University of Bath

PHD

Autoimmune aspects of diabetes mellitus.

Quilley, John

Award date:
1979

Awarding institution:
University of Bath

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AUTOIMMUNE ASPECTS OF DIABETES MELLITUS

Submitted by

John Quilley

for the Degree of Ph.D.

at the University of Bath

1979

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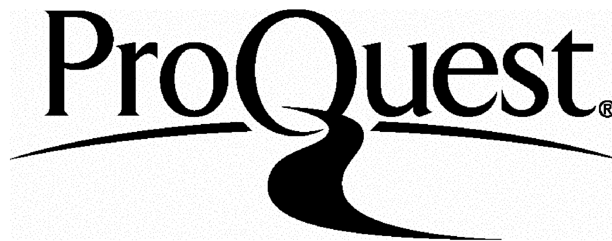

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Acknowledgments.

I should like to thank Dr. Elizabeth Richens for her supervision and perseverance, Dr. Martin Hartog for his criticism, Professor R. J. Ancill for making available facilities at Bath University and the Royal United Hospital, Mrs. J. Russel for her technical assistance, and the Public Health Laboratory for the culture and use of Coxsackie B_4 virus.

This work was supported by a grant from the Science Research Council.

Summary.

In this study induction of diabetes in CD_1 mice using Coxsackie B_4 virus was attempted with a view to developing an adequate animal model. However, in a series of three experiments no diabetic-like state was observed.

In clinical studies insulin-dependent diabetics (I.D.D.), insulin-independent diabetics (I.I.D.), and control subjects were tested for leucocyte migration inhibition against pancreatic antigens, Coxsackie B_4 virus, and rat liver mitochondria. Inhibition of migration was observed more often against these antigens in the I.D.D. In another study of cell-mediated function lymphocytes from 10 I.D.D. were tested for PHA-induced transformation. Two patients showed depression of this response compared to individual control responses.

An indirect immunofluorescence technique was used to assess the incidence of autoantibodies. In 54 I.D.D. islet cell antibodies were found in 32%, whilst there was a greater incidence of other autoantibodies (thyroid, gastric parietal cell, antinuclear, reticulin, smooth muscle and mitochondrial) in the sera of this group compared to I.I.D. and the control subjects. Preliminary absorption studies of islet cell antibody (ICA) positive sera from 4 I.D.D. incubated with Coxsackie B_4 virus produced a decrease in intensity of fluorescence in 3 cases, whilst an increase in the intensity was observed in some cases when pancreatic homogenate, microsomes, and mitochondria were used.

HLA studies revealed a higher incidence of HLA-B8 and B15 in I.D.D. with a protective effect conferred by HLA-B7. No correlation was found between any HLA antigens and immune phenomena.

Finally, 13 I.D.D. were examined for T-cell cytotoxicity against chicken erythrocytes coated with pancreatic fractions, and 17 I.D.D. for antibody-dependent cell-mediated (K-cell) cytotoxicity. No evidence of T-cell cytotoxicity was found, whilst 4/17 patients showed depression of K-cell activity by more than 10% when compared to control subjects.

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CHAPTER I.

Introduction.

Definition. Diabetes mellitus is a chronic disease which, in the uncontrolled state, is manifested by multiple disturbances of metabolic processes which are attributable to an insufficient insulin supply. The rate of utilisation of sugar declines and hyperglycaemia and glycosuria ensue. As the loss of sugar increases, protein is broken down in an attempt to supply energy and muscle wasting becomes apparent. In addition, there is a decrease in the synthesis of fat from carbohydrate and an increase in fat catabolism which leads to the production of ketones in excess of tissue utilisation. Eventually, without treatment, there is a massive build-up of ketones in the blood which leads to coma and finally death.

Complications. Complications arising from diabetes mellitus include retinopathy, neuropathy, nephropathy, and atherosclerosis. The life expectancy of the diabetic is thus reduced. In the diabetic population the most common cause of death is heart disease or kidney disease. In the U.S.A. diabetes mellitus is the third most common cause of death behind dissociated heart disease and cancer. It is also a major cause of blindness, being the second most common cause in the U.S.A. (Maugh, 1975).

Incidence. Diabetes mellitus is a common disease and has traditionally been divided into two forms, the maturity- and juvenile-onset types.

The majority of cases belong to the maturity-onset type. Baird and Strong (1975) quote the prevalence of diabetes as being 1% in the United Kingdom, but of these only 10% are juvenile-onset diabetics. Drash (1975) estimated that 1% of the adult population in the United States are diabetic, and that there are an equal number with chemical diabetes or asymptomatic glucose intolerance. He further states that diabetes occurs in approximately 1 of 2,500 children under fifteen years of age. Farquhar (1973) estimated the prevalence to be slightly lower in Britain with diabetes affecting approximately 35 of every 100,000 children under the age of sixteen.

Steinke and Soeldner (1977) estimated that there are over 200 million diabetics in the world. This data, taken from the U.S. Public Health Service, shows that for children up to the age of seventeen years there are 130 diabetics/1000,000 population, for people aged between 25-44 years, 1,700/100,000, and for people between 45-64 years of age there are 4,300 diabetics per 100,000 population, whilst in the group over 60 years of age there are 7,900/100,000 population.

A 1975 survey of new diabetics in the United Kingdom between the ages of 0-15 years revealed an annual incidence rate of 7.67/100,000 (Bloom, Hayes, and Gamble, 1975). Mann, Thorogood, and Smith (1978) reported on new cases of juvenile-onset diabetes where development of the disease occurred before the age of sixteen years, and found the incidence ranged from 4.6/100,000 to 14.8/100,000 according to the district. Recent surveys in Denmark have revealed an incidence of 13.2/100,000 per year for juvenile-onset diabetics aged between 0 and 29 years (Cristau et al., 1977) and an incidence of 11.3/100,000 in the Copenhagen area (Andersen et al., 1977).

The prevalence of diabetes mellitus varies considerably between populations. Thus, Bennett reports a prevalence of 30.6% in American

Pima Indians aged fifteen years or more, while the prevalence in Eskimos is reported to be low (Scott and Griffiths, 1957). A survey on the Central Pacific island of Nauru revealed a prevalence of 34.4% for maturity-onset diabetes (Zimmet et al., 1977), whilst juvenile diabetes is virtually unknown. Other population groups showing low prevalence of juvenile-onset diabetes include Indians and Negroes in Cape Town and Trinidad, Cook Island Maoris, Japanese, and Maltese (Keen and Jarrett, 1976). However, Keen and Jarrett (1976) point out that comparative prevalence studies have limited value where the duration of disease varies from group to group and that true incidence studies would be of more value.

Classification. Diabetes mellitus is usually divided into two forms, the juvenile-onset, insulin-dependent, ketosis prone type, and the maturity-onset, nonketotic form. The former is characterised by a severe insulin deficiency, whilst in the latter type insulin is present, but its action or secretion is impaired. Sonksen (1972) considers diabetes mellitus as a syndrome rather than as a specific disease. He divides clinical diabetes into acute or juvenile-onset and chronic or maturity-onset diabetes. Craighead (1975) states that the syndrome of diabetes mellitus may represent a complex of diseases having several aetiologies and different forms of clinical expression. Other investigators classify diabetes mellitus in different ways. Irvine (1977) divides idiopathic diabetes mellitus into types I and II:

I. Insulin-dependent juvenile-onset.

Insulin-dependent maturity-onset.

Islet cell antibody-positive diabetics initially controlled by oral hypoglycaemic agents but requiring insulin in later years.

II. Insulin-independent juvenile-onset.

Insulin-independent maturity-onset.

Islet cell antibody negative at diagnosis.

He further subdivides type I according to pathogenesis:

(a) severe autoimmune/non-viral (rare).

(b) moderate autoimmune/moderate viral (common).

(c) severe viral/non-autoimmune (rare).

Cudworth and Woodrow (1977) regard type I as equivalent to juvenile-onset diabetes irrespective of age and showing a significant association with the major histocompatibility system, whereas type II corresponds to maturity-onset, insulin-independent diabetes and has no association with the major histocompatibility system. They question Irvine's classification, pointing out that the use of oral hypoglycaemic agents to middle-aged or elderly diabetics within three months of onset is probably meaningless in terms of pathogenesis of the two types of diabetes and is much more likely to reflect differences in clinical practice in the management of such patients. They also state that there is no evidence for the subgroups (b) and (c) of type I diabetes which are based upon those patients who show islet cell antibody for a limited period after diagnosis and those in whom antibodies are not found. As there is uncertainty in the time lapse between the pathological event and diagnosis, the latter group may initially have had islet cell antibodies.

Bottazzo and Doniach (1976) divide type I diabetes mellitus into IA and IB, the IA subgroup being the classical juvenile-onset form with no associated autoimmune disorders and high titres of islet cell antibodies at the onset which tend to disappear with time. The IB subgroup is associated with well established autoimmune disorders and

a high incidence of other autoantibodies. In this type islet cell antibodies, when found, remain stable for several years. Bottazzo and Doniach's (1976) separation of diabetes into types IA and IB is on the basis of whether islet cell antibodies are temporary and perhaps secondary to viral islet disease or permanent and associated with polyendocrine disorders and HLA-B8. Type IA of this system corresponds to Ib of Irvine's classification, although Bottazzo does not take into account diabetics initially treated with oral hypoglycaemic agents who are islet cell antibody positive.

In the present report the diabetic groups are divided into juvenile-onset, insulin-dependent diabetics independent of age of onset, and maturity-onset, insulin-independent diabetics. When comparisons are made with work presented by other investigators, however, the juvenile-onset type diabetics may be subdivided according to age.

Aetiology and Pathogenesis. The aetiology of diabetes mellitus has been the subject of much speculation. Since viruses were originally suggested (Adams, 1926), a large number have been implicated. These include mumps, hepatitis, rubella, Coxsackie and influenza viruses, adenoviruses, enteroviruses, and cytomegalovirus (Maugh, 1975). The fact that many viruses have been shown to be diabetogenic in various animal species has lent support to the theory of a viral aetiology. The viral implications of diabetes mellitus will be fully discussed in Chapter II.

Other environmental factors have also been suggested, such as diet. Thus, Trowell (1974) related the incidence of childhood diabetes to dietary factors and suggested that high fibre carbohydrate foods are protective against diabetes.

Recent studies of the HLA system have provided evidence for the existence of genetic heterogeneity in diabetes. Cudworth (1978) stated that, irrespective of age of onset, the major susceptibility to chemical insulin-dependent diabetes is conferred by genes in the HLA chromosomal region. Other genes outside the HLA region may also contribute to overall susceptibility. Thus, HLA-associated immune response genes may affect susceptibility to environmental pathogens such as viruses. Furthermore, Cudworth (1978) has suggested that strong genetic factors, not associated with the HLA system, operate to produce susceptibility to maturity-onset, insulin-independent (type II) diabetes.

Immune response genes, possibly associated with the HLA system, might play a part in conferring susceptibility or protection to particular viruses which have an affinity for the islet cells of the pancreas (Notkins, 1977; Cudworth, 1978). The association of diabetes and the HLA system will be more fully discussed in Chapter 6.

For several years diabetes mellitus has been thought of as an autoimmune disease. Considerable evidence has been compiled in favor of this hypothesis:

1. An association with other autoimmune endocrinopathies (Irvine, 1970; Nerup and Binder, 1973; MacCuish et al., 1974).
2. The presence of circulating autoantibodies to the gastric parietal cell, thyroid cytoplasm, and adrenal tissue (Nerup and Binder, 1973; MacCuish et al., 1974).
3. The presence of islet cell antibodies (Bottazzo, Florin-Christensen, and Doniach, 1974; MacCuish et al., 1974; Lendrum, Walker, and Gamble, 1975).

4. The presence of cell-mediated immunity to pancreatic antigens (Nerup et al., 1971, 1973; MacCuish et al., 1974a; Richens, Hartog, and Ancill, 1976).
5. The induction of experimental diabetes in animals (Craighead and Steinke, 1971; Coleman et al., 1974).
6. The infiltration of the pancreas by lymphocytes (Gepts, 1965; Junker et al., 1977).
7. The association with certain HLA antigens (Nerup et al., 1974; Cudworth and Woodrow, 1974, 1975, 1976; Cudworth, 1978).

Irvine et al. (1970) found an increased frequency of circulating thyroid and gastric parietal cell antibodies in patients with diabetes mellitus. These antibodies were more frequently found in insulin-dependent diabetics than in insulin-independent diabetics. Nerup and Binder (1973) confirmed Irvine's work but were unable to find any difference between juvenile-onset and maturity-onset diabetics when the frequencies of autoantibodies were compared. They also found that the simultaneous occurrence of two or more autoantibodies to thyroid, adrenal cortex, or gastric parietal cell cytoplasm was seen only in the sera of diabetic patients.

According to Bottazzo et al. (1974) the prevalence of thyroid and gastric antibodies is three times greater in juvenile diabetics than in age and sex matched controls. The association of diabetes with autoimmune adrenalitis was investigated and Bottazzo et al. (1974) found that the incidence of overt diabetes in idiopathic Addison's disease was approximately 10% as compared to 1% in the overall population. They also reported that adrenal antibodies are found thirty times more often in insulin-dependent diabetics than in control

subjects. They additionally noted that the combination of adrenalitis and thyroiditis has been reported in association with insulin-dependent diabetes. Bottazzo et al. (1974) concluded that the presence of several autoimmune disorders in the same patient indicates a strong hereditary predisposition to autoimmunity.

MacCuish et al. (1974) supported the presence of an autoimmune process in some cases of diabetes and quoted as evidence an association between diabetes mellitus and other autoimmune disorders such as pernicious anaemia, thyrotoxicosis, Hashimoto's thyroiditis, idiopathic hypoparathyroidism, and myasthenia gravis.

Islet cell antibodies were first detected by Bottazzo et al. in 1974 and they found an increased incidence in patients with diabetes mellitus. They were also found by MacCuish et al. (1974). Both groups of workers suggested that islet cell antibodies were confined to patients with one or more overt autoimmune diseases in addition to diabetes mellitus. However, Lendrum et al. (1975) found islet cell antibodies were more common in juvenile-onset diabetics of recent onset and suggested that autoimmune activity directed against islet cells was likely in a high proportion of cases of childhood diabetes. This idea is not compatible with the age distribution and seasonal variation found in juvenile diabetes, and these are unlikely to be the result of a purely immunologic pathogenesis. Lendrum et al. (1975) suggested that perhaps autoimmune damage in combination with a seasonal factor such as a virus was a more probable explanation.

MacLaren, Huang, and Fogh (1975), using human insulinoma cells, found a high incidence of antibodies showing cell surface immunofluorescence in the sera of insulin-dependent diabetics. They were uncertain whether autoimmunity is of primary aetiological significance

and suggested that β-cell damage, possibly by a virus, might expose sequestered β-cell antigens and initiate an immune response as a secondary event.

Further evidence that diabetes mellitus has an autoimmune basis comes from reports of insulitis and infiltration by lymphocytes of the islet cells at post-mortem of diabetic children (Gepts, 1965; Junker et al., 1977) and a report by Le Compte and Legg (1972) of lymphocytic infiltration of the pancreatic islets in two insulin-dependent late onset diabetics. This, combined with lack of plasma cells and lymphoid follicle formation, is a feature of accepted organ-specific autoimmune disorders (Editorial, Lancet, 1976). However, Doniach and Morgan (1973) found no lymphocytic infiltration in thirteen young diabetics, whilst Maclean and Ogilvie (1959) reported only three cases out of twenty-two diabetics.

Delayed hypersensitivity as assessed by the leucocyte migration inhibition test has yielded further evidence. Nerup et al. (1973) performed leucocyte migration tests using foetal calf pancreatic extract as antigen. They concluded that in diabetes there is an organ-specific, species nonspecific, antipancreatic hypersensitivity. Nerup et al. (1973a) reported, in further experiments, that antipancreatic, cellular hypersensitivity was predominantly found in patients with juvenile diabetes of recent onset. MacCuish et al. (1974a) using human pancreatic antigen found a significant difference in migration inhibition between diabetics and control subjects.

Other experiments performed by Nerup et al. (1973a) showed that antipancreatic delayed hypersensitivity could be induced in rats by immunisation with a foetal calf pancreatic extract. Histological examination of the pancreas revealed mononuclear cell infiltration of

islets in these animals but not those immunised with insulin and Freund's adjuvant. Wright et al. (1976) induced pancreatic lesions in rabbits and guinea pigs with various pancreatic antigens (whole bovine insulin and bovine insulin A component, and homogenates of the islets of Langerhans) but none of the animals developed defined signs of diabetes mellitus.

Other evidence for diabetes being an autoimmune disease comes from HLA associations. According to Bodmer and Bodmer (1974), diseases showing the most significant association with histocompatibility antigens are autoimmune. It is possible that immune response genes linked to HLA genes may provide the basis for disease associations. Many investigators have shown an association of juvenile-onset insulin-dependent diabetes with certain HLA antigens, particularly HLA B8 and B15 (Nerup et al., 1974; Cudworth and Woodrow, 1974, 1975, 1976; Singal and Blajchman, 1973). Recent studies show a positive association with DW3, DRW3 and DRW4 antigens (Thomsen et al., 1975; Nerup et al., 1976; De Moerloose et al., 1978).

The evidence in favor of an autoimmune basis for juvenile-onset diabetes mellitus is thus considerable. However, this does not preclude the involvement of viruses and other environmental factors. It is possible that immune response genes linked to the HLA system confer susceptibility to viruses which have an affinity for the pancreatic islet cells and this in turn leads to β-cell damage which may then trigger an autoimmune response in susceptible individuals. The mechanisms whereby viruses may trigger autoimmune reactions will be discussed in Chapter II.

