.4 \pm 2.1$  yr). Ten were newly diagnosed insulin-dependent diabetic patients (IDDM) and five had been treated previously with oral antidiabetic drugs. Patients in this group were treated with human monocomponent insulins; 5 received only the Monotard (Novo Industri, Copenhagen, Denmark) preparation and 10 the combination Monotard + Actrapid (Novo).

**Group 2 (28 subjects).** Sixteen male and 12 female subjects

TABLE 1  
Insulin requirement (U/kg/day) in 68 newly treated IDDM from 2 wk to 6 mo after the beginning of insulin treatment

| Type of insulin | No. of subjects | Months |      |      |      |      |
|-----------------|-----------------|--------|------|------|------|------|
|                 |                 | 0*     | 1    | 2    | 3    | 6    |
| Human           | 15              | 0.41†  | 0.48 | 0.44 | 0.42 | 0.44 |
|                 |                 | 0.03‡  | 0.03 | 0.04 | 0.02 | 0.05 |
| Porcine         | 28              | 0.42†  | 0.46 | 0.50 | 0.43 | 0.52 |
|                 |                 | 0.02‡  | 0.04 | 0.05 | 0.04 | 0.03 |
| Conventional    | 25              | 0.40†  | 0.45 | 0.51 | 0.55 | 0.62 |
|                 |                 | 0.03‡  | 0.05 | 0.05 | 0.07 | 0.07 |

\*Fifteen days after the beginning of insulin therapy.

†Mean; ‡SEM.

comprised group 2 (mean age,  $22.5 \pm 3.3$  yr; mean duration of diabetes,  $2.6 \pm 1.2$  yr). Twenty-one were newly diagnosed IDDM and seven had been treated previously with oral antidiabetic drugs. Patients in this group were treated with porcine monocomponent insulins; 10 received only the Monotard (Novo) preparation and 18 the combination Monotard + Actrapid (Novo).

Group 3 (25 subjects). Fourteen male and 11 female subjects comprised group 3 (mean age,  $21.4 \pm 4.1$  yr; mean duration of diabetes,  $3.3 \pm 1.4$  yr). Nineteen were newly IDDM and five had been treated previously with oral antidiabetic drugs. Patients in this group were treated with conventional insulins; 9 received only long-acting preparations and 16 the regular + long-acting.

Blood samples were collected before insulin treatment and 1, 2, 3, and 6 mo after the beginning of insulin treatment. Serum from blood samples was stored at  $-25^{\circ}\text{C}$  until assay for (1) islet cell antibodies (ICA<sub>b</sub>) using the indirect immunofluorescence techniques;<sup>12</sup> (2) insulin antibodies (IBC) following the immunoelectrophoretic method described by Christiansen<sup>13</sup> using a Novo Industri kit; (3) circulating immune complexes using the solid phase C1q method (C1q-AgAb) described by Hay et al.<sup>14</sup> with minor modifications;<sup>15</sup>

and (4) circulating immune complexes using the conglutinin method (Kg-AgAb) described by Casali et al.<sup>16</sup> with minor modifications.<sup>15</sup>

The determination of immune complexes and the characteristics and criteria used to establish positive limits of the technique have been described elsewhere.<sup>8</sup>

Metabolic control was evaluated by assay of glycosylated hemoglobin (HbA<sub>1c</sub>) with column chromatography using the Bio Rad kit (Bio-Rad, Richmond, California). The upper limit of normal values in our laboratory was 9%, intraassay variation was 0.3% (HbA<sub>1c</sub> < 7%) and 0.6% (HbA<sub>1c</sub> > 7%), and interassay variation was 0.8% and 1.4%, respectively. Student's *t* test and Cox's trend test were used for statistical evaluation of the data.