Development of Autoimmunity. Irvine (1974) lists six distinct immune mechanisms, each of which, with the exception of type I, may be involved in various endocrine disorders:

I. Anaphylactic (immediate hypersensitivity)

II. Immune complex (Arthus).

III. Cytotoxic antibody.

IV. T-cell mediated (delayed hypersensitivity)

V. Stimulating antibody

VI. Lymphoid (K) cell antibody mediated cytotoxicity.

From the foregoing discussion it is seen that both the T and B cells of the immune system have been implicated in the aetiology and pathogenesis of diabetes mellitus. Recently, a third type of cell, the K cell, has been described which Irvine (1974) considers may have a major role in autoimmunity. These cells have receptors for activated Fc fragments of antibodies, i.e., complexed antibodies. If the antigen-antibody complex is on the surface of a target cell, then the K-cell will kill that cell. Also, immune complexes may be free in the circulation and taken up by K cells reacting with the Fc fragment. Provided antibody is in excess, the K cell will acquire specificity to kill any cell which has the corresponding antigen on its surface and to which access is afforded. Antibody produced B cells may, in the presence of complement, have a direct cytotoxic effect on target cells but it may also form complexes, either on the surface of the target cell or free in the circulation. There is evidence that B, T, and K cells are operative in Hashimoto's thyroiditis (Irvine, 1974). Calder et al. (1974) proposed three possible mechanisms of damage to the thyroid gland: the production of lymphokines, a cytotoxic mechanism (sensitised T-cells), non-immune lymphoid cells and antibody and Fc

receptor activation. The cytotoxic mechanism she describes involves the cellular and humoral components of the immune system in the pathogenesis of autoimmune disease. It is possible that the same mechanisms apply in juvenile diabetes mellitus.

Investigations into the development of autoimmunity (Marx, 1975; Asherson and Zembata, 1976; Turk, Polak, and Parker, 1976) have stressed the importance of the loss of self-tolerance and of suppressor T cells. Burnet's clonal selection theory (1959) states that contact of lymphocytes and body constituents during embryonic life or in early postnatal development might destroy or permanently inactivate the complementary clones of lymphocytes which have the potential of reacting with an individual's own antigens. He attributed autoimmunity to the regeneration of "forbidden" clones of lymphocytes with specificities directed against self-antigens by a process of somatic mutation.

Another explanation of autoimmune damage is that it takes place against antigens hidden from the immune system. Sensitive tests, however, have shown the presence of certain proteins in the circulating blood (Allison, 1974). Tolerance has now been clarified by the demonstration of T and B lymphocytes and the helper effect of T cells in the formation of antibodies, and the induction of tolerance to proteins that circulate in the blood in high concentrations. Low dose tolerance specifically inhibits the capacity of T cells to respond to antigen whilst high dose tolerance induces unresponsiveness in both T and B cells (Allison, 1974). On the basis of this, Allison suggests that many body proteins such as thyroglobulin and some glycoproteins shed from cell membranes are present in the circulation in low doses, so T cells are unresponsive whilst B cells with receptors for these

antigens can be stimulated to proliferate and secrete antibody. Such stimulation follows exposure to autoantigen in the presence of adjuvant which causes T call stimulation. With antigens circulating in high doses, complementary B and T cells are inactivated and autoantibodies would not be elicited by the above method. Tolerance maintained by specific unresponsiveness of helper T cells can be easily overcome, for example, by certain infections and Allison suggests that the "fail-safe mechanism" is the suppressor T-cell which gives a balance in preventing autoimmunity.

The importance of suppressor T-cells is verified by other workers. Thus, Marx (1975) suggests that suppression is essential for the normal control of immune responses. Asherson and Zembata (1976) postulate that suppressor cells may play a role in the orderly sequence of different classes of immune response as: (i) some are damaging, (ii) some interfere with the effectiveness of others, e.g., antibody and immune complexes interfere with cell-mediated responses, (iii) the type of immune response required depends upon the invading organism. Turk et al. (1976) consider the immune response to be the resultant of a balance between effector and suppressor elements, and that immunological tolerance can be thought of as the generation of a population of suppressor cells. If malfunction of this delicate balance occurs, then an excess of antibodies as in allergic reactions, or production of aberrant antibodies such as in autoimmune disorders, might prevail. Alternatively, there could be a lack of antibodies and consequent immunodeficiency (Marx, 1975). Many investigators consider that suppressor T cells normally prevent the production of autoantibodies and a deficiency may contribute to autoimmunity. Marx (1975) supports this view with the analogy of the progressive disease in NZB mice where

there is a decrease of suppressor effects with age and the spontaneous development of a condition similar to systemic lupus erythmatosus in man. The cause of the loss of suppressor T cells is unknown, but viruses and genetic factors probably play a role (Marx, 1975). Williams and Messner (1975) suggest that a decline in T cells and T cell function, particularly suppressor T cells, in conjunction with an increase in B cells, might relate to the known increased incidence of autoantibodies with old age.

Allison and Denman (1976) also postulate the involvement of viruses in the development of autoimmunity suggesting that viral antigens and autoantigens could form common immunologic units which function in a manner analogous to the hapten-carrier system, and that by-passing the requirement for T cells responsive against autoantigens, autoantibody formation might be elicited, e.g., by a viral infection.

Methods for Investigating Autoimmunity. The foregoing account outlines evidence for juvenile-onset diabetes being an autoimmune disease, the different types of immune reactions possibly involved, and the concepts as to the development of autoimmunity. Methods used in the investigation of autoimmune diseases may be considered under three main headings as proposed by Roitt and Doniach (1965):

(i) Evidence of abnormal immunological reactivity.

(ii) Association with known autoimmune disorders.

(iii) Study of experimental models.

The methods used to study abnormal immunological reactivity are: immunofluorescence; passive haemagglutination, e.g., thyroglobulin antibody; agglutination of coated inert particles, e.g., latex particles coated with γ-globulin react with rheumatoid factors; complement fixation is well suited for estimations of antibodies to particulate tissue antigens

e.g., subcellular fractions and crude extracts can be used; gel precipitation where formation of precipitating complexes between antibody and soluble antigen can be visualised in Ouchterhony plates, makes possible analysis of a complex mixture of antigens or antibodies; mixed agglutination where suitably coated red cells are used as indicators for antigen present on living cells in tissue culture or on histological sections; cytotoxicity tests. Other methods not included by Roitt and Doniach are the leucocyte migration test and phytohaemagglutinin transformation of lymphocytes which are both tests of cell-mediated (T-cell) function.

Scope of the Project. The intention in this investigation was, initially, to study an animal model in which diabetes was induced by a virus based on previous results presented by Gamble et al. (1969, 1973); Coleman, Gamble, and Taylor (1973, 1974) and others who reported the production of diabetes in mice after infection by various viruses (Craighead and McLane, 1968; From et al., 1968; Burch et al., 1971; Craighead and Steinke, 1971; Wellman et al., 1972; Boucher and Notkins, 1973; Kanich, Craighead, and Kessler, 1973). Once a diabetic state had been induced it was intended to undertake further investigations to try to determine whether damage to the islet tissue was directly viral or involved an autoimmune component. The proposed plan of research was to study the effect of virus inoculation in neonatally thymectomised mice which were later to be sublethally irradicated. Owing to the failure to induce a diabetic state after several experiments, the animal model idea was abandoned and all further work was the result of clinical investigation which was to have run in parallel with the animal studies. The clinical investigations were performed using lymphocytes from juvenile-onset diabetics and control

subjects and testing for leucocyte migration, phytohaemagglutinin transformation, and cytotoxicity. In addition, patients and their families, when available, were HLA typed and their sera tested for the presence of autoantibodies.

CHAPTER II.

Viruses and Diabetes Mellitus.

Introduction

Viral Infection and Autoimmunity. Before reviewing the evidence for the implication of viruses in the aetiology of diabetes mellitus, the mechanisms whereby a viral infection could precipitate an autoimmune attack will be considered. Several authors have proposed theories to account for this process, including van Loghem (1965); Isacson (1967); Notkins, Mergenhagen and Howard (1970); and Allison (1972).

van Loghem (1965) associates the thymus gland with autoimmune conditions quoting the frequent finding of thymoma in patients with myasthenia gravis. He implicates viruses on the basis of the following evidence:

(a) The presence of a pathogenic hepatotropic virus in mice thymectomised at birth. This is not present in non-thymectomised or sham-operated animals.

(b) The wasting syndrome (neonatally thymectomised mice waste after 1-3 months, probably as the result of being unable to combat infection) does not occur in germ-free thymectomised mice. It is possible that in normal mice a latent viral infection, otherwise suppressed, becomes active when the thymus is removed.

(c) The wasting syndrome in mice can be prevented by the injection of syngeneic adult spleen cells. This indicates that the wasting

syndrome and autoimmunity are not the direct result of thymectomy but could depend on viral infection.

(d) Normal mice thymectomised at birth and grafted with the thymus gland from NZB/BL mice suffering from autoimmune disease develop the same disease.

van Loghem (1965) considers three mechanisms whereby viral infections may lead to autoimmunity:

(i) Viral infection may be limited to the thymus, where regulating function may be impaired and result in a lack of self-recognition by immunologically competent cells, with subsequent autoantibody formation.

(ii) Viral disease may start in the thymus and spread throughout the immune system. Autoantibody might then be produced by viral interference with the DNA or RNA of clones of immunologically competent cells.

(iii) Viral infections may start in the thymus and spread to other organs evoking the transformation of target cells. Antibodies may then be formed against antigen coating the altered cells and these might cross-react with normal body constituents. van Loghem (1965) suggests that selective affinity of different viruses for organs and tissues could explain the large variety of autoimmune diseases.

It has been shown that viruses affect the immune system, in some cases, by depression of both primary and secondary responses, and in other cases by enhancing humoral immunity and by preventing immunological tolerance (Notkins et al., 1970). Notkins et al. suggest that the latter two effects might be important factors in the aetiology of autoimmune diseases, i.e., stimulation of the production of autoantibody. In addition, Levy and Notkins (1971) report that, in mice, under certain conditions viruses can act as immunologic adjuvant, preventing

the development of tolerance and accelerating immunologic disease. They also state that in addition to the direct effect of viral replication on cellular function, the immune response may result in damage whereby antigens of the host are released or incorporated into the envelope of the maturing virion. These antigens might reach immunologically competent cells and stimulate the production of antibody. If these antigens come from hormone-producing cells, the immune response might lead to endocrine dysfunction and might account for the autoantibodies found in certain endocrine disorders.

Autoimmune diseases could also result from the host's immune response to virus-induced antigens, i.e., if viral antigens are located on the surface of hormone-producing cells, the interaction of sensitised lymphocytes or viral antibody plus complement, with these neoantigens might lead to cell destruction (Levy and Notkins, 1971). In support of this Wiktor, Kuwert, and Koprowski (1968) showed that tissue culture cells infected with rabies virus were lysed after exposure to antirabies antibody and complement, and that the binding of viral antibody to the cell surface and cytolysis occurred before the release of the virions by budding.

Endocrine dysfunction could also result from the effects of circulating virus-antibody complexes in the same way as the deposition of complexes in the kidney can result in glomerulonephritis. These circulating virus-antibody complexes have been shown to persist in mice chronically infected with anti-lactic dehydrogenase virus (Notkins et al., 1966). Levy and Notkins (1971) suggested that complexes become lodged in the endocrine organs and cause lesions due to disseminated intravascular coagulation. McKay and Margaretten (1967) provided evidence that many viruses, including rubella and arbovirus,

can trigger the blood clotting mechanism. The proposed mechanisms are multiple:

1. Complexes may agglutinate platelets in vivo as they do in vitro.

2. Viruses invade and damage endothelial cells leading to thrombus formation. Simultaneous activation of the fibrinolytic system may lead to dissolution and platelets may be swept into circulation to produce coagulation at a distant site.

3. Some viruses cause haemolysis which promotes intravascular coagulation.

4. Antigen-antibody complexes in antigen excess are capable of triggering the clotting mechanism. It is conceivable that under circumstances of continual viral proliferation antibody might combine with the virus to form a procoagulant complex.

5. Antiplatelet antibody.

6. Multiple mechanisms may be responsible.

Isacson (1967) proposed three mechanisms by which an autoimmune condition may arise as the result of a viral infection:

1. Antigenic modification of host cells.

2. Release of host antigens from concealed sites.

3. Antigenic similarity between host tissue and immunising agent. Thus, cross-reactive antigens may induce formation of antibody capable of reacting with antigen and host tissues.

He considered myxoviruses to be ideal inducers of autoimmunity by the first two and, possibly, the third mechanism. Neuramidase is present in the influenza and mump-NDV-parainfluenza viruses and is capable of antigenically modifying host cells. The other important feature of these viruses is their maturation at or near the cell

surface. Influenza viruses are known to incorporate antigens derived from cells in which they are grown; Lindenmann and Klein (1967) demonstrated in the mouse that cellular structures of low immunogenecity become potent immunogens when integrated into the substance of viral particles.

Similarly, Allison et al. (1972) list five mechanisms whereby viruses could theoretically precipiate an autoimmune reaction. In addition to those proposed by Isaacson, there are the possibilities that viruses might derepress host cell antigens, e.g., embryonic antigens, or that they might affect the proliferation or responses of immunocompetent cells or their precursors.

Viral Associations With Diabetes Mellitus. Many viruses have been implicated in the aetiology of diabetes mellitus. These include mumps, hepatitus, rubella, Coxsackie and influenza viruses, adenoviruses, enteroviruses, and cytomegaloviruses (Maugh, 1975). However, Maugh (1975) states that any association can be dismissed as a coincidence because of the high incidence of both viral infections and diabetes mellitus. Brown (1956) was more convinced of the viral infective aetiology of some cases of juvenile diabetes and listed the evidence as follows:

1. A history of infection preceding diabetes, reported in 15-98% of cases.

2. A seasonal variation in onset with fewer cases presenting in the summer.

3. The highest incidence of diabetes occurs in climates where streptococcal infections are greatest. Conversely, diabetes is rare in the tropics.

4. Increased sedimentation rates and capillary fragility are found in diabetics which are indicative of infection.

The finding of families in which several members simultaneously develop diabetes is also suggestive of islet cell damage being initiated by an infectious agent. Thus, Nelson et al. (1977) reported one family in which three of five siblings with diabetic parents developed diabetes within a three-month period in the autumn.

Mumps has been the virus most frequently associated with the onset of diabetes. Maugh (1975) quotes a study in an isolated Swedish community where forty people contracted mumps and, of these, four developed diabetes the following year. This is a much higher incidence rate than expected. A more extensive study was carried out by Sultz, Hart, and Zielezny (1975) who collected data over a twenty-five year period. Their results were assessed, allowing a four-year period after the infection. They found that the incidence of diabetes paralleled that of mumps and mumps encephalitis. In addition, a sharp rise in the incidence of diabetes occurred in boys between 1950 and 1960. Sultz et al. (1975) suggested that this could be due to the deliberate exposure of young boys to mumps to avoid adult orchitis. They also interviewed 112 parents of diabetic children and found that in 50% of the cases mumps or exposure to mumps had preceded diabetes; furthermore, an additional 11% had received mumps vaccine prior to the onset of diabetes mellitus. The time lag period between infection and the onset of diabetes was 3.8 years. This agreed with results reported by Gundersen (1927) who found that the mortality rate from diabetes among young people reached a peak from two to four years after each mumps epidemic. McCrae (1963) found no time lag period and reported a single case of diabetes in a ten-

month-old baby in whom the onset of diabetes was immediately preceded by mumps pancreatitis. He suggested that there might be a more significant relationship between diabetes mellitus and those cases of mumps where the main site of infection is the pancreas. However, Gamble et al. (1969) in a study of antibody titre to a number of common viruses concluded that there was no evidence for the development of diabetes after a mumps infection.

Rubella virus has often been associated with diabetes mellitus especially in the maturity-onset form. Forrest, Menser, and Burgess (1971) investigated fifty young adults who had sustained a congenital rubella infection and found only one with diabetes mellitus. In a survey four years later, when forty-four of these patients were still available for study, diabetes mellitus was diagnosed in five patients (11%) and latent diabetes in four (9%), giving a combined frequency of 20% which was much higher than normal. Dudgeon (1970) suggested that foetal damage in patients with congenital rubella might be due to the inhibition of cell growth, cytolytic activity, or interference in the blood supply. Forrest et al. (1971) cite these mechanisms as of possible relevance to the development of diabetes. In contrast, in a later study quoted by Smithells et al. (1978) there were only two cases of diabetes in 482 children aged between 0-7 years who had had confirmed or suspected congenital rubella. The same group quote an earlier survey of 83 patients aged 3-19 years retrospectively diagnosed as having had rubella, but none of whom had diabetes mellitus. However, Smithells et al. (1978) concluded that although the incidence of diabetes mellitus in individuals exposed to rubella in utero is low in the U.K., follow-up in the second or third decades of life may reveal a higher incidence of diabetes in these subjects.

Of the other viruses implicated in the cause of diabetes most attention has been focused upon a group of picornaviruses, the Coxsackie viruses, especially Coxsackie B_4. The Coxsackie viruses are a group of small, common, RNA containing viruses which produce upper respiratory infections in man. Gamble et al. (1969) cited a previous study where two cases of Coxsackie B_4 virus infection resulted in necrotic lesions of the pancreas. They also quote unpublished results where the Coxsackie B_4 neutralising antibody titre rose from 1:512 to 1:1024 during the course of pancreatitis. Further studies of antibody titres to Coxsackie viruses B_{1-6} in diabetics of recent onset, diabetics of more than two years' duration, and control subjects revealed higher Coxsackie B_4 virus antibody titres in the diabetics, and furthermore these titres were inversely proportional to the duration of diabetes (Gamble et al., 1969). Similar patterns were found for Coxsackie B_1 and B_4 viruses. Gamble, Taylor, and Cumming (1973) conducted a further survey of neutralising antibodies to Coxsackie B viruses, types 1-5, in 162 patients with insulin-dependent diabetes and 314 control subjects. This revealed a higher proportion of diabetics with neutralising antibody to Coxsackie B_4 virus. In addition, Capner et al. (1975) found a higher incidence of elevated neutralising antibody titres against Coxsackie B_3 and B_4 viruses in a group of patients with acute pancreatitis compared to control subjects.

However, these observations have been challenged. Hadden et al. (1972) studied 58 newly diagnosed diabetic patients in Northern Ireland. Thirty-four of these subsequently required insulin, 15 required OHA, and 9 were controlled by diet alone. No predominance of any one Coxsackie virus was found, and there were no significant

differences in the neutralising antibody titres among diabetics, hospital control subjects, and laboratory control subjects. In addition, Nelson, Pyke, and Gamble (1975) in a study of identical twins, measured viral antibody titres to mumps, cytomegalovirus, rubella, Coxsackie B_{1-5} virus, and Mycoplasma pneumoniae. They found that diabetic co-twins had no greater incidence of antibodies to any of the viruses than the non-diabetic co-twins of discordant pairs.

Seasonal Incidence of Diabetes Mellitus. Further evidence of a viral cause of diabetes mellitus arises from the observation that diabetes is subject to seasonal variation. Gamble and Taylor (1969) suggested that such variations in the incidence of diseases may indicate that specific infectious agents are implicated, by correlation with their prevalence.

Many investigators have reported a seasonal variation in the incidence of diabetes mellitus since Adams in 1926, who showed a peak incidence in September and the lowest incidence in May and June. These results are in agreement with those of Brown (1956) who found the incidence to be lowest in the summer, Danowski (1957) who claimed there were slightly more new cases of diabetes in the winter months, and Christau et al. (1977) who found a reduction in incidence in May, June, and July. Gamble and Taylor (1969) reported pronounced variation in the incidence of insulin-dependent diabetes in patients 0-19 years old, with a broad peak around October and the lowest incidence in June. This seasonal variation was much less noticeable in patients with insulin-independent diabetes. In addition to the seasonal variation, Gamble and Taylor (1969) showed a remarkable similarity in the incidence of insulin-dependent diabetes and Coxsackie B_4 viral

infections in patients between 0-19 years. In a further study, Gamble et al. (1973) found a seasonal variation with peaks in October and January, but in this study the October peak was not mainly composed of patients who had antibody to Coxsackie B_4 virus. This is incompatible with the idea that diabetes is a direct sequel to Coxsackie virus infection since its peak incidence would be expected to occur in September or October. Gamble et al. (1973) suggested that a second or anamnestic response to another virus might be needed, and that this virus would have a similar seasonal incidence. An alternative explanation may be that delayed hypersensitivity reactions may play a part (Gamble and Taylor, 1977). The incidence of diabetes may reflect the incidence of an infection but be delayed by a short period. Thus, it is possible that diabetes is the result of an immunological event triggered in the autumn, the effects of which might become apparent over several months.

Virus-induced Diabetes in Animals. Several experimental studies have shown that animals may develop diabetes when exposed to certain viral infections. Thus, Maugh (1975a) quotes a report from Italy where cows developed pancreatic lesions and hyperglycaemia after foot and mouth disease, a condition which is caused by a picornavirus, the group that contains the Coxsackie viruses.

Another picornavirus, the encephalomyocarditis (EMC) virus has been shown to cause diabetes in mice. From et al. (1968) inoculated mice subcutaneously with the M variant of EMC virus and produced hyperglycaemia associated with insulin deficiency 8, 12, and 24 days after inoculation. Histologic examination of tissues from the infected mice showed pathological changes in the islet cells but not

the acinar cells of the pancreas. Craighead and McLane (1968) also used the M variant of EMC virus and reported hyperglycaemia with pancreatic lesions, and the recovery of large amounts of virus from the pancreas during the acute stage of infection when the islets of Langerhans exhibited focal necrosis and degranulation. In further experiments Craighead and Steinke (1971) injected male CD_1 mice subcutaneously with the M variant of EMC virus and found that virus multiplication is associated with insulin release after which the level drops and hyperglycaemia ensues. They also discovered a sex and strain difference in susceptibility of mice to EMC virus, the incidence of diabetes being significantly lower in female mice and varying with different strains of inbred mice. Boucher and Notkins (1973) also found sex and strain differences, inducing hyperglycaemia with the M variant of EMC virus in only DBA/2N and CD_1 mice from six strains of mice. This was explained on a genetic basis. Craighead and Steinke (1971) suggested that the predisposition to diabetes may possibly consist of an inability to replace defunct β-cells and that damage may cause permanent diabetes, the severity of which reflects the extent of the lesion.

Wellman et al. (1972) inoculated CD_1 mice with the M variant of EMC virus and produced the expected hyperglycaemia but also observed, after two days, disruption of the islets with β-cell necrosis, degranulation, and mononuclear cell infiltration. They suggested that the rapid effect noted with the virus is likely to be mediated by immune factors. Also using the M variant of EMC virus Kanich, Craighead, and Kessler (1973) produced a diabetic-like syndrome in mice and in addition found lesions of the renal glomeruli.

Coxsackie viruses have been reported to replicate in the pancreas and induce hyperglycaemia in mice. This is of interest when

considered in conjunction with the work of Gamble et al. (1969, 1973) who found high neutralising antibody titres to Coxsackie viruses in diabetics of recent onset. Burch et al. (1971) inoculated HaM/ICR mice intraperitoneally with 0.2 cc. Coxsackie B_4 virus, TC ID_{50} 10^3 and found pathologic changes in both endocrine and exocrine tissues of the pancreas. These changes ranged from focal and mild degeneration to widespread necrosis and inflammation. They suggested an analogous situation in man where there is a high frequency of viral infections in childhood and early adulthood. If islet cell damage is extensive and irreversible, diabetes mellitus would be expected in the early part of life, whilst milder damage may not lead to diabetes or only later in life. Further experiments by Burch, Tsui, and Harb (1972) demonstrated EMC and Coxsackie B_1 viral crystals in the β-cells and mild to severe pancreatic islet cell damage in HaM/ICR mice, and they claim that these results provide strong evidence for a viral aetiology in diabetes mellitus since direct viral invasion of the β-cells followed by cell degeneration and necrosis, and later atrophic changes, can reduce the mass of functional β-cells. This work was supported by Coleman et al. (1973) who inoculated male CD_1 mice intraperitoneally with Coxsackie B_4 virus. By day 12 some mice were obviously diabetic with a blood sugar level greater than 160 mg/ 100 ml., although this was most noticeable between day 17-21 after inoculation. Microscopy revealed β-cell degranulation and mononuclear cell infiltation occurred around many damaged islets while acinar tissue degeneration was very limited. Further work by Coleman et al. (1974) using CD_1 mice inoculated with unadapted Coxsackie B_4 virus led them to believe that Coxsackie virus-induced diabetes in mice might serve as an appropriate model for diabetes in

man, especially as Coxsackie B_4 virus is a common human pathogen. Coleman et al. (1974) induced diabetes in 20-30% of CD_1 mice 15-20 days after infection and found that, as before, β-cell degeneration was accompanied by mononuclear infiltation. It was suggested that the delay between inoculation and the onset of diabetic symptoms might indicate a mechanism involving some general response to viral infection rather than a direct attack on the islet cells by the virus.

From the foregoing review it is apparent that considerable differences of opinion exist as to which virus or viruses may play a part in the aetiology of diabetes mellitus. However, it is generally accepted that viruses may play some part in initiating some forms of diabetes.

Of the viruses proposed, Coxsackie B_4 seems to be the most likely candidate because it has been shown to produce hyperglycaemia in mice; it is a common pathogen of man; and elevated antibody titres to type B_4 have been found in newly diagnosed diabetics. In view of this, mice were inoculated with Coxsackie B_4 virus to produce diabetes and to investigate the mechanism whereby diabetes was caused.

Materials and Methods

A series of experiments were performed using 8-9 week old, male CD_1 mice. The experimental protocol in each case was as follows:

Experiment I. Forty mice were inoculated with 0-2 ml. Coxsackie B_4 virus, TC ID_{50} 10^{-4-5} intraperitoneally (i.p.). Forty control mice were given an equivalent volume of saline i.p. Seven mice from each group were tested for glucose intolerance 4, 8, 11, 18, and 28 days after inoculation.

Experiment II. This procedure was identical to that of the previous experiment, but the mice were tested for glucose intolerance 3, 7, 14, and 37 days after inoculation with Coxsackie B_4 virus.

Experiment III. Ten CD_1 mice were given an intravenous (i.v.) injection of streptozotocin (Upjohn, Ltd.) 60 mg/kg. in 0.2 ml. citrate buffer. Control mice were given an equivalent volume of buffer. Blood sugar levels were measured 2, 4, 8, 14, and 30 days after injection.

Experiment IV. Five CD_1 mice were given streptozotocin, 100 mg/kg. i.v. Control mice were given an equivalent volume of buffer. The animals' blood sugar levels were measured 3 hours and 48 hours after injection.

Experiment V. Four groups of 15 mice were treated as follows:

(a) Controls. 0.2 ml. saline i.p.

(b) Cyclophosphamide 50 mg/kg. in 0.2 ml. saline i.p. on days 0, 2, and 4.

(c) 0.2 ml. Coxsackie B_4 virus, TC ID_{50} 10^{-4-5} i.p. on day 7.

(d) Cyclophosphamide 50 mg/kg. i.p. on days 0, 2, and 4, followed by 0.2 m. Coxsackie B_4 virus i.p. on day 7.

Animals from each group were tested for glucose intolerance on day 14. The remaining virus treated animals were given a further injection of 0.2 ml. Coxsackie B_4 virus i.p. on day 14 and tested for glucose intolerance on day 28.

The reagents required for glucose estimates are:

Protein diluent, 10 g $Na_2WO_4 2H_2O$, 10 g Na_2HPO_4 and 9gNaCl dissolved in 800 mls. distilled water. The pH was adjusted to 3.0 with concentrated HCl; 1 gm. of phenol was added and the volume made up to 1 litre with distilled water.

Colour reagent. 42 gm. Na_2HPO_4 dissolved into 215 mls. of warm distilled water and 1.4 g. aminophenazone added.

Fermcozyme (952 DM).

Mice were fasted overnight, anaethetised with ether and 0.02 mls. blood were taken from the tail into a blood pipette. Blood was transferred into 2.5 mls. protein diluent and mixed thoroughly. The mixture was then centrifuged at 400 g for 5 mins. and 2 ml. of the supernatant removed and added to 0.5 ml. of a colour reagent and fermcozyme mixture which was prepared immediately before use, 2 ml. fermcozyme being used for every 15 ml. colour reagent. The solution was left at room temperature for 15 minutes and read in a spectrophotometer at 500 nm. against a blank containing no glucose. Standard solutions of glucose were prepared containing 50, 100, 200, 300, and 400 mg/100 ml. From the readings obtained a calibration curve was drawn and the glucose in the unknown blood samples determined.

Where glucose tolerance tests were performed, the animals were given an i.p. injection of 50 mg. glucose and blood sugar levels determined at time 0 and at 30, 90, and 120 minutes after injection. In addition to glucose tolerance tests, routine histology of the pancreas was performed in experiments I, II, and V.

Results

In experiment I mice were injected i.p. with Coxsackie B_4 virus while control mice were injected with an equivalent volume of saline. The results of glucose tolerance tests performed at various times after the inoculation of virus are shown in Figure 2.1. The shaded areas on the graphs represent the control values, mean $\pm$ standard deviation.

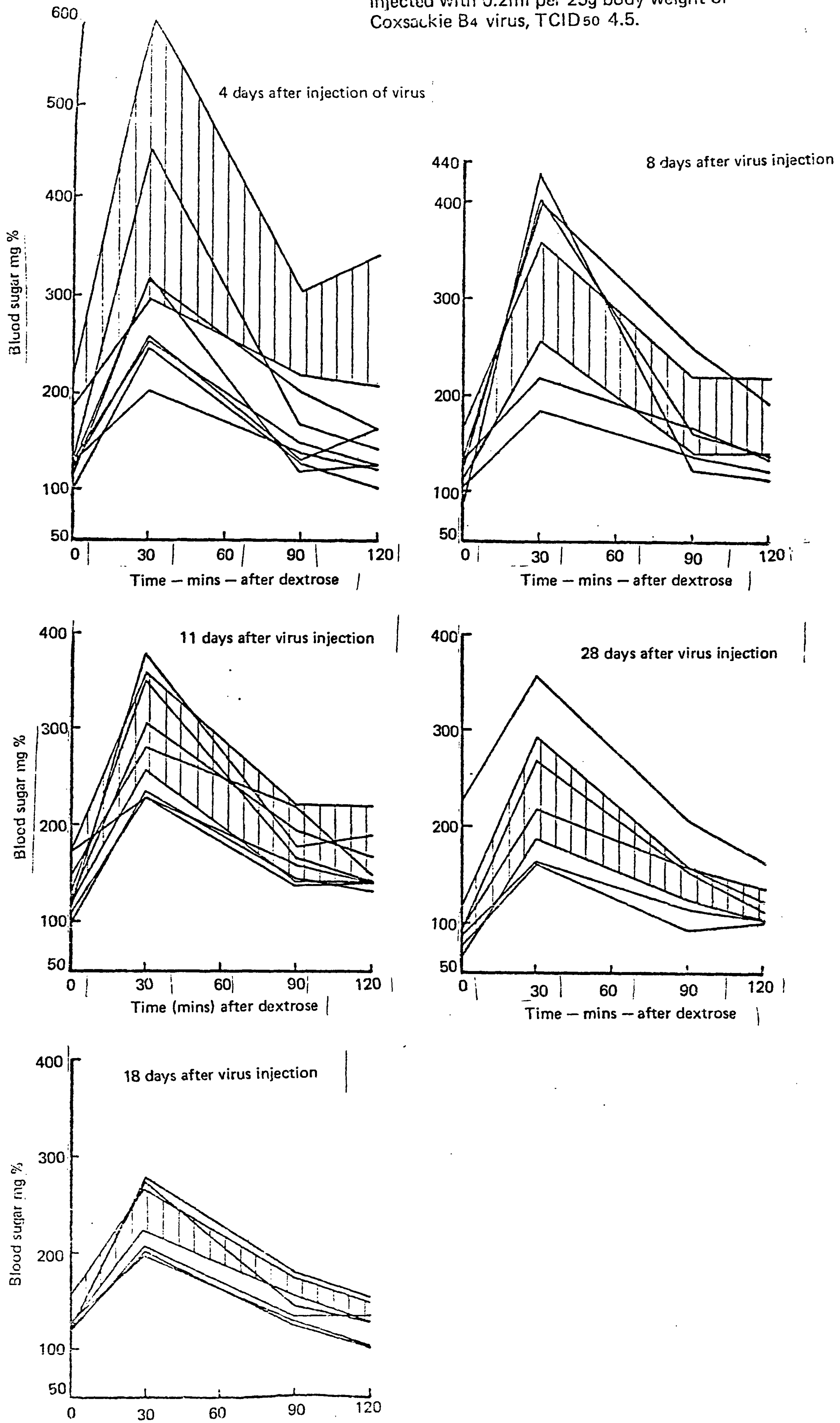


Fig. 2.1 Glucose tolerance tests on control mice and mice injected with 0.2ml per 25g body weight of Coxsackie B4 virus, TCID50 4.5.

The results from day 4 showed considerable variation, with the blood sugar levels of the control mice being approximately double those of the virus treated mice at all stages of the test.

The blood sugar levels of mice tested on day 8 again showed variation. Three virus-treated animals revealed high blood sugar levels 30 minutes after the administration of dextrose. However, two mice from this group had comparatively low blood sugar levels.

The results obtained on days 11, 18, and 28 show greater reproducibility but do not give any indication of glucose intolerance in the virus-treated animals compared with the controls. When the mean blood sugar levels of the test and control mice were compared, there was no difference.

In the second experiment mice were treated in the same way to that described for experiment I. However, the mice were tested for glucose intolerance 3, 7, 14, and 37 days after inoculation with virus or injection of saline. Some signs of glucose intolerance were observed in two virus-treated animals on day 7 and one on day 14. However, there was no difference in the mean blood sugar levels of either group at the various stages of the glucose tolerance test (Figure 2.2).

Experiment III. Streptozotocin was administered to mice in order to induce diabetes through a direct pancreatic effect and thereby have a control diabetic animal against which virus-induced diabetic animals could be compared. Blood sugar levels of streptozotocin-treated and control mice were measured 2, 4, 8, 14, and 30 days after injection. The results are shown in Table 2.1. The blood sugar levels varied with time after injection, but the only appreciable difference between the test and control levels was on day 8. Thereafter there was very little difference.

Fig 2.2 Glucose tolerance tests on control mice and mice injected with 0.2ml per 25g body weight of Coxsackie B4 virus, $TCID_{50}$ 4.5.