## RESULTS

The prevalence of ICA<sub>b</sub> at the beginning of the investigation was comparable in the three groups of patients (52% in group 1, 57% in group 2, and 52% in group 3). The metabolic control after 6 mo treatment was similar in all three groups: the mean HbA<sub>1c</sub> was 9.4%, 9.2%, and 10.1%, respectively, in groups 1, 2, and 3. The insulin requirement (Table 1) was

TABLE 2  
Insulin binding capacity (mU/ml) in 68 newly treated IDDM from the first to the sixth month after the beginning of insulin therapy

| Type of insulin | No. of subjects | Months   |           |           |           |
|-----------------|-----------------|----------|-----------|-----------|-----------|
|                 |                 | 1        | 2         | 3         | 6         |
| Human           | 15              | 0.07*    | 0.1       | 0.1       | 0.05      |
|                 |                 | 0.03†    | 0.07      | 0.05      | 0.02      |
| Porcine         | 28              | 0.07*    | 0.2       | 0.3       | 0.4       |
|                 |                 | 0.02†    | 0.08      | 0.07      | 0.09      |
| Conventional    | 25              | 0.5*     | 0.8       | 1.2       | 1.7       |
|                 |                 | 0.2†     | 0.2       | 0.3       | 0.3       |
| H-P             |                 | NS       | NS        | NS        | P < 0.05  |
| H-C             |                 | P < 0.05 | P < 0.05  | P < 0.025 | P < 0.01  |
| P-C             |                 | P < 0.05 | P < 0.002 | P < 0.002 | P < 0.005 |

H = human insulin; P = porcine insulin; C = conventional insulin. Statistical evaluation by Student's *t* test.

\*Mean; †SEM.



TABLE 3  
Prevalence of circulating immune complexes (C1q-AgAb) (%) in 68 newly treated IDDM from the beginning of insulin treatment to the sixth month of therapy

| Type of insulin | No. of subjects | Months |    |    |    |    |
|-----------------|-----------------|--------|----|----|----|----|
|                 |                 | 0      | 1  | 2  | 3  | 6  |
| Human           | 15              | 40     | 40 | 27 | 20 | 13 |
| Porcine         | 28              | 53     | 42 | 22 | 22 | 16 |
| Conventional    | 25              | 46     | 33 | 25 | 25 | 14 |

similar in all three groups 15 days after the beginning of insulin treatment, whereas after 6 mo a progressive rise was observed from group 1 to group 3, but these differences were not statistically significant.

Anti-insulin antibodies (IBC) (Table 2) at 6 mo were significantly lower ( $P < 0.05$ ) in patients in group 1 than in group 2. The levels were significantly higher ( $P < 0.05-0.002$ ) in group 3 than in groups 1 and 2 from the end of the first month onward. The prevalence of C1q-AgAb was similar in the three groups of patients before the beginning of insulin treatment, thereafter decreasing progressively to reach, by 6 mo, values ranging between 13% and 16% (Table 3).

The prevalence of Kg-AgAb was similar in the three groups before the beginning of insulin treatment (Table 4). At 6 mo the presence of Kg-AgAb remained practically constant in groups 1 and 2, and rose significantly ( $P < 0.001$ ) from 24% at the beginning of treatment to 44% at 6 mo of observation in group 3.

#### DISCUSSION

Before the beginning of insulin treatment the three groups of patients displayed similar prevalence of ICAb, C1q-AgAb, and Kg-AgAb showing the same immunologic background. Two weeks after the beginning of insulin treatment, insulin requirement was also comparable in the three groups. The present study confirms a previous report<sup>17</sup> indicating that human monocomponent insulin produces a lower insulin antibody response than porcine monocomponent insulin in subjects never treated previously with insulin.

TABLE 4  
Prevalence of circulating immune complexes Kg-AgAb (%) in 68 newly treated IDDM from the beginning of insulin treatment to the sixth month of therapy

| Type of insulin | No. of subjects | Months |     |     |     |     |
|-----------------|-----------------|--------|-----|-----|-----|-----|
|                 |                 | 0      | 1   | 2   | 3   | 6   |
| Human           | 15              | 27     | 27  | 33  | 27  | 20  |
| Porcine         | 28              | 25     | 27  | 24  | 24  | 28  |
| Conventional    | 25              | 29*    | 33* | 33* | 38* | 44* |

\* $P < 0.001$  (Cox's trend test).