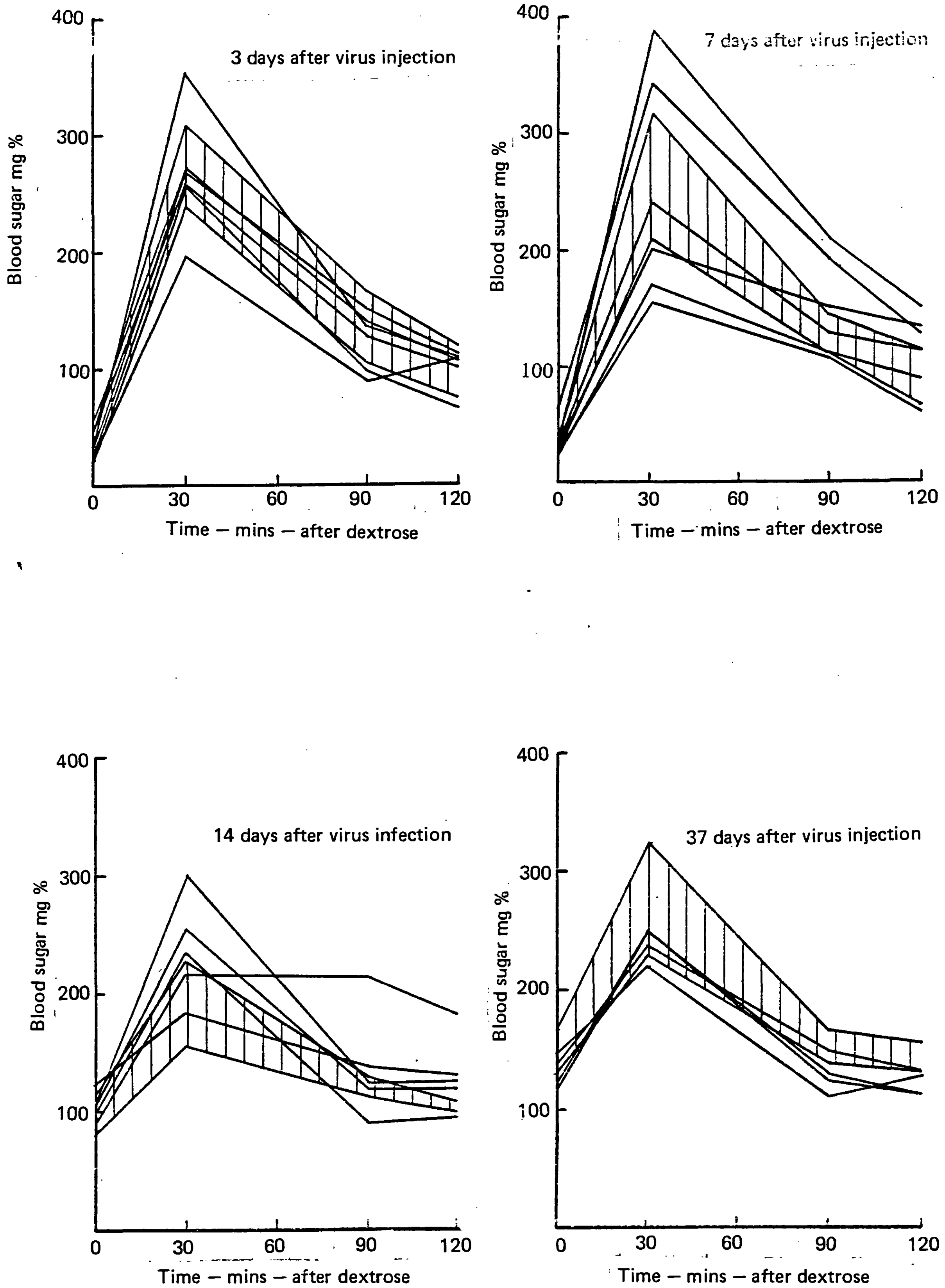


Table 2.1. Mean blood sugar levels of mice treated with 60 mg/kg streptozotocin i.v. at various intervals after injection compared to control mice

Time After Injection (Days)	Mean Blood Sugar Level mg/100 ml.	
	Control	Test
2	173 (not fasted)	143 (not fasted)
4	77.4	73.8
8	78	129
14	80.6	99.3
30	128	141

As no permanent elevation of blood sugar level resulted from 60 mg/kg streptozotocin, a further experiment was performed where mice were injected i.v. with 100 mg/kg streptozotocin and the blood sugar levels measured 3 hours and 48 hours later. Four of five mice injected with streptozotocin revealed markedly elevated blood sugar levels two days later, while the control values remained the same (Table 2.2).

Table 2.2. Blood sugar levels of 5 mice treated with streptozotocin (100 mg/kg i.v.) and 5 saline-treated controls, measured 3 and 48 hours after injection

Blood Sugar Level mg/100 ml.			
3 hrs.		48 hrs.	
Control	Test	Control	Test
70	97	79	390
102	125	89	370
102	98	77	94
76	88	82	325
82	72	118	201

The blood sugar levels measured 3 hours after injection of streptozotocin revealed no difference from control values.

Experiment V. All animals were tested for glucose intolerance 14 days after the beginning of treatment. No abnormalities in blood sugar levels were noted in any of the three groups treated with virus and cyclosphosmide, virus only, and cyclophosphamide only when compared to control values. (Figure 2.3.1). As there was no evidence of glucose intolerance in the virus treated animals, the experiment was modified. Those animals receiving virus plus cyclophosphamide or virus alone were given another i.p. injection of Coxsackie B_4 virus on day 14 of the treatment and tested for glucose intolerance 28 days after the beginning of the treatment. The results are shown in Figure 2.3.2. No glucose intolerance was observed compared to the controls in either of the virus-treated mice.

The histological examinations of mouse pancreas performed on animals in experiments I, II, and V revealed no changes in the structure of the acinar or islet tissue.

Discussion

In the experiments described, Coxsackie B_4 virus failed to induce diabetes in CD_1 mice. The absence of any abnormal glucose tolerance curves was apparent. Coleman et al. (1974) reported diabetes in 20-30% of CD_1 mice 15-20 days after inoculation with Coxsackie B_4 virus, in association with β-cell degranulation and mononuclear infiltration. In the experiments carried out here using the same strain of mice, treated at the same age with Coxsackie B_4 virus, no diabetes was observed and histology showed the islet cells to be normal with no mononuclear infiltration. These results are in agreement with those reported by Ross, Hayashi, and Notkins (1974) and Craighead (1975) who were unable to reproduce Coleman's results. Ross

Fig. 2.3.1 Glucose tolerance tests on mice injected with 0.2ml per 25g body weight Coxsackie B4 virus, TCID50, estimated 14 days after cyclophosphamide injection and 7 days after viral injection with:
(1) Virus only
(2) Virus and cyclophosphamide
(3) Cyclophosphamide only.

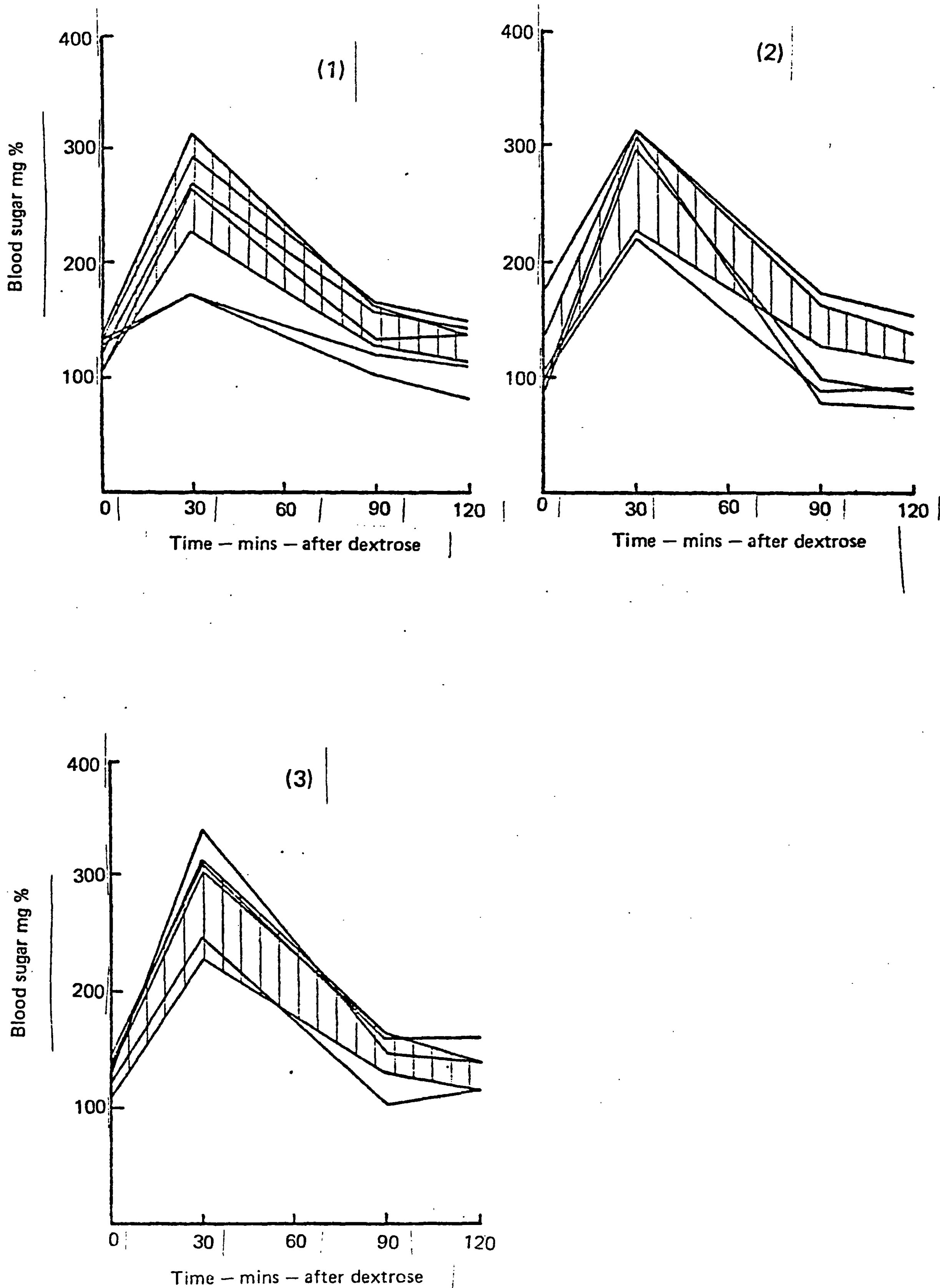
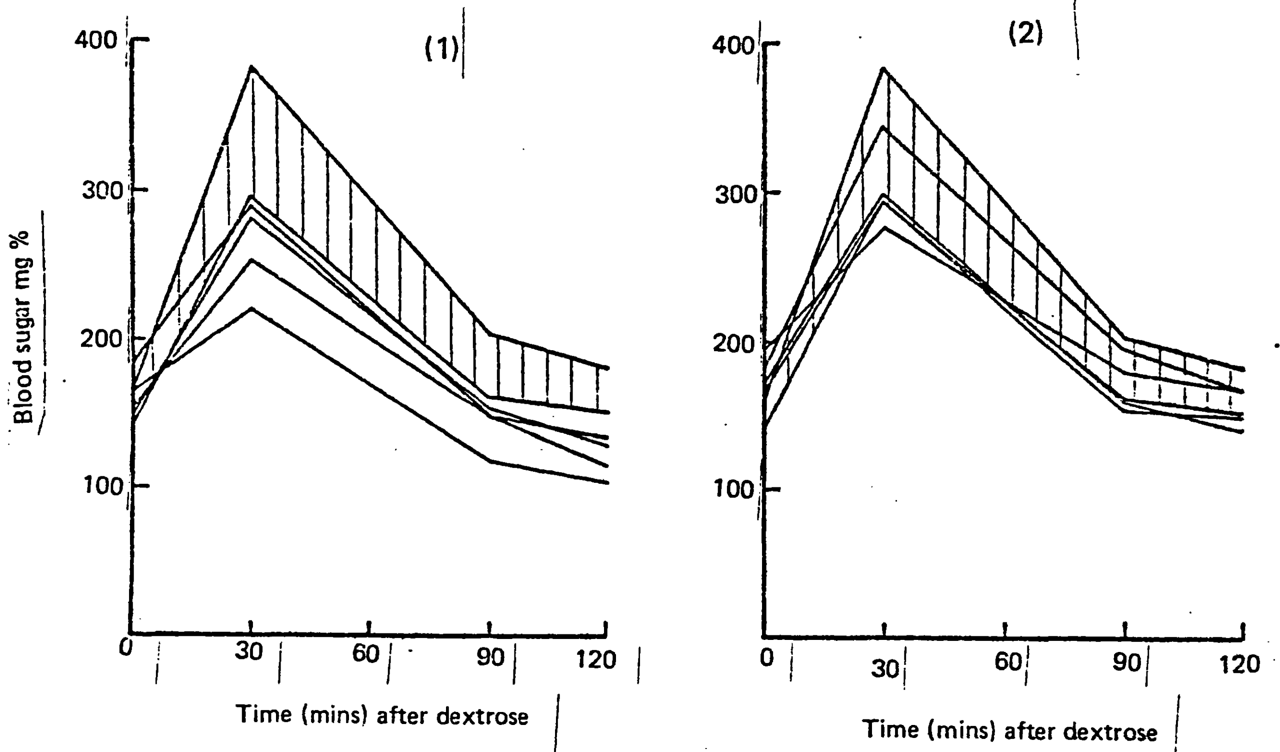


Fig. 2.3.2 Glucose tolerance tests on control mice and mice injected with 0.2ml per 25g body weight Coxsackie B4 virus, $TCID_{50}$, estimated 28 days after cyclophosphamide injection and 14 days after a further inoculation of Coxsackie virus with:

(1) Double inoculation of virus
(2) Double inoculation of virus and pre-treatment with cyclophosphamide.



et al. (1974) inoculated various strains of mice, including DBA/2 and CD_1 strains, with Coxsackie B viruses types 1-5, and the M variant of encephalomyocarditis virus. They found that the Coxsackie B viruses produced pancreatic acinar damage with little or no effect on the islets and no resulting hyperglycaemia. Passage of Coxsackie B_4 virus via pancreatic homogenates, in order to enhance any diabetogenic potential proved ineffective in the induction of hyperglycaemia. Mice infected with EMC virus, however, became hyperglycaemic with damage to the β-cells but not to the acinar cells of the pancreas. Maugh (1975 a) reported that Notkins had examined fifteen strains of Coxsackie viruses and found no β-cell damage or abnormal glucose tolerance even when using the same strain of virus and the same type of mice as Coleman et al. (1973).

In the present study no evidence of diabetes was found in response to Coxsackie B_4 virus after cyclophosphamide pretreatment. If the virus were acting directly on the islet cells, then this pretreatment would be expected to enhance the action and result in more severe hyperglycaemia. Alternatively, if the virus triggers an immune response which is effective in damaging the islet cells, then pretreatment with cyclophosphamide would be expected to result in less damage to the islets. However, from these experiments it is not possible to postulate either mechanism of action.

Double inoculation of mice with Coxsackie B_4 virus also produced no diabetes which further supports the claim that Coxsackie B_4 virus does not normally cause hyperglycaemia by pancreatic islet cell damage in CD_1 mice (Notkins, 1977). Whether the virus is acting directly or triggering an immune response, double inoculation within 14 days would be expected in the first instance to have an additive effect and in the latter case, a magnified effect corresponding to a secondary immune response.

There are several possible reasons for not being able to reproduce the work of Coleman et al. (1973, 1974). Maugh (1975a) suggests that diet may play a part as CD_1 have been known to spontaneously develop diabetes mellitus when fed on a high fat diet. It is also possible that the virus became attenuated, although this is unlikely in view of the fact that Notkins used the same strain as Coleman et al. (1973). The other possibility is genetic variation in the mice used as CD_1 mice are not an inbred strain. Notkins (1977) suggested that conflicting reports might be due to the fact that, in general, mouse β-cells are resistant to infection by Coxsackie B viruses and that if viral damage occurs, it is usually insufficient to cause persistent hyperglycaemia.

Evidence from studies in animals (Coleman et al. 1973, 1974) and in man (Gamble et al., 1969) suggested that Coxsackie B_4 virus might be a causative agent in diabetes mellitus and that CD_1 mice infected with this virus might provide a good animal model. However, the experiments described here and those reported by Ross et al. (1974) and Craighead (1975) show that CD_1 mice infected with Coxsackie B_4 virus cannot be used as a reliable animal model. In fact, considerable evidence has been published against the involvement of Coxsackie B_4 virus in diabetes mellitus. Since Gamble et al. (1969) work showing high neutralising antibody titres to Coxsackie B_4 virus, many workers have found no connection between diabetes and this virus. Wales and Hambling (1973) followed-up 24 patients who were diagnosed for Coxsackie B_2 or B_4 virus infection in the period 1968-1972 and found that no patients or members of their families had developed diabetes. They suggested that any connection of Coxsackie B_4 virus as an aetiological agent must be in the nature of an abnormal

or delayed response to such infection. Hierholzer and Farris (1974) reported that of 94 children who were infected with Coxsackie B_3 and B_4 virus in 1968, none showed any signs of diabetes four years later. They concluded that their results showed no association between juvenile-onset diabetes and Coxsackie viruses. Nevertheless, it is possible that diabetes may become apparent in genetically predisposed individuals after a multitude of random infectious, chemical, or immunologic insults to the pancreas. In a similar study, Dippe et al. (1975) found no evidence of an increase in diabetes mellitus during a five-year period after a Coxsackie B_4 virus epidemic in the Pribiloff islands, even though diabetes was as common in the islanders as in the Caucasian population. They found no cases of diabetes in anyone under 20 years of age even though 77% of this group had antibodies to Coxsackie B_4 virus. In addition, there is a lack of evidence that newly diagnosed diabetics transmit to others an agent that induces the disease within a short period (Mann et al.,1978).

Notkins (1977) reviewed the subject of a viral aetiology for diabetes mellitus and suggested that since the incidence of both diabetes and acute viral infections is high in children, any other relationship might be coincidental.

The results obtained from this study and those obtained by other workers militate against the involvement of Coxsackie B_4 virus as a causative agent of diabetes mellitus in man. The discrepancies from animal studies support this view. However, it is possible that Coxsackie B_4 virus, in susceptible individuals, may be one of many environmental factors that initiate diabetes mellitus.

CHAPTER III.

Leucocyte Migration Inhibition Test.

Introduction

The leucocyte migration inhibition test has been used for a number of years as an in vitro correlate of delayed or cell-mediated hypersensitivity (Bendixen and Soborg, 1969; Dumonde et al., 1969; Brostoff, 1970; Roitt, 1971). According to Brostoff (1970) thymus-dependent lymphocytes, on contact with antigen, transform to blast cells and release soluble factors, including migration inhibition factors. Lomnitzer, Rabson, and Koornhof (1975) suggested that the leucocyte migration test is dependent upon the production of a lymphokine which acts specifically on polymorphonuclear leucocytes. This factor can be demonstrated using a capillary tube migration technique first proposed by Bendixen and Soborg (1969).

Leucocyte migration inhibition has been shown in a number of autoimmune diseases including pernicious anaemia (Brostoff, 1970; Goldstone et al., 1973), primary biliary cirrhosis (Brostoff, 1970), ulcerative colitis (Bendixen and Soborg, 1969), Hashimoto thyroiditis (Brostoff, 1970; Calder et al., 1972), and in juvenile-onset diabetes (Nerup et al., 1971, 1973; Richens et al., 1973, 1974); MacCuish et al., 1974a).

In addition to producing inhibition by the use of specific antigens in this test, e.g., thyroglobulin in Hashimoto thyroiditis

and intrinsic factor in pernicious anaemia, many workers have reported migration inhibition using liver mitochondrial extracts (Brostoff, 1970; Goldstone et al., 1973; Calder et al., 1972; Richens et al., 1973, 1974). Leucocyte migration inhibition is considered to be a generalised marker of autoimmune disease (Brostoff, 1970; Calder et al., 1972; MacCuish et al., 1974) since it has been variously reported to be organ non-specific (Calder et al., 1972) and species non-specific but organ specific (Richens et al., 1973).

The leucocyte migration inhibition test has been used in studying juvenile-onset diabetes. Hence, Nerup et al. (1971) found typical delayed hypersensitivity in four of seven diabetics where six had low migration indices. They used, as antigen, subcellular fractions of porcine islet tissue obtained by ligation of the pancreatic duct, a procedure which results in atrophy of the acinar tissue but leaves the islet cells intact. Nerup et al. (1971) results suggested an organ specific, species non-specific, antipancreatic hypersensitivity. In experiments using rats in which antipancreatic delayed hypersensitivity was induced using foetal calf pancreas in complete Freund's adjuvant, Nerup et al. (1973a) found that spleen cell migration was inhibited in the majority of treated animals and that islet cell changes were detectable when migration inhibition factor was maximal. In further experiments, Nerup et al. (1973) performed migration inhibition tests on ninety-seven diabetics and forty-five control subjects using a foetal calf pancreatic extract as antigen, and found a significant reduction in the mean migration index in the diabetic patient group. Within this group of diabetic subjects, it was found that the newly diagnosed patients showed significantly greater inhibition than the other groups.

MacCuish et al. (1974a) also carried out leucocyte migration tests on one hundred and one diabetic patients and fifty control subjects using human pancreatic extract as antigen. They found inhibition in seventeen of thirty-one young diabetics aged under 42 years, compared to four of twenty-seven age-matched control subjects.

When MacCuish et al. (1974a) used rat liver mitochondria as antigen in the leucocyte migration test, they found no difference in inhibition among young diabetics, control subjects, and older diabetics. Richens et al. (1973), however, using rat liver mitochondria and human liver mitochondria as antigens, found marked differences in inhibition of leucocytes from insulin-dependent diabetics, insulin-independent diabetics, and control subjects. In further experiments using inner and outer mitochondrial membranes, Richens et al. (1974) found inner membranes inhibited migration more than outer membranes in a group of eighteen insulin-dependent diabetics. These results suggest that diabetes may be related to other conditions considered to be of autoimmune origin, and Richens et al. (1974) proposed that hypersensitivity to mitochondria might reflect altered T-cell reactivity.

The following experiments were carried out on blood from diabetic patients, when available, with a view to verifying previous results and correlating this immune reaction with other immunological and genetical aspects of diabetes such as phytohaemagglutinin transformation, islet cell antibodies, and histocompatibility antigens.

Materials and Methods

The method used here was based upon that of Bendixen and Soborg (1969). This test is based upon the supposition that lymphocytes

from sensitised donors, on contact with specific antigen, produce soluble factors which affect leucocyte migration, whilst in the absence of antigen or in the presence of lymphocytes from a non-sensitised donor, migration remains unaffected.

For the test 20 ml. of venous blood were taken and heparinised (20 U/ml.). The blood was allowed to sediment at 37° C for 30 minutes when the leucocyte rich plasma was removed and centrifuged at 400 g for 5 minutes. The cell pellet was washed three times in Eagles medium M.E.M. (Wellcome Laboratories, Beckenham) supplemented with 10% foetal calf serum, resuspended and the cells counted. The concentration was adjusted to 5 x 10^6 cells/ml. and then 25 µl. capillary tubes were filled with this suspension. These were sealed at one end and centifuged at 400 g for 5 minutes. The tubes were then cut just below the cell/medium interface and the pellet positioned in a Sterilin leucocyte migration chamber with silicone grease. The chambers were then filled with culture medium (Eagles M.E.M. and foetal calf serum) supplemented with penicillin and streptomycin (100 U/ml.) and one of several antigens at a concentration of 100 µg/ml. The antigens used were Coxsackie B_4 virus, rat liver mitochondria, and pancreatic cell fractions. Control chambers were filled medium alone.

Cultures were performed in quadruplicate for each given antigen and the chambers sealed with cover slips using silicone grease. They were incubated for 24 hours at 37° C on a flat surface. The pattern of migration was then projected onto paper, the outline drawn, and the area measured by planimetry. The effect of antigen on migration was then expressed as a migration index calculated as:

$$\frac{\text{mean migration with antigen}}{\text{mean migration without antigen}}.$$

A figure of less than 0.80 was taken to indicate significant inhibition and a figure greater than 1.20 significant stimulation.

Preparation of Antigens and Protein Estimation. Coxsackie B_4 virus was obtained from the Public Health Laboratory, Royal United Hospital, Bath.

Pancreatic tissue was obtained from a cadaver at the time of renal transplant, usually within half an hour of death, and rat liver from a freshly killed rat. The tissue was diced into small pieces and diluted ten times with 0.25 M ice cold sucrose solution. The mixture was then homogenised with a Potter-Elvehjem homogeniser and filtered through muslin to remove the fibrous tissue. All procedures were carried out at 0° C. In the preparation of the pancreatic homogenate two enzyme inhibitors were added to the sucrose according to the method of Lendrum and Walker (1975). The inhibitors were epsilon aminocaproic acid (0.6% w/v) and trasylol (100 U/ml.). The tissue homogenates were centrifuged differentially to obtain various subcellular fractions according to methods quoted by Murray, Suss, and Pitot (1967) and Lendrum and Walker (1975):

Preparation	G Force	Time (mins.)	Resultant Fraction	Supernatant
Homogenate	600 g	10	Nuclei + debris	A
Supernatant A	1000 g	10	Nuclei	B
Supernatant B	6000 g	20	Mitochondria	C
Supernatant C	105000 g	60	Microsomes	

The pellets were resuspended in the original volume of 0.25 M sucrose, aliquoted into 0.5 cc. volumes and frozen at -20° C until required. The final suspension was used once and any remaining in the tube discarded.

Protein estimation was determined by the method of Lowry et al. (1951). The following reagents were used:

A. 5% $CuSO_4$ } Combined Reagent
B. 10% Na/K tartrate } Combined Reagent
C. 2% Na_2CO_3 in 0 IN NaOH } Combined Reagent
D. Folin-Ciocalteaux Reagent diluted 1:1 with water
E. Standard protein solution - bovine serum albumin 60 μg/ml.

The combined reagent was prepared by adding 1 ml. of B to 1 ml. of A and diluting to 10 ml. with distilled water, and then taking 1 ml. of the resulting solution and diluting to 50 ml. with solution C.

For the calibration curve the tubes were prepared as follows:

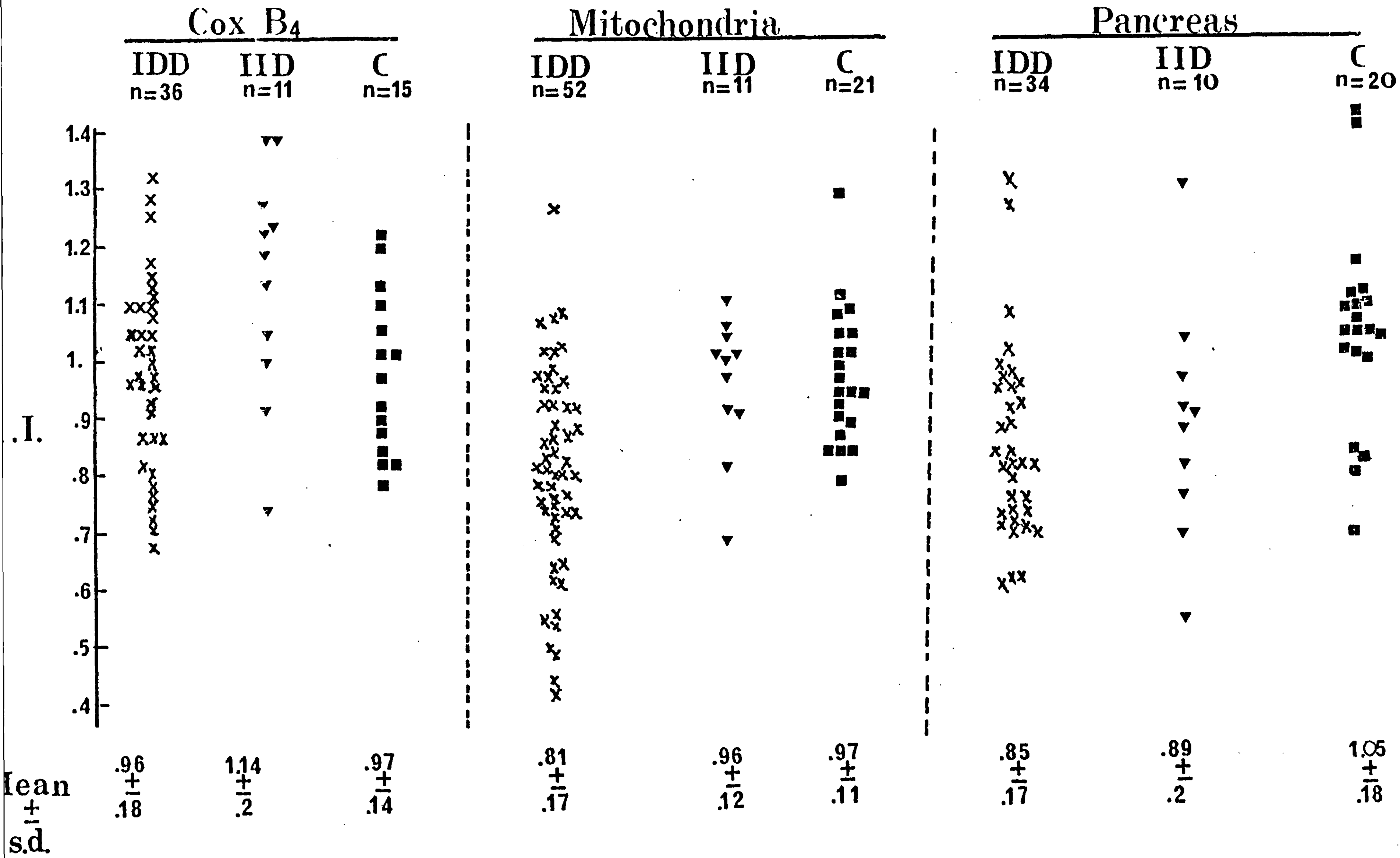
Tube	H_2O (ml.)	Protein (60 μg/ml.)	Combined Reagent	F+C Reagent
1	1.00	0.00 ml.	5 ml.	0.5 ml.
2	0.75	0.25 ml.	5 ml.	0.5 ml.
3	0.50	0.50 ml.	5 ml.	0.5 ml.
4	0.25	0.75 ml.	5 ml.	0.5 ml.
5	0.00	1.00 ml.	5 ml.	0.5 ml.

The test solution was usually diluted 1:50 and 1:100 in order to obtain a reading within the range of the calibration curve. One ml. of the diluted test solutions was added to further tubes instead of 1 ml. of standard protein solution. All samples were duplicated and read after 30 minutes in a spectrophotometer at 700 mμ against a water blank.

Results

Figure 3.1 shows the results of migration inhibition tests against antigens Coxsackie B_4 virus, rat liver mitochondria, and human pancreatic fractions in insulin-dependent diabetics, insulin-independent diabetics and control subjects. The mean migration

Fig. 3.1. Migration indices from insulin-dependent diabetic (IDD), insulin-independent diabetic (IID), and control leucocytes when migrated against various antigenic preparations. 0.80 to 1.20 represents the normal range of migration indices.



indices all lie within the 0.80-1.20 range in each of the groups tested, but migrations against Coxsackie B_4 virus, mitochondria, and pancreatic fractions in the insulin-dependent group show migration values outside this range, 19%, 42%, and 44% of those subjects tested, respectively. For the insulin-independent group these values are 9%, 9%, and 30%, respectively, and for the control group 7%, 5%, and 5%, respectively.

Stimulation was shown in 8.5%, 2%, and 6% of insulin-dependent diabetics against Coxsackie B_4 virus, mitochondria, and pancreatic fractions, respectively, compared with 45%, 0%, and 10% in insulin-independent diabetics, and 7%, 5%, and 10% in the control group.

Table 3.1 shows the mean migration indices and standard deviations from insulin-dependent diabetics who were tested at different times after diagnosis. Inhibition of migration as indicated by the mean migration index was found against mitochondria and pancreas in diabetics tested one year or later after diagnosis. In the group tested 0-6 months after diagnosis 21% showed inhibition of migration against Coxsackie B_4 virus, 33% against mitochondria, and 35% against pancreatic fractions. In the patients tested one year or more after diagnosis 63% showed inhibition against mitochondria and pancreatic fractions, whereas none showed inhibition against Coxsackie B_4 virus although the number tested here was very small.

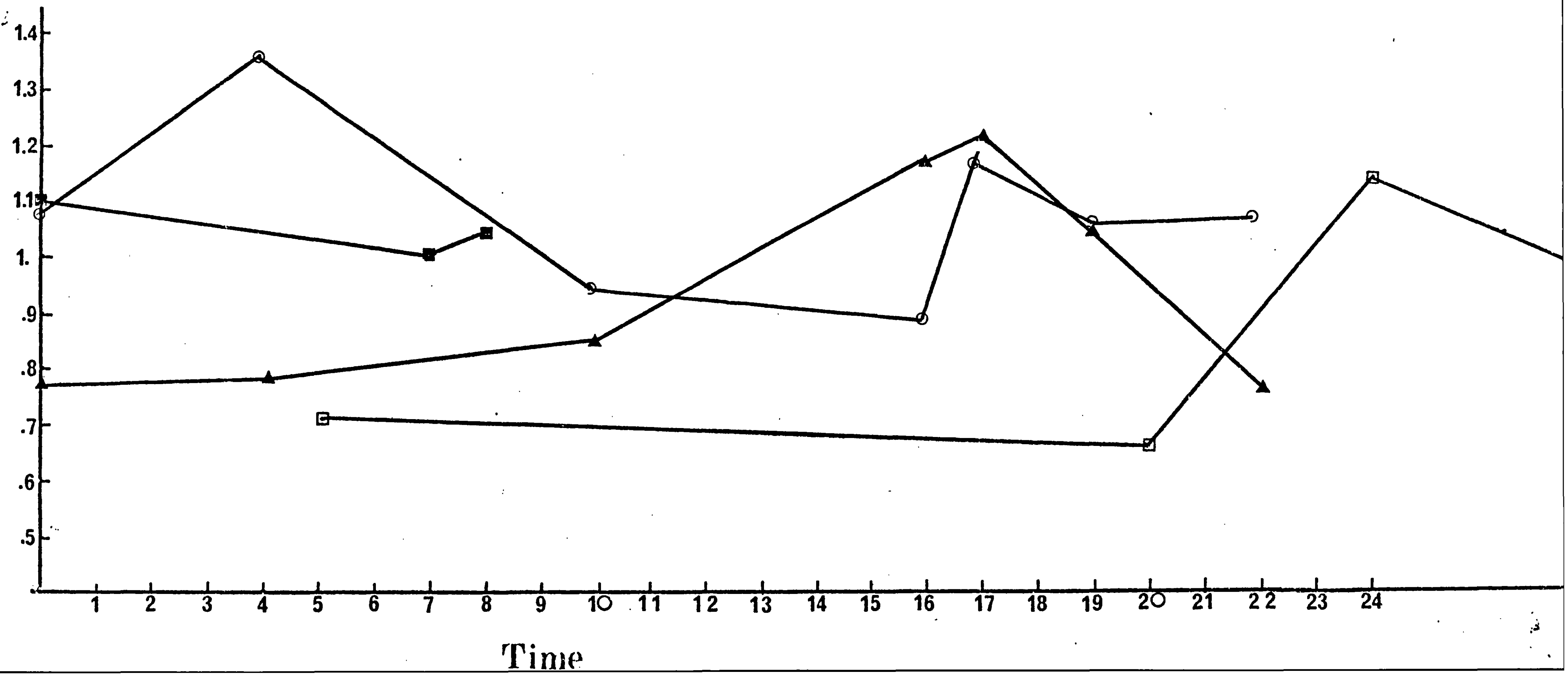
Figure 3.2 shows the variation in migration indices when tested at various times after diagnosis. Over relatively short periods the migration index varies considerably, in one case by 0.48 over a period of 4 months, the patient showing inhibition with an index of 0.67, which rises to 1.15 and can almost be called stimulation. In the case of the control only three sequential results were

Table 3.1. Mean migration indices of insulin-dependent diabetics.

Time Tested After Diagnosis	Antigen			
	Cox B_4	Mitochondria	Pancreas	
0 - 6 months	0.97 ± 0.18 (33)	0.85 ± 0.17 (33)	0.88 ± 0.18 (26)	1
1 - 5 years	0.88 ± 0.04 (2)	0.71 ± 0.17 (5)	0.72 ± 0.21 (2)	2
> 5 years	0.81 (1)	0.77 ± 0.17 (14)	0.77 ± 0.05 (6)	3
All diabetics	0.96 ± 0.18 (36)	0.81 ± 0.17 (52)	0.85 ± 0.17 (34)	

Figures in brackets are the number of subjects tested.

Fig. 3.2. Leucocyte migration indices obtained from three insulin-dependent diabetics at various times after diagnosis, and one control subject tested three times, against a given antigenic preparation.



obtained from which very little can be deduced, although the values were virtually identical.

Discussion

Altered in vitro reactivity of leucocytes probably reflects cellular hypersensitivity against components of rat liver mitochondria and pancreatic fractions in the case of insulin-dependent diabetics, and cellular hypersensitivity against a pancreatic component in the case of insulin-independent diabetics. It is interesting that this hypersensitivity is more pronounced in the insulin-dependent diabetics and may possibly indicate a greater autoimmune component involved in the aetiology of this form of diabetes.