The observed differences may be due to the primary structure of these insulins. However, it should be noted that the amino acid in position 30 of the B-chain, which differentiates human from porcine insulin, does not appear to be directly involved in the immunogenic activity of the hormone. On the other hand, since not only the primary structure but also the stereoisometric configuration define the immunogenic characteristics of a polypeptide, the difference in only one amino acid may change the spatial structure of a protein. This modification may change the insulin capacity to induce the formation of specific antibodies and to bind with them.

The difference in insulin antibody formation between groups 1 and 2 could also be explained by a difference in the genetic background of the diabetic population studied. In effect, subjects with HLA-D4 antigens produce more IgG insulin antibodies to animal insulin than do patients with other HLA antigens.<sup>18,19</sup> Of interest, however, Schernthaner<sup>17</sup> found a high prevalence of HLA-DR3 in patients producing IgG insulin antibodies against human monocomponent insulin. The relevance of these observations to the current study remains to be determined.

Circulating immune complexes assayed with C1q, which have been previously shown to be correlated with the onset of type 1 diabetes<sup>15</sup> and with the severe forms of microangiopathy in both types of diabetes,<sup>8,20</sup> do not appear to be influenced by the type of insulin treatment employed. This observation would confirm earlier findings indicating that insulin is not a component of C1q-AgAb. The prevalence of circulating immune complexes determined with the KgBt technique appears, in contrast, to show a course dependent on the type of treatment used. This behavior is probably due to the positive correlation between Kg-AgAb and anti-insulin antibodies demonstrated elsewhere.<sup>21</sup>

Nevertheless, since there is no definite evidence to show that insulin is to be found among the antigens present in the Kg-AgAb complexes, we may only suspect that some anti-insulin-insulin antibody immune complexes may be revealed with KgBt. Whatever mechanism is involved, human insulins injected in man appear to induce a slight formation of immune complexes when compared with the heterologous insulins. The clinical importance of Kg-AgAb in diabetes mellitus still remains to be defined inasmuch as the microangiopathy processes have been shown to be correlated only with the C1q-AgAb.

In conclusion, the present 6-mo trial suggests that equivalent glycemic control may be achieved with human monocomponent insulins at similar doses to those required with porcine monocomponent insulins. Furthermore, human insulin is the least immunogenic of the present available insulins. No evidence of undesired side effects of human monocomponent insulin was present.

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## Insulin-anti-insulin complexes in diabetic women and their neonates

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**Summary.** It is known that insulin does not cross the placenta, whereas maternal anti-insulin antibodies do. We have therefore investigated insulin antibodies and insulin-anti-insulin complexes both in pregnant diabetic women during pregnancy and in umbilical cord blood from their new-born infants. Forty-seven diabetic pregnant women and 23 new-born infants of these diabetic women were studied. All the pregnant patients were studied at the end of pregnancy and, in 27, at least on one other occasion during pregnancy. All the patients were treated with insulin during pregnancy: 26 had Type 1 (insulin-dependent) diabetes, 14 Type 2 (non-insulin-dependent) diabetes and seven had gestational diabetes. Insulin antibodies were found in 62% of the Type 1 diabetic patients, in 71% of the Type 2 diabetic patients and in 43% of the gestational

diabetic patients. They were present in 48% of the infants studied. Insulin-anti-insulin complexes were found in 37% of the women with Type 1 diabetes, in 21% of those with Type 2 diabetes and in 14% of those with gestational diabetes. Complexes were found in 38% of the new-born infants. The presence of these complexes in the babies was more strongly correlated with their occurrence in their mothers at the beginning than at the end of pregnancy. Insulin-anti-insulin complexes are thus present in the neonatal circulation. They may differ from those in their mothers and they may have pathophysiological and clinical importance.

**Key words:** Insulin antibodies, insulin-anti-insulin complexes, diabetic pregnancy.

Diabetic pregnancy is useful for the study of several immune phenomena. The immune system in normal pregnancy is thought to be in a state of 'activation' rather than in a state of 'depression' [1]. In diabetes, several immunological abnormalities may be present and these may be related to pathogenic events, metabolic derangement and insulin therapy [2].