The results support the theory that diabetes has an autoimmune component in its aetiology by linking this disease with other conditions thought to have an autoimmune basis. Cellular hypersensitivity to liver mitochondria has also been shown in several other conditions such as Addison's disease (Nerup and Bendixen, 1969), and primary biliary cirrhosis (Brostoff, 1970) where inhibition is considered to be organ-specific. Calder et al. (1972) found inhibition by mitochondria in Hashimoto thyroiditis to be organ non-specific, and Goldstone et al. (1973) results of inhibition by mitochondria in pernicious anaemia suggest that this is also non-specific. Richens et al. (1973) in migration inhibition studies in diabetics found that the reaction was apparently specific to liver mitochondria but species non-specific. It is generally accepted that inhibition by mitochondria in these conditions is non-specific and a general manifestation of autoimmune disease (Brostoff, 1970; Calder et al., 1972; Richens et al., 1973; MacCuish et al., 1974a). The results presented here support those found by Richens et al.

(1973, 1974) but not those of MacCuish et al. (1974) who found no inhibition by mitochondria in diabetics. Irvine et al. (1977) were also unable to confirm Richens et al. (1973, 1974) findings but suggested the difference in results may be due to the preparation of the liver mitochondrial fraction.

Stimulation of migration has been reported to be associated with weak sensitisation to an antigen whilst inhibition is considered to be the result of strong sensitisation (Soborg, 1967).

Stimulation of migration occurred in both the diabetic and control groups to a small degree. The only exception was the insulin-independent group against Coxsackie B_4 virus where stimulation was pronounced. Forty-five percent of this group showed stimulation, although the number tested was only eleven. However, if this stimulation is considered as weak sensitisation, then combining the results with those showing inhibition gives a total of 55% sensitised to Coxsackie B_4 virus compared to 28% of the insulin-dependent diabetics and 13% of the control subjects. However, there was only one subject whose leucocytes showed inhibition when cultured with Coxsackie B_4 virus. This mild sensitisation found in the insulin-independent group might possibly reflect a mild infection with little resulting damage to the pancreas compared to the insulin-dependent diabetics where a more severe infection leads to greater damage and consequent dependence on insulin.

The results obtained using pancreatic fractions as antigen showed sensitisation in both the insulin-dependent and insulin-independent group, although this was more pronounced in the former group. The results are in agreement with those of Nerup et al. (1974), MacCuish et al. (1974), and Irvine et al. (1977) who found

antipancreatic cellular hypersensitivity in juvenile-onset diabetics. However, when the insulin-dependent diabetic group was subdivided into untreated juvenile diabetics or those treated for less than a year and those treated for a year or more, Nerup et al. (1974) found a significant difference in migration inhibition. When the group studied here was subdivided, the mean migration indices were below the 0.80 mark in diabetics tested a year or more after diagnosis but above 0.80 in the group tested within six months of diagnosis. Nerup et al. (1974) state that their results agree with previous findings and the lymphocytic infiltration found in the pancreases of recent onset juvenile diabetics.

CHAPTER IV.

Phytohaemagglutinin (PHA)-induced Transformation of Lymphocytes

Introduction

Roitt et al. (1969) state that on contact with antigen, T-lymphocytes transform into large blast cells and divide both *in vivo* and in tissue culture. This proliferation of sensitised cells on contact with specific antigen has frequently been used as an in vitro test for cell-mediated hypersensitivity (Roitt, 1974).

Roitt et al. (1969) proposed that these T-lymphoblasts subserve several functions:

1. They may divide further to give a larger population of primed antigen-sensitive cells which provide immunological memory;

2. They may serve as "killer" cells which are cytotoxic for graft cells;

3. They may release a number of soluble factors;

4. They may cooperate by stimulating antigen-sensitive B-lymphocytes.

The induction of mitosis in cultured small lymphocytes and their transformation to blast cells can also be effected by non-immunological stimuli such as the non-specific mitogens PHA and Concanavalin A (Con A) (Roitt et al., 1969).

PHA is commonly utilised for its mitogenic activity in vitro for the estimation of thymus-dependent (T) lymphocyte reactivity

(Koch and Nielsen, 1975). PHA probably stimulates T-cells non-specifically (Roitt, 1974, p. 153), but it may also stimulate B-cells by an indirect mechanism. Jacobson and Blomgren (1975), working with mouse lymphoid preparations exposed to phytomitogens, showed the release of soluble factors from T-cells which are mitogenic for both T- and B-cells. In a further study Blomgren (1976) showed that T- and B-cells are both able to produce mitogenic factors. This is in agreement with the finding that the classical T-cell product, migration inhibition factor is released by T- and B-cells (Bryceson,1974). In addition, Potter and Moore (1977) report that highly purified T-cell populations respond poorly to PHA and that responsiveness can be enhanced by the addition of a small number of cells from a monocyte-rich population. Blomgren (1976) also notes that the DNA synthetic responses of human T-cells, cultured with phytomitogens, may be strongly enhanced by non-T-cells. However, it is generally accepted that PHA transformation is primarily a response of the T-cell population. Roitt et al. (1969) states that the transformed cells contain no intracellular immunoglobulin suggesting that they come from the T-cell population and that the known correlation of transformation with delayed hypersensitivity accords with this view. Roitt (1974, p. 152) states that PHA transformation has been frequently used as an in vitro test for cell-mediated hypersensitivity and several studies have shown reasonable correlation with in vivo results. Thus, Greaves and Janossy (1972) carried out experiments in mice and with reference to humans, the evidence was compatible with the view that PHA stimulates only T-cells, whilst pokeweed mitogen stimulates both T- and B-cells. Kreeftenberg,

Leerling, and Loggen (1975) found that PHA predominantly activates T-cells, although 10-20% of the blast cells are derived from B-cells.

PHA is a macromolecule with a molecular weight > 30,000 (Greaves and Janossy, 1972), the chemical composition of which is unknown (Koch and Nielsen, 1975). It reacts non-specifically with the lymphocyte cell surface triggering the same series of events as does antigen combining to a specific surface receptor (Roitt, 1974, p. 153). With PHA the majority of T-cells are transformed, whereas with antigen stimulation only a small number of cells are sensitive (Roitt, 1969, 1974).

The first report of PHA responsiveness in diabetes came from Brody and Merlie (1970). These workers compared five insulin-dependent and one insulin-independent diabetics with seven patients with chronic lymphocytic leukaemia. Both of these diseases show a lack of resistance to infection, and Brody and Merlie (1970) found an overall significant depression in PHA responsiveness in both diabetics and leukaemics when compared to control subjects. They suggested that the lymphocyte may be metabolically abnormal in diabetes and that this could ultimately lead to defective DNA synthesis, failure of antigenic recognition, and clinically deficient immunity. Ragab, Hazlett, and Cowan (1972) determined the responsiveness of cells from the peripheral blood of diabetics (seventeen taking insulin, three chlorpropamide, and three treated by diet alone) to PHA and found no difference from the control subjects. However, in this case the diabetics were in good clinical control with only four patients with blood sugar in excess of 250 mg/100 ml. In Brody and Merlie (1970) study four of the six diabetics had blood sugar above 300 mg/100 ml., although these were the highest values observed during

treatment. MacCuish et al. (1974b) measured PHA responsiveness in forty well controlled, insulin-dependent diabetics, fourteen poorly controlled, insulin-dependent diabetics, and forty control subjects and found a significantly decreased response in the poorly controlled diabetics compared to both other groups. MacCuish et al. (1974b) also reported that there were no differences in circulating subpopulations of lymphocytes in any of the groups described, a finding supported by Hann, Kaye, and Falkner (1976). MacCuish et al. (1974b) concluded that the depression in PHA responsiveness reported by Brody and Merlie (1970) reflects a metabolic disturbance rather than any inherent immunologic abnormality. Further support for the results of MacCuish et al. comes from Mahmoud et al. (1976) who found cell-mediated responses were reduced in streptozotocin-induced diabetic mice and concluded that this is due to alteration in the metabolism and function of the lymphocyte in diabetes.

This study was undertaken to investigate cell-mediated immunity in insulin-dependent diabetics with a view to correlating this with other immunological features and HLA antigens.

Materials and Methods

Twenty cc. of venous blood were taken and mixed with preservative-free heparin at 20 U/ml. (Weddel Laboratories). The lymphocytes were separated on a Ficoll-Triosil column, specific gravity 1.073, and washed in phosphate buffered saline and then a further three times in culture medium consisting of:

TC 199 with Earles Salts buffered with 20 mM Hepes (Wellcome Labs)
Penicillin and Streptomycin, 150 U/ml.
Foetal Calf Serum, 10% by volume of medium
2 mM L-glutamine

Phosphate buffered saline was prepared in the following way:

NaCl	32 g.
KCl	0.80 g.
$Na_2HPO_4 2H_2O$	5.10 g.
KH_2PO_4	0.80 g.

in a volume of four litres of distilled water which gives a final solution pH 7.35 $\pm$ 0.05. The lymphocytes were counted and adjusted to 0.75 x 10^6 cells/ml. in culture medium.

Two cc. of culture medium and lymphocytes were pipetted in round bottomed plastic tubes (112 mm. x 13 mm.) and 0.10 cc. of PHA of various dilutions were added. A dose-response curve was carried out in each case using the equivalent of 0, 0.01, 0.02, and 0.04 cc. of the original PHA in the final volume of medium. Each determination was carried out in triplicate. The cells were cultured in stoppered tubes for 72 hours at 37° C. A radioactive pulse, consisting of 0.1 cc. of tritiated thymidine, 2 µ Ci. (Radiochemical Centre, Amersham) was then added to each tube. Cultures were terminated four hours later by cooling to 0° C. The cells were harvested onto glass fibre filter paper (Whatman AF/C). Material remaining in the tube was washed onto the pad with 40 cc. ice-cold phosphate buffered saline, and the protein precipitated with 20 cc. of ice-cold 5% trichloroacetic acid. The pads were finally washed with 20 cc. methanol and dried in an oven at 120° C. for four hours. They were then placed in vials containing 10 cc. scintillation fluid)0.4% PPO, 0.005% POPOP in toluene) and counted in a scintillation counter, the results being given in c.p.m. or d.p.m.

All procedures prior to culturing of the cells were carried out in sterile conditions under an ultra-violet lamp. Glassware was sterilised by wrapping in tin foil and heating at 200° C. for three hours. Phosphate buffered saline was autoclaved and the medium

sterilised by passage through a 0.22 μ filter, and Ficoll-Triosil by passage through a 0.45 μfilter.

Ten insulin-dependent diabetics, mean age 30 years 1 month, were compared with 10 control subjects, mean age 28 years, who were age and sex-matched where possible.

Results

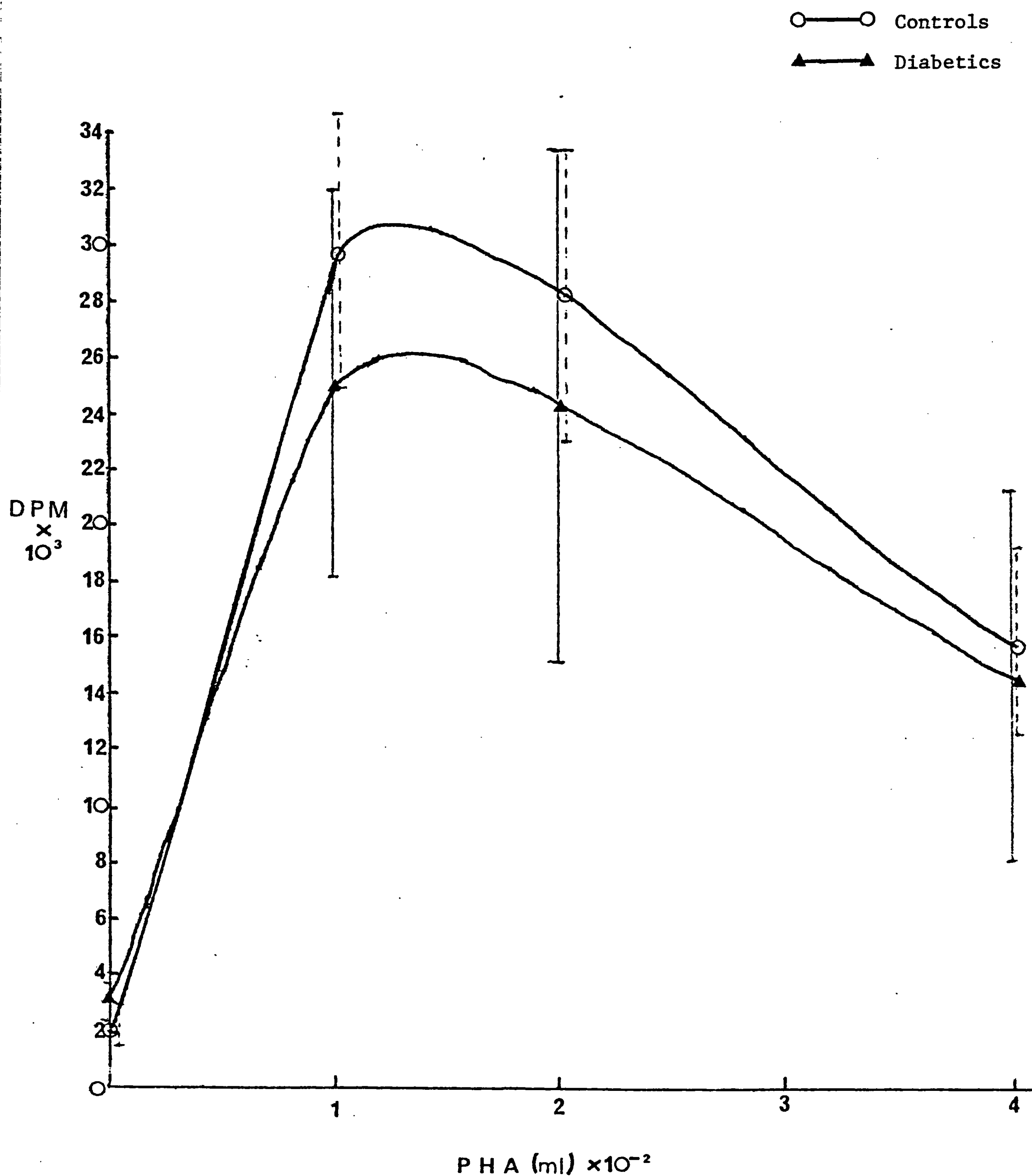
In order to determine the optimal quantities of PHA to be used in routine transformation experiments, general dose-response curves were performed initially. The results of these are seen in Table 4.1, and it was decided to use 0, 0.01, 0.02, and 0.04 cc. PHA in future experiments.

Table 4.1. Mean DPM obtained on adding ^{3}H-thymidine to cultures of 1.5 x 10^6 lymphocytes from control subjects in the presence of varying quantities of PHA.

	DPM (mean)		
PHA (ml.)	Expt. 1	Expt. 2	Expt. 3
0	1542	718	4678
0.01		59018	36286
0.02			46971
0.025	43929		
0.03		55050	
0.04			25189
0.05	30977		
0.10	6543	48386	

Figure 4.1 shows the results of experiments using lymphocytes from 10 control subjects and 10 insulin-dependent diabetics. The results shown here are the mean DPM values for each PHA volume. The diabetic group shows a slight depression in PHA response when compared to the control group.

Fig. 4.1. 1.5 x 10^6 lymphocytes from each of 10 insulin-dependent diabetic patients (mean age 30 years 1 month) and 10 control subjects (mean age 28 years) were incubated in the presence of varying quantities of PHA. Transformation was estimated by measuring the uptake of ^{3}H-thymidine. The results are expressed as DPM $\pm$ SEM for both groups of subjects.



Figures 4.2 and 4.3 show the results of individual experiments when the mean DPM values from triplicate cultures were plotted against PHA volume for one control subject and one diabetic patient. In both cases the diabetic lymphocyte response to PHA was greatly depressed. However, in other experiments there was little difference between the responsiveness of lymphocytes from the control subjects and the diabetic patients; in some cases the diabetic lymphocyte response was greater than that of the control subject.

There was considerable variation in responses to PHA when individual subjects were compared. This is shown by the large standard errors of the mean obtained when the results were aggregated. The standard error of the mean of the control responses was less than that of the diabetic responses.

Discussion

The results presented here of the mean PHA transformation from 10 diabetic patients show a small depression in response. This is in agreement with both Brody and Merlie (1970) and MacCuish et al. (1974b). However, both these groups of investigators found significant differences only between the control subjects and poorly controlled diabetic patients. MacCuish et al. (1974b) suggest that this reflects a metabolic disturbance rather than an abnormality in the lymphocyte, as suggested by Brody and Merlie (1970).

Of the ten diabetic patients tested here only two showed any significant depression in the lymphocyte response to PHA. There were both recent diabetics tested within four months of diagnosis, and of late onset for juvenile-type diabetes. However, at the time of testing both had satisfactory blood sugar levels. Consequently,

Fig. 4.2. Transformation of lymphocytes from one insulin-dependent diabetic and one control subject incubated with varying quantities of PHA. Results are expressed as mean DPM $\pm$ standard deviation.

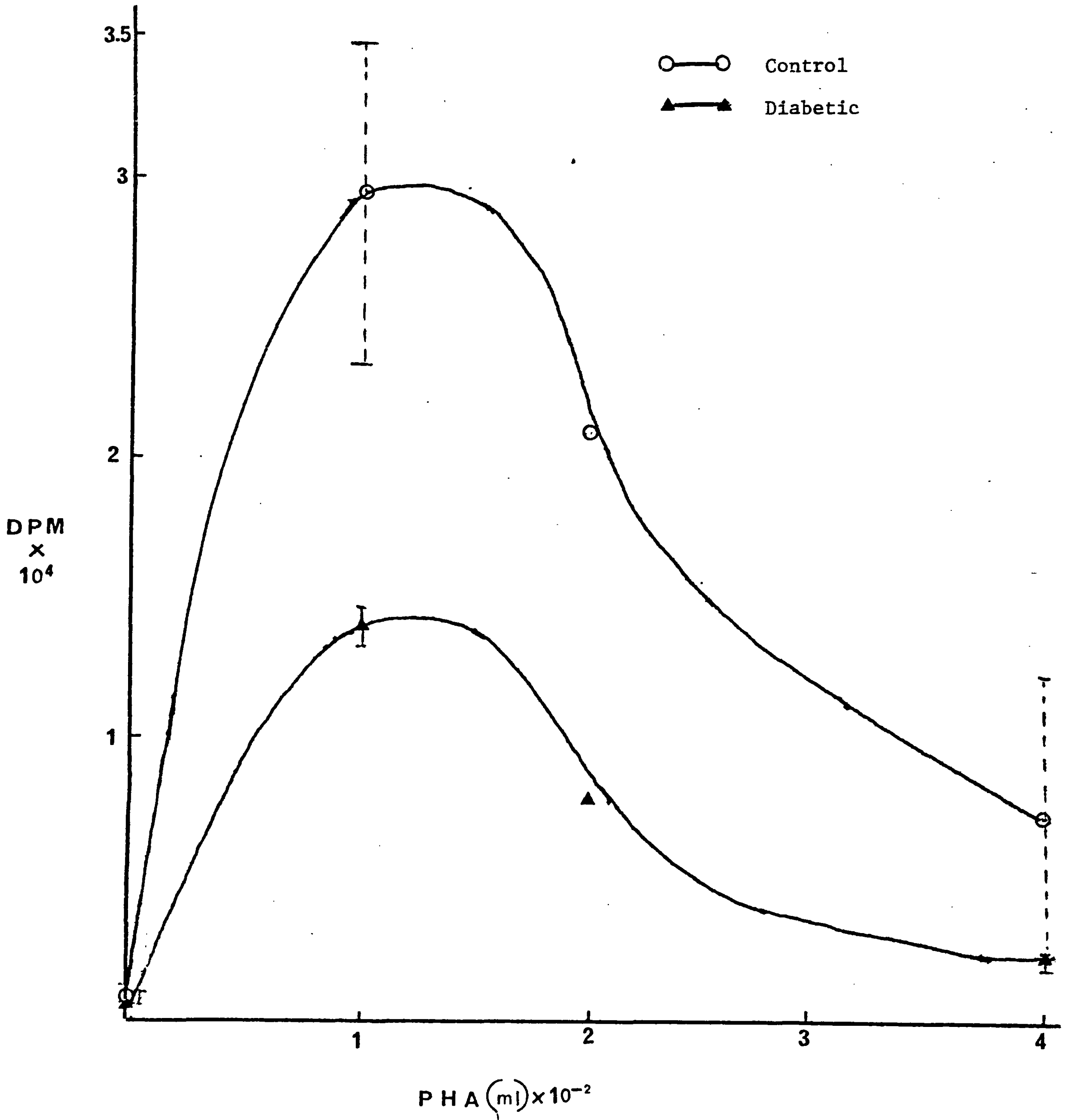
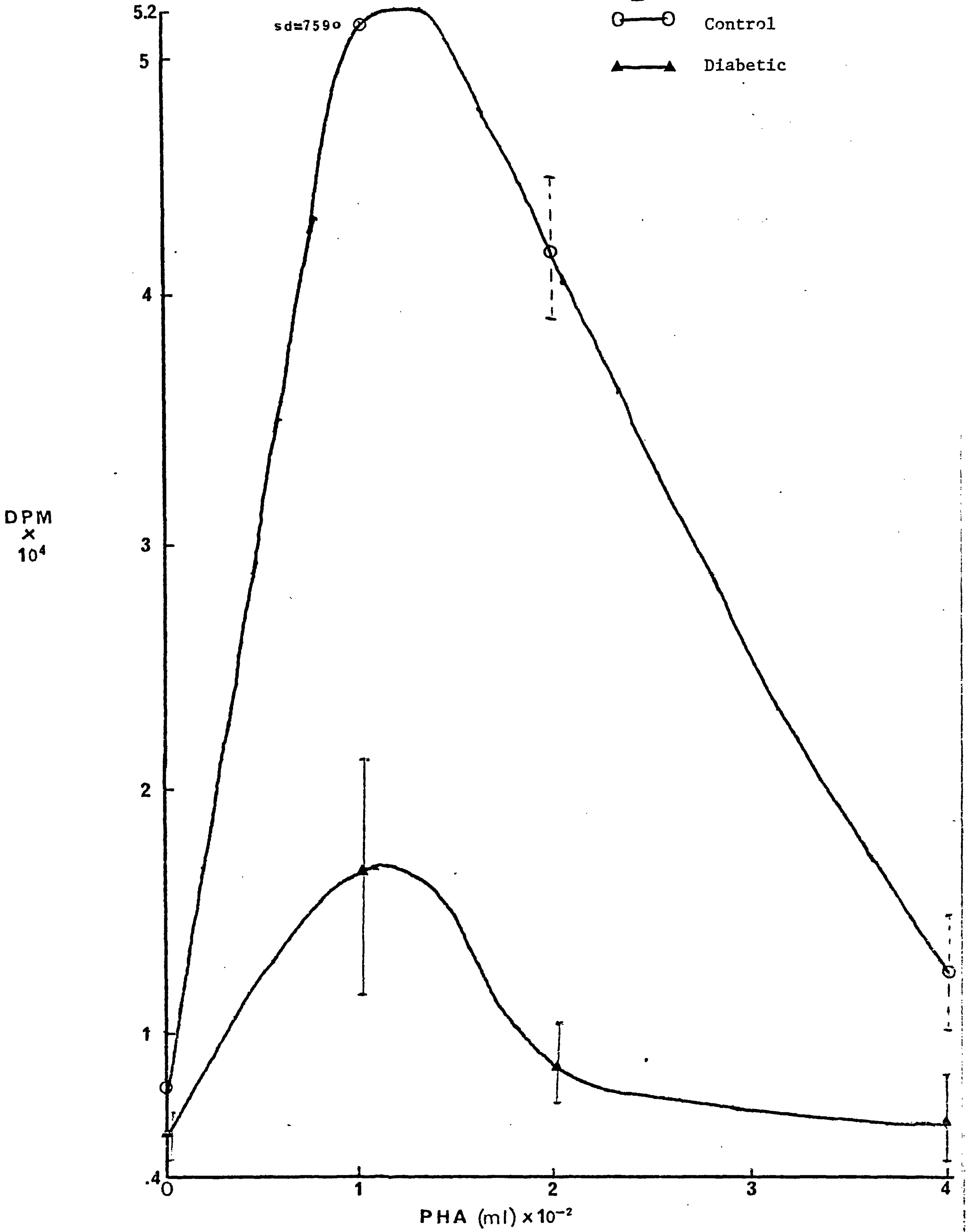


Fig. 4.3. Transformation of lymphocytes from one insulin-dependent diabetic and one control subject incubated with varying quantities of PHA. Results are expressed as mean DPM ± standard deviation.



these results tend to support the suggestion of Brody and Merlie (1970). Their ages were 35 and 45 years, respectively. The control subjects against whom these two patients were compared were 24 and 26 years, respectively. According to Girard et al. (1977) responsiveness to PHA shows a progressive decrease from the age of 40. This may be an explanation of the depression found in one of the two experiments presented here. Another factor to be considered is the reported decrease in the absolute number of T-lymphocytes with age (Girard et al., 1977). In the experiments reported here lymphocytes were separated on a Ficoll-Triosil column. Hence no distinction was made between T- and B-cells. The reduced DPM's obtained with the older subjects may be a direct reflection of T-cell number. In addition, results presented by Cattaneo, Saibene, and Pozza (1976) show a decrease in the absolute number and percentage of E-rosette-forming cells (T-cells) in the peripheral blood of juvenile-onset diabetics in comparison with maturity-onset diabetics and control subjects. On this basis PHA transformation of diabetic lymphocytes, obtained by separation of whole blood on a Ficoll-Triosil column, would be expected to be lower than in control subjects, as there would be an overall decrease in the number of T-lymphocytes obtained from diabetic patients. However, both MacCuish et al. (1974b) and Hann et al. (1976) report normal numbers of circulating subpopulations of lymphocytes in insulin-dependent diabetics.

Diurnal rhythms may also cause fluctuations in PHA responsiveness. Thus, Eskola et al. (1976), using whole blood, report low responses from blood taken at 08.00 hours and high response from blood taken at 20.00 hours.

It has also been shown that high circulating levels of oestrogenic and progestagenic steriods in women cause a significant depression of PHA response (Barnes et al., 1974). Therefore, those women who are pregnant or taking oral contraceptives would be expected to show a decreased PHA response.

In order to eliminate as many factors as possible, the diabetic and control populations should be carefully age- and sex-matched, venopuncture should be performed at a constant time, and the E-rosetting procedure should be adopted to ensure response from a known number of T-cells.

Pooled data from the ten diabetic patients and ten control subjects showed great variation between individual experiments. This is reflected by the large standard error of the mean. At a PHA volume of 0.01 cc., the control values varied from 12000 to 51000 DPM, while the test values varied from 8000 to 77000. This causes the large standard error in a group so small as ten. The only way to compare diabetic and control lymphocyte responsiveness to PHA is on an individual basis, taking into account the factors described above.

CHAPTER V.

Autoantibodies in Diabetes Mellitus.

Introduction

An increased incidence of autoantibodies in diabetics compared to control subjects has been reported by several workers (Irvine et al., 1970; Nerup and Binder, 1973; Bottazzo et al., 1974; MacCuish et al., 1974). Nerup and Binder (1973) reported an association between diabetes and other autoimmune disorders and found that antibodies to thyroid cytoplasm and gastric parietal cell cytoplasm occurred with significantly increased frequency in diabetics. These results confirmed those of Irvine et al. (1970) who found an increased frequency of thyroid and gastric parietal cell antibodies in both insulin-dependent and insulin-independent diabetics regardless of the duration of diabetes. Lendrum et al. (1975) found twenty of one hundred and five diabetic patients possessed either thyroid or gastric parietal cell antibody or both, whereas thyroid antibody was found in only one control subject and gastric parietal antibody in two. Bottazzo et al. (1974) state that the prevalence of thyroid and gastric antibodies is three times higher in juvenile diabetics than in age and sex matched controls. They point out, however, that in the older population diabetes and thyroiditis are common conditions and are present in subclinical form in up to 20% of this group. When this is considered, the association between diseases is less impressive.

However, when autoimmune adrenalitis and insulin-dependent diabetes are considered, the association is far more striking. Bottazzo et al. (1974) report the incidence of overt diabetes in idiopathic Addison's disease as being 10% compared to 1% in the general population, and that adrenal antibodies are found thirty times more often in insulin-dependent diabetics than in control subjects.

The simultaneous occurrence of two or more antibodies to thyroid, adrenal cortex, or gastric parietal cell cytoplasm has been reported in patients with diabetes but not in control subjects (Nerup and Binder, 1973). In addition to the three organ specific autoantibodies described above, MacCuish et al. (1974) claim that antibodies to intrinsic factor are increased four times in diabetics compared with controls.

The increased incidence of these organ specific antibodies in diabetes supports autoimmune involvement. Further evidence comes from an association with other autoimmune disorders such as pernicious anaemia, thyrotoxicosis, Hashimoto thyroiditis, idiopathic hypoparathyroidism and myasthenia gravis (MacCuish et al., 1974). The concept of autoimmunity in diabetes mellitus was further strengthened by the discovery of islet cell antibodies (ICA) in patients with polyendocrine disorders (Bottazzo et al., 1974; MacCuish et al., 1974). Bottazzo et al. (1974) reported the presence of lgG, complement fixing islet cell antibodies in thirteen of seventy-one sera; ten of these thirteen positive patents were diabetic. MacCuish et al. (1974) found islet cell antibodies in five of one hundred and five sera; the positive cases were juvenile-onset, insulin-dependent diabetics with co-existent Addison's disease and adrenal antibodies. Nerup and Binder (1973) failed to demonstrate antibodies to the islets of

Langerhans in patients with diabetes and associated autoimmune disorders. Bottazzo et al. (1977) proposed that these antibodies were not seen by several observers because of technical factors including low light transmission, the comparatively weak fluorescence produced by these antibodies, and the possible interference of blood group substances normally secreted by the acini so that group A or B organs give fluorescence with many sera (Lendrum and Walker, 1975).

Since the first report of islet cell antibodies in patients with polyendocrine disorders, many investigators have detected them with increasing frequency. Lendrum et al. (1975) found fifty-one of one hundred and five juvenile diabetics under the age of eighteen and less than one year's duration possessed ICAs. Among the ICA positive patients, six had thyroid antibody, two gastric parietal cell antibody, and two possessed both. They suggested that ICAs were present for a limited period only after the onset of diabetes and that their presence in adults occurred only when accompanied by other autoimmune disorders. This was based on the finding that sera from patients obtained a year or more after diagnosis were negative. This view has since been confirmed by Lendrum et al. (1976) who found that in almost every case of newly diagnosed juvenile diabetes weak ICAs are present in the sera and that this incidence falls to 50% within seven weeks and less than 20% within a year. Studies in identical twins also revealed a decline in positive ICA patients with increasing time after diagnosis (Lendrum et al., 1976). Irvine et al. (1977) found ICAs in 65% of newly diagnosed insulin-dependent diabetics, but within one to twelve months the prevalence was 55% and 40% with one to two years, 20% within two to five years, 10% within five to ten years, and 5% within ten to twenty years. Bottazzo et al.

(1977) tested ten ICA positive diabetics twelve to fourteen months later and found much lower titres with five being completely negative.

In newly diagnosed patients ICAs are found in 60-85% of juvenile diabetics (Lendrum et al., 1975). Irvine et al. (1977) report that 70% of juvenile-onset diabetics possess ICAs at the time of diagnosis compared to 0.5% of the general population. Lendrum et al. (1976) found 59% of diabetics of less than one year duration had ICAs. They had previously reported (Lendrum et al., 1975) only one out of seventy-two control subjects were positive.

MacLaren and Huang (1975) performed immunofluorescent tests using cultured human insulinoma cells. These are considered to be closely related to the islet β-cell. They found 87% of insulin-dependent diabetics were ICA positive. Sorenson, Shank, and Elde (1975), using an immunoperoxidase method tested sera for the presence of islet binding immunoglobulins and found that juvenile diabetics have an elevated titre over age matched control subjects. Lendrum et al. (1975) estimation of 60-85% of newly diagnosed diabetics are ICA positive was further confirmed by Bottazzo et al. (1978) who found 74% of diabetics studied within three months of diagnosis had ICA. When patients were studied three or more years after diagnosis, less than 20% were ICA positive.

The incidence and titre of ICA is higher in diabetics with other autoimmune disorders and the ICA persists for many years (Bottazzo et al., 1977). Irvine et al. (1977) found the incidence of ICA in insulin-treated diabetics with other autoimmune diseases was 38% and without other autoimmune diseases was 22%. Non-diabetics with autoimmune disease had a higher incidence of ICAs, 5.6%

compared to 0.5% of control subjects. Bottazzo et al. (1978) found that persistence of ICA was associated with a high prevalence of thyrogastric autoimmunity.

ICAs are often found in patients who have not developed diabetes. Bottazzo et al. (1977) report a case of a woman with polyendocrine disease who had a high titre of ICA and who developed diabetes two years later. Lendrum et al. (1976) show that ICAs may be present in people with normal glucose tolerance and may precede clinical diabetes by as much as three and one half years. Irvine, Gray, and McCallum (1976) proposed that ICAs may be a marker for asymptomatic and latent diabetes after carrying out glucose tolerance tests in thirty-six subjects who had ICAs but not diabetes. Most ICAs detected so far are lgG and fix complement (Bottazzo et al., 1974). MacLaren et al. (1975) found that the antibodies to cultured human insulinoma cells were of the lgG and lgM class. ICAs are organ specific and appear to react with all the cells of the islets, probably directed against the cytoplasmic organelles concerned in the synthesis or transport of hormones (Bottazzo et al., 1977). Other autoantibodies, reacting to pancreatic glucagon and somatostatin cells independently of ICAs have also been found (Bottazzo and Lendrum, 1976) mainly in insulin-dependent diabetics but also in insulin-independent diabetics and control subjects. A specific glucagon antibody has also been reported in a diabetic patient given no insulin (Baba et al., 1976).

In this study ICAS and other autoantibodies were studied by immunofluorescent techniques in insulin-dependent and insulin-independent diabetics. It was intended to correlate these with the incidence of other autoimmune phenomena under study.

Materials and Methods

The tissues used as substrates in these studies came from various sources. Human pancreas was obtained at death from blood group O renal transplant donors; thyroid tissue was obtained from thyroidectomy operations. Other tissues used were obtained fresh from mice and stomach, liver, and kidney were mounted together to form a composite block. Once removed the tissues were cut into approximately 0.4 cm. cubes with a sharp scalpel and snap frozen in liquid nitrogen. The tissues were then mounted on liquid nitrogen precooled chucks, using a drop of saline. They were then stored in liquid nitrogen until required.

Cryostat sections were cut at -22° C at a thickness of 6 μ, whereupon they were placed on standard microscope slides and allowed to dry under an electric fan for at least half an hour. Slides prepared in this way can be stored at -70° C and used for several weeks.

The immunofluorescent technique used here was based on the double layer method for antibody detection. Sera were obtained from insulin-dependent and insulin-independent diabetics and from control subjects and stored at -70° C until required.

The cryostat sections were covered with sera preheated to 37° C to avoid misleading antinuclear antibody (ANA) reactions. They were left for 30 minutes at 37° C and then washed for 20 minutes in phosphate buffered saline (PBS) on a rotatable. The PBS was carefully wiped off the slide leaving the sections well moistened, and one drop of conjugate (anti-human lgG fluorescein conjugate for the detection of ICAs and a polyclonal immunoglobulin fluorescein conjugate for the detection of other autoantibodies) was placed on each

section. The slides were left in a moist box for a further 30 minutes and the sections then washed for 40 minutes in PBS. The sections were then mounted in glycerol buffer which was prepared as follows:

Glycine	14.0 gm.
Sodium hydroxide (solid)	0.7 gm.
Sodium chloride	17.0 gm.
Sodium Azide	1.0 gm.

made up to 1 litre with distilled water. For mounting 1 part buffer to 2 parts glycerol were used which gave a buffer of pH 8.6 at which fluorescence is maximal (1969).

The conjugates used in these tests were obtained from Burroughs Wellcome Ltd. Anti lgG was used at a dilution of 1:8 for detection of ICAs (Lendrum and Walker, 1976) and polyclonal immunoglobulin at a dilution of 1:80 for the detection of other autoantibodies. Neat sera was used on pancreatic sections whilst a 1:10 dilution was used on thyroid and composite block sections.