In diabetic pregnancy, several immune phenomena may be present temporarily as a consequence of both diabetes and pregnancy. Moreover, some abnormal immune factors can cross the placenta and interfere with fetal metabolism and development. In particular, maternal antibodies to exogenous insulin may be transferred to the fetal circulation, whereas maternal insulin does not cross the placenta. The significance of insulin antibodies in fetal blood during the first months of fetal development and their possible pathophysiological effects and interactions with fetal insulin are unclear and these antibodies have not received the attention they merit.

In this study, we looked for the possible presence of insulin-anti-insulin complexes in cord blood and examined the relationships between these complexes in new-born infants with maternal insulin antibodies and insulin-anti-insulin complexes.

### Subjects and methods

#### Subjects

A total of 47 pregnant diabetic patients, all attending the same clinic, were studied. Twenty-three were investigated with their babies. All the pregnant patients were tested at the end of pregnancy; 27 were also tested in the first trimester of pregnancy. Twenty-six had Type 1 diabetes, 14 Type 2 diabetes and seven gestational diabetes. All patients were treated with insulin, either monocomponent or semi-synthetic human, except in two cases. Infant cord blood was also studied.

#### Methods

Insulin antibodies, estimated as insulin binding capacity, were evaluated by the method of Ortvad Andersen et al. [3] as modified by Mustaffa et al. [4] with a few further minor modifications [5]. Values above 10 mU/l were considered indicative of the presence of antibodies.

Insulin-anti-insulin complexes were measured by a modification of the techniques described by Jayarao et

al. [6] and Virella et al. [7]. Serum to be tested was split into aliquots. One aliquot was dialysed overnight in buffer glycine-HCl (pH 3), added to dextran-charcoal in glycine-HCl, incubated for 30 min at room temperature, centrifuged and finally the supernatant was dialyzed against barbital buffer, pH 7.4. To this mixture and the other aliquot, adequately diluted, radiolabelled insulin was added. After 2 h incubation at 37 °C, the addition of dextran-charcoal and centrifugation, the supernatants and precipitates were counted.

The insulin-anti-insulin complexes were calculated as the difference between 'total' antibody and 'free' antibody. 'Total' and 'free' antibodies were calculated as follows:  $\frac{100}{\text{cpm in the supernatant} / \text{total cpm}} \times 100 - \text{background}$ . Positive values were taken as  $> \text{mean} + 2 \text{ SD}$  of normal values and are expressed as a percentage (i.e.  $> 10\%$ ). Positive values of insulin-anti-insulin complexes were  $> \text{mean} + 2 \text{ SD}$  of values found in normal subjects (i.e.  $> 1\%$ ). The sensitivity of this technique in assaying the sole presence of insulin antibodies was slightly different from Andersen's method [3] (Table 1).

Fisher's exact test was used for statistical evaluation.

## Results

### Insulin antibodies

**Diabetic mothers:** Insulin antibodies were found at the end of pregnancy in 62% of the Type 1 diabetic patients (median 15.5 mU/l, interquartial range 10–45 mU/l), in 71% of the Type 2 diabetic patients (median 26.5 mU/l; interquartial range 10–79 mU/l) and in 43% of the gestational diabetic patients (median 10 mU/l, interquartial range 10–13 mU/l). No significant difference was found when the levels of insulin antibodies in the Type 1 diabetic patients at the beginning of pregnancy (median 18 mU/l, interquartial range 10–43 mU/l) were compared with those at the end of pregnancy.

**Neonates:** Insulin antibodies were found in 48% of the neonates studied. Their levels were similar in the neonates (median 10 mU/l, interquartial range 10–28 mU/l) and in the mothers at the end of pregnancy (median 10 mU/l, interquartial range 10–30 mU/l; Table 1).