Fluorescence was detected using a Reichert microscope and viewing the sections with an ultraviolet light source.

Blocking and specificity tests were performed using sera positive for ICAs. In the specificity test serum was added to the pancreatic section for 30 minutes, washed for 20 minutes, and fluorescein labelled sheep antirabbit lgG (1:8) was added instead of sheep antihuman lgG, i.e., specific conjugate was replaced by a conjugate against an unrelated antigen.

In the blocking test serum was added to the pancreatic section as before and then unlabelled specific antibody (antihuman lgG, 1:8) was added. After washing, specific labelled antibody (sheep antihuman lgG, 1:8 d:1) was added. The principle of this control is

based upon the combination of the unlabelled antisera (blocking antibody) with the corresponding human immunoglobulin, thus blocking the antigen sites which are normally available for reaction with fluorescein labelled antibody. This leads to diminution or inhibition of fluorescence in preparations incubated with the blocking reagent, of the same immunological specificity as that of the fluorescent conjugate, prior to conjugate application.

As a control the double layer immunofluorescence technique, as described, was performed.

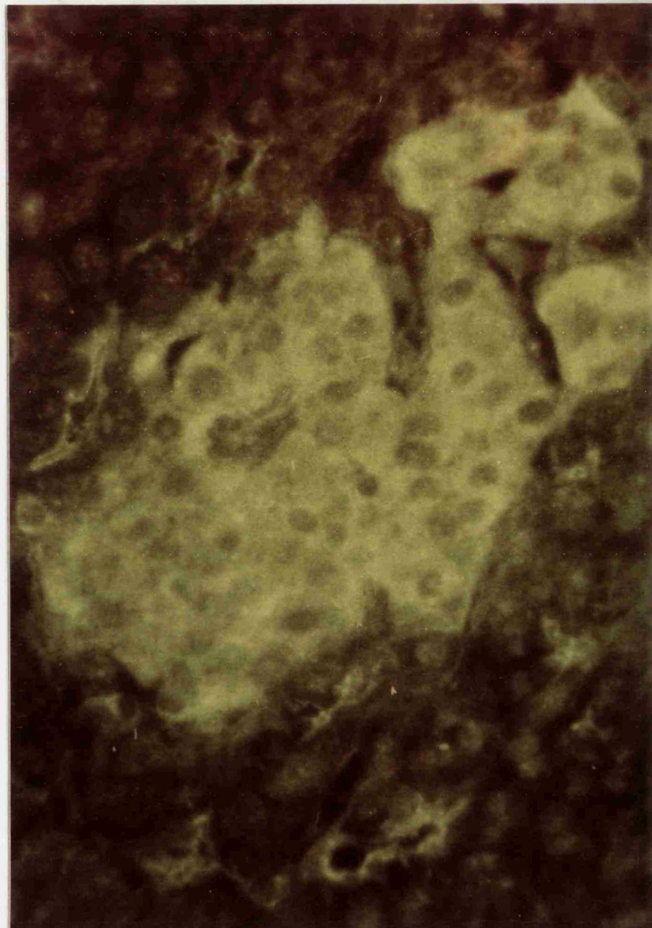
Absorption studies were initiated in order to determine the antigen against which ICA is reacting. These studies were performed on ICA positive sera with the following antigens: pancreatic homogenate, mitochondria, and microsomes and Coxsackie B_4 virus, prepared as previously described. Coxsackie B_4 virus was included as there is evidence of its involvement in initiating diabetes and reports of high neutralising antibody titres in newly diagnosed juvenile diabetics (reviewed in Chapter II).

The method used here was based on that of Lendrum and Walker (1976). Sera were incubated with equal volumes of either the antigen or PBS for 24 hours at 4° C. The mixtures were then centrifuged at 100,000 G for 1 hour, the supernatant removed and tested for ICAs as above.

Results

Sixty juvenile-onset insulin-dependent diabetics with a mean age of 24 years 6 months and a mean duration of diabetes of 5 years 1 month were tested for the presence of ICAs. Figure 5.1 shows typical islet cell staining with positive ICA serum. Of the 60 diabetic

Fig. 5.1. Typical pancreatic islet cell staining with the serum of a newly diagnosed juvenile-onset diabetic, using human pancreas in the indirect immunofluorescent technique.



patients tested 33% were positive for ICA. However, the incidence of ICAs was found to decline with the duration of disease, from 62% in those patients studied within 3 months of diagnosis to zero in those patients of more than 5 years duration (Table 5.1).

Table 5.1. Incidence of ICAs in 60 juvenile-onset insulin-dependent diabetics at various times after diagnosis.

Time after diagnosis	ICA +ve		ICA -ve	
0 - 3 months	13/21	62%	8/21	38%
3 - 6 months	--		--	
6 - 12 months	2/2	100%	0/2	0%
0 - 1 year	15/23	65%	8/23	35%
1 - 5 years	5/19	26%	14/19	74%
> 5 years	0/18	0%	18/18	100%

Thirty-three maturity-onset insulin-independent diabetics were also tested. They had a mean age of 63 years 3 months and a mean duration of diabetes of 3 years 5 months. None were ICA positive.

None of the 10 control subjects, mean age 28 years 6 months, were ICA positive.

In an additional group of 14 first degree relatives of insulin-dependent diabetics, 3 (21%) were ICA positive. The results are shown in Table 5.2.

The presence of other autoantibodies such as thyroid, gastric parietal cell, antinuclear, reticulin, smooth muscle, and mitochondrial were compared in the four groups (Table 5.2). One of 8 controls (12.5%), who were tested on all tissues, revealed the presence of at least one autoantibody compared to 40/59 (68%) of juvenile-onset insulin-dependent diabetics and 13/21 (62%) of mature-onset insulin-independent diabetics.

Table 5.2. Incidence of autoantibodies in 60 insulin-dependent diabetics, 14 first degree relatives, 33 insulin-independent diabetics and 10 control subjects.

Tissue Autoantibody	Insulin-Dependent Diabetics	1st Degree Relatives	Insulin-Independent Diabetics	Control Subjects
Islet Cell Antibody	20/60 (33%)	3/14 (21%)	0/33 (0%)	0/10 (0%)
Thyroid	11/58 (19%)	2/14 (14%)	3/29 (10%)	0/8 (0%)
G.P.C.	12/57 (21%)	0/14 (0%)	5/21 (24%)	0/8 (0%)
Others	17/58 (29%)	6/14 (43%)	9/21 (43%)	1/8 (12 1/2%)

Antibodies to thyroid cytoplasm were found in 19% of insulin-dependent diabetics compared to 10% of insulin-independent diabetics and 0% of control subjects.

Gastric parietal cell antibody was found in 21% and 24% of patients with insulin-dependent diabetics and insulin-independent diabetics, respectively, and none of the controls.

Other autoantibodies (antinuclear, mitochondrial, reticulin and smooth muscle) were found in 29% of insulin-dependent diabetics, 43% of insulin-independent diabetics, and 12 1/2% of control subjects.

When the first degree relatives were considered for thyroid, gastric parietal cell and other autoantibodies, the figures were 14%, 0%, and 43%, respectively.

Fifteen of 59 or 25% of insulin-dependent diabetics studied possessed at least two autoantibodies compared to 14% of the insulin-independent diabetics and none of the controls. Of the first degree relatives, 36% possessed at least two autoantibodies.

Results of the blocking and specificity tests performed with ICA positive sera from 4 insulin-dependent diabetics are shown in Table 5.3. Where sheep antirabbit lgG was used instead of sheep

antihuman lgG, no islet cell fluorescence was observed with any of the sera. Similar results were obtained when unlabelled specific antibody was used.

Table 5.3. Fluorescence (+) or lack of fluorescence (-) observed from 4 ICA positive diabetic sera on pancreatic sections when nonspecific antibody, unlabelled specific antibody, and labelled specific antibody were used.

Diabetics	Serum + Antihuman lgG	Serum + Antirabbit lgG (Specificity)	Serum + Unlabelled Antihuman lgG (Blocking)
1	+	-	-
2	+	-	-
3	+	-	-
4	+	-	-

Absorption studies with positive ICA sera from 4 insulin-dependent diabetics using pancreatic antigens and Coxsackie B_4 virus revealed the results shown in Table 5.4. In 3/4 cases there was increased intensity of fluorescence with the pancreatic homogenate, whilst 3/4 sera absorbed with Coxsackie B_4 virus showed less intense fluorescence than control sera incubated with PBS. Sera absorbed with pancreatic mitochondria and microsomes produced mixed results with some of the sera showing increased fluorescence and others showing no fluorescence.

Table 5.4. Intensity of fluorescence (0 to +++) obtained from 4 ICA positive sera when incubated with various antigens and PBS

Patients Serum	PBS	Pancreatic Homogenate	Pancreatic Mitochondria	Pancreatic Microsomes	Cox B_4 Virus
1	++	+++	++	0	+
2	++	+++	+++	0	++
3	++	+++	+++	++	+
4	++	++	0	+++	+

Discussion

The results presented here are in agreement with those of Irvine et al. (1970), Nerup et al. (1973), Bottazzo et al. (1974), MacCuish et al. (1974), and Lendrum et al. (1975) with respect to the increased incidence of thyroid and gastric parietal cell antibody in insulin-dependent diabetics. The incidence of thyroid antibody was 19% and that of gastric parietal cell antibody was 21% compared with the figures of 20% and 16%, respectively, reported by Nerup and Binder (1973) and the 20% for either thyroid or gastric parietal cell antibody, or both, reported by Lendrum et al. (1975). An increased incidence in thyroid and gastric parietal cell antibody was also found in insulin-independent diabetics compared with control subjects and this is in agreement with the results of Irvine et al. (1970). However, the insulin-independent group used in the present study had a mean age of 63 years 3 months compared to the control group mean age of 28 years 6 months. It is well known that there is an increase in autoantibody production with age and, consequently, it would be expected to find an increased incidence of autoantibodies in this group, where the age of onset of diabetes is much greater than that of the insulin-dependent group. This appears to be confirmed by the finding that 43% of the insulin-independent mature-onset diabetics possessed antibody other than those to islet cells, thyroid, or gastric parietal cells compared with 29% of insulin-dependent diabetics and 12 1/2% of control subjects.

The simultaneous occurrence of two or more autoantibodies in 25% of insulin-dependent diabetics is also in agreement with results published by Nerup and Binder (1973) and emphasises an autoimmune component in this type of diabetes. In accordance with this is the

finding that 36% of first degree relatives of insulin-dependent diabetics possess two or more autoantibodies and may point to a genetic predisposition to autoimmunity in these families.

Confirmation that islet cell antibodies are common in newly diagnosed juvenile-onset insulin-dependent diabetics is supplied here. Thus, 65% of patients tested within one year of diagnosis showed the presence of ICAs; this is comparable to the figures of Lendrum et al. (1975) of 60-85%. That these antibodies tend to disappear is also apparent as only 26% of diabetics tested 1-5 years after diagnosis and none of those tested over 5 years after diagnosis possess ICAs. Persistence of ICA in diabetes is usually associated with other autoimmune disorders (Bottazzo et al., 1977). In this study 2/5 juvenile diabetics with ICA present one year or more after diagnosis revealed the presence of thyroid or gastric parietal cell antibody whereas only 1/14 who were negative for ICA in this group possessed thyroid antibody. However, there was no clinical manifestation of other autoimmune endocrine disorders. One patient whose serum was tested at the time of diagnosis still showed the presence of ICA over one year later. In addition, he possessed antibodies to both thyroid and gastric parietal cell cytoplasm.

Islet cell antibody appears to be almost exclusive to newly diagnosed juvenile-onset diabetes and was not seen in any of the mature-onset diabetics suggesting that ICAs are of significance in the aetiology of type I diabetes. When ICAs are found in patients other than juvenile-onset diabetics, it is usually in association with polyendocrine disorders (Bottazzo et al., 1974). Bottazzo et al. (1974) suggested that it may be of value in predicting diabetes in these patients as the ICA is often present before the onset of diabetes.

The role of ICA in the pathogenesis of diabetes mellitus is unknown. Bottazzo et al. (1977) think that they are useful as markers which separate two types of insulin-dependent diabetes, IA and IB. The IA type includes uncomplicated juvenile, insulin-dependent diabetes where the disorder could be due to viral infection. The fact that ICAs are transient is compatible with this view as transient autoimmune reactions are known to occur in viral infections. Type IB diabetes includes the polyendocrine cases with onset at any age. Bottazzo et al. (1977) subdivide these as follows:

	Type IA	Type IB
Aetiology	Viral	Related to organ-specific autoimmunity
Overall frequency of DM	10%	< 1%
Insulin-dependent	Yes	Yes
Sex	M = F	F > M
Age	< 30	Any age
Associated autoimmune disorders	None	Adrenalitis, gastritis, thyroiditis
Incidence of other autoantibodies	Low	High
Frequency of ICA	At onset 85% After 1 year 20% Tend to disappear	Not known 38% Remain stable for years
First appearance of ICA	Time of viral infection	Years before onset of diabetes mellitus

However, Irvine et al. (1976) regard the presence of ICAs in patients with polyendocrine autoimmune disorders as a marker for asymptomatic prediabetes.

In this study no distinction has been made between type IA and type IB, the diabetics being divided into insulin-dependent (the majority of whom were diagnosed before the age of 30) and insulin-independent or mature onset, so it is not possible to confirm this

subdivision of insulin-dependent diabetes according to the frequency and association of autoantibodies. In addition, the numbers in the Bath area were low and there are insufficient data for any meaningful analyses.

MacLaren et al. (1975) carried out immunofluorescent tests using insulinoma cells instead of pancreatic sections. They found positive fluorescence in 34 of 39 diabetic patients but were uncertain whether this marker of autoimmunity was primary (involved in the pathogenesis) or secondary (as a result of diabetes). They suggested that β-cell damage, possibly by a virus, might initiate an immune response by releasing sequestered antigens. Alternatively, immune β-cell damage might result from either complement-fixing antibody cytotoxicity or antibody which could coat the cell and permit antibody dependent lymphocyte cytotoxicity.

However, both mechanisms require interaction with the target cell membrane and yet the classic ICA gives intracytoplasmic and not cell membrane staining (Kaldany, 1979). It is, therefore, difficult to understand their role in the pathogenesis of islet cell damage by cytotoxic mechanisms.

In this study, islet cell antibody was further investigated using various putative antigens in absorption experiments. The results obtained from 4 juvenile-onset, insulin-dependent diabetics, strongly positive for ICAs, are shown in Table 5.4. The increased intensity of fluorescence on absorption with pancreatic homogenate suggests that a factor in the serum that was inhibiting the binding of ICAs to the section was being absorbed out. The decreased fluorescence seen on absorption with Coxsackie B_4 virus may be caused by the antibody reacting with antigenic determinants common to both

the virus and the islet cell. This might provide an explanation for the possible mechanism of the virus causing β-cell destruction by an autoimmune process.

These preliminary results await confirmation by a more quantitative technique. This could possibly be achieved by noting the antibody titre prior to and after absorption. Alternatively, it may be possible to quantitate ICAs by using other techniques for their estimation, e.g., immunoprecipitation procedures with a presumptive antigen.

CHAPTER VI.

HLA Antigens in Juvenile Diabetics and Family Studies.

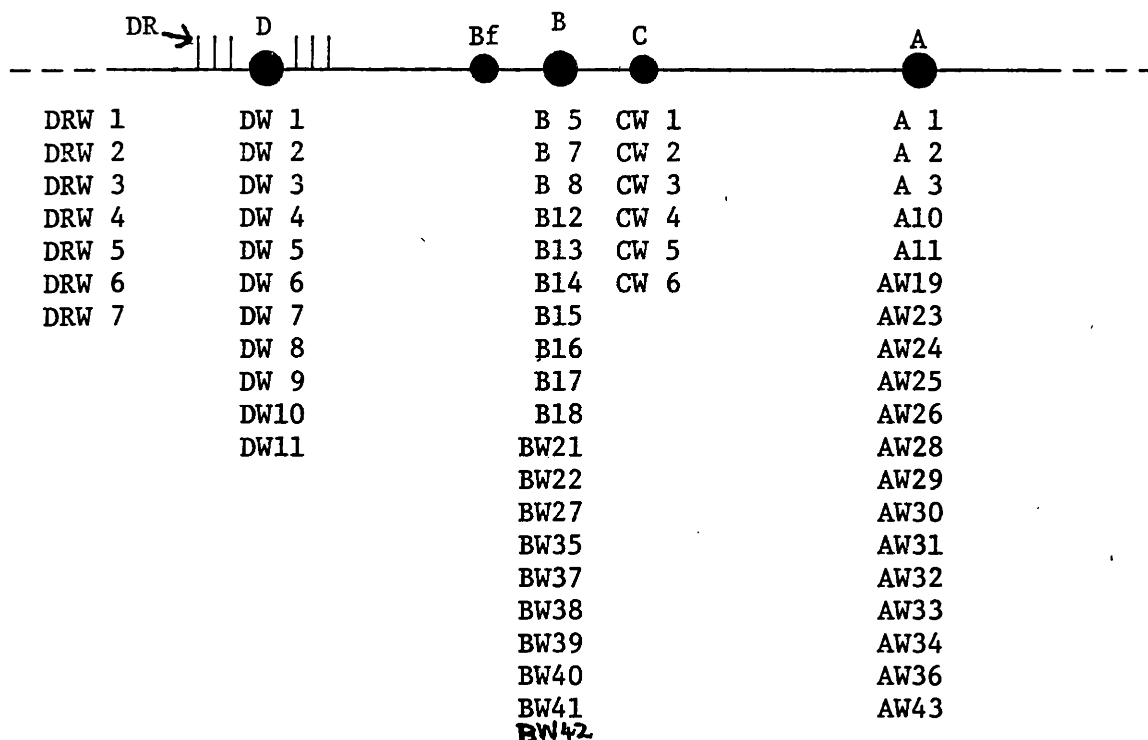
Introduction

The major histocompatibility complex is a system of closely linked multiple genes on the short arm of chromosome number six, which