### Insulin-anti-insulin complexes

**Mothers:** Quantifiable insulin-anti-insulin complexes were found in 37% of the Type 1 diabetic patients at the beginning of pregnancy (median 7%, interquartial range 7–13.3%) and in 27% of the Type 1 diabetic patients at the end of pregnancy (median 7%, interquartial range 7–7.1%). Complexes were also present in 21% of the Type 2 diabetic mothers at the end of preg-

nancy (three patients: 8%, 9%, 82%) and in 14% of gestational diabetic patients (one patient: 8%).

**Neonates:** Insulin-anti-insulin complexes were present in the infants of diabetic mothers (38% of infants of Type 1 and 43% of Type 2 diabetic mothers; Table 1). There was a significant correlation between the presence of the complexes in neonates and their occurrence in their mothers, and this correlation was much stronger when the data in neonates were correlated with data from the mothers in the first trimester of pregnancy ( $p < 0.0005$ ) than at the end of pregnancy ( $p < 0.01$ ).

## Discussion

This study shows that insulin-anti-insulin complexes are present in the circulation of some infants of Type 1 diabetic mothers. The amount of complexed anti-insulin antibody is related not only to the total amount of the antibody, but is also related to the affinity and avidity of the maternal insulin antibody.

There was a strong correlation between the presence of complexes in cord blood and in the maternal circulation. However, their presence in the fetus does not merely represent passive transfer of insulin antibodies across the placenta. The levels of the complexes in the neonate do not reflect those of the mother at the end of pregnancy, but the average complex levels throughout pregnancy.

Insulin antibodies can easily cross the placenta, whereas insulin is not transferred in significant amounts. In the fetus, these antibodies react with a different antigen, fetal endogenous insulin, rather than exogenous heterologous insulin as in the mother. The antigen/antibody molar ratio is substantially different. In the mother, the antibody reacts with massive doses of insulin, of either high or low immunogenicity, administered in subcutaneous boluses, often twice a day, while in the fetus, it reacts with continuously produced pancreatic insulin. The amount of insulin produced by the fetal pancreas varies greatly between the first months of gestation until term, whereas the amount of insulin-antibodies crossing the placenta does not usually vary significantly during pregnancy. From a situation probably characterized by antibody excess at the beginning of pregnancy, the proportion between antigen and antibody becomes more balanced, as the fetal pancreas produces increasing amounts of insulin.

It is likely that both the fate and the significance of the fetal insulin-anti-insulin complexes are different from those of the mother.

Clearance of fetal complexes seems slower than that in the mother. It is not known whether insulin antibodies in fetal circulation may be easily retransferred to the maternal circulation, but it is unlikely that the complexes are freely exchanged.

Whereas, in the mother, small to medium size com-

Table 1. Insulin antibodies and immune complexes in diabetic pregnant women and their neonates

| Patient groups                            | Duration of diabetes (years) | Insulin treatment before pregnancy | Insulin treatment during pregnancy | Maternal insulin binding |                         | Insulin-anti-insulin complexes (%) |                  | Neonate insulin binding (mU/l) | Neonatal insulin-anti-insulin complexes (%) |          |
|---|------------------------------|------------------------------------|------------------------------------|--------------------------|-------------------------|------------------------------------|------------------|--------------------------------|---|----------|
|   |                              |                                    |                                    | First trimester (mU/l)   | End of pregnancy (mU/l) | First trimester                    | End of pregnancy |                                | Total                                       | Autobody |
| <i>Type 1 diabetic patients</i>           |                              |                                    |                                    |                          |                         |                                    |                  |                                |   |          |
| 1   | 15                           | C                                  | Human                              | 18                       | 20                      | <10                                | <7               | <10                            | <7  | <10      |
| 2   | 10                           | C                                  | Human                              | 18                       | 27                      | <10                                | <7               | <10                            | <7  | <10      |
| 3   | 12                           | MC                                 | Human                              | 158                      | 167                     | 94                                 | 19               | 46                             | 28  | 41       |
| 4   | 6                            | MC                                 | MC                                 | <10                      | <10                     | <10                                | <7               | <10                            | <7  | <10      |
| 5   | 6                            | MC                                 | MC                                 | <10                      | <10                     | <10                                | <7               | <10                            | <7  | <10      |
| 6   | 4                            | MC                                 | MC                                 | <10                      | <10                     | <10                                | <7               | <10                            | <7  | <10      |
| 7   | 2                            | C                                  | C                                  | <10                      | <10                     | <10                                | <7               | <10                            | <7  | <10      |
| 8   | 3                            | C                                  | MC                                 | 35                       | <10                     | 30                                 | 20               | <10                            | <7  | 15       |
| 9   | 16                           | C                                  | MC                                 | 14                       | 14                      | 17                                 | 8                | 15                             | 7   | 15       |
| 10  | 5                            | C                                  | MC                                 | <10                      | <10                     | <10                                | <7               | <10                            | <7  | <10      |
| 11  | 10                           | C                                  | MC                                 | 11                       | <10                     | <10                                | <7               | <10                            | <7  | <10      |
| 12  | 16                           | C                                  | MC                                 | 37                       | 74                      | 18                                 | 10               | 30                             | <7  | 28       |
| 13  | 9                            | C                                  | MC                                 | 44                       | 11                      | 33                                 | 21               | <10                            | <7  | 35       |
| <i>Type 2 diabetic patients</i>           |                              |                                    |                                    |                          |                         |                                    |                  |                                |   |          |
| 14  | 1                            | *                                  | C                                  | 153                      | 202                     | 42                                 | <7               | 63                             | <7  | 39       |
| 15  | 6                            | *                                  | MC                                 | <10                      | 28                      | <10                                | <7               | 16                             | <7  | <10      |
| 16  | 11                           | *                                  | MC                                 | 207                      | 236                     | 104                                | 67               | 126                            | 82  | 50       |
| 17  | 7                            |                                    | MC                                 | <10                      | 30                      | <10                                | <7               | <10                            | <7  | <10      |
| 18  | 7                            |                                    | Human                              | <10                      | <10                     | <10                                | <7               | <10                            | <7  | <10      |
| 19  | 16                           | *                                  | Human                              | <10                      | 18                      | <10                                | <7               | <10                            | <7  | <10      |
| 20  | 14                           | *                                  | Human                              | 198                      | 79                      | 47                                 | 23               | 18                             | 8   | 32       |
| <i>Patients with gestational diabetes</i> |                              |                                    |                                    |                          |                         |                                    |                  |                                |   |          |
| 21  | -                            |                                    | MC                                 | <10                      | <10                     | <10                                | <7               | <10                            | <7  | <10      |
| 22  | -                            |                                    | Human                              | <10                      | 10                      | <10                                | <7               | <10                            | <7  | <10      |
| 23  | -                            |                                    | Human                              | 17                       | 13                      | <10                                | <7               | <10                            | <7  | <10      |

C = Conventional non-highly purified insulin; MC = monocomponent insulin; \* The patient was treated with conventional insulin in previous pregnancies

plexes are rapidly removed by the kidney and the reticulo-endothelial system [8], in the fetus, clearance is not as efficient, particularly in the infants of diabetic mothers in whom the duration of gestation is often shortened. This could explain why, in those few cases in which the insulin antibody levels are high in the mother at the beginning of pregnancy and low at the end, the insulin-anti-insulin complex levels in the fetus are comparable to those found in the mother in the first months of pregnancy.

Our group and others have found a correlation between the presence of insulin antibodies and a lack of neonatal complications [9-13]. On the other hand, the possible damage induced by the deposition and presence of immune complexes is well known, both in diabetic patients [6, 14] and in pregnant women with clinical complications [15, 16]. Moreover, it is well known that the injection of insulin antibodies may induce a diabetic-like syndrome in normal animals [17]. The presence of insulin-anti-insulin complexes may theoretically interfere with the placental circulation and explain some of the neonatal complications of diabetic pregnancy, in particular macrosomia and neonatal hypoglycaemia with neutralising or abrupt release of substantial amounts of neonatal insulin.

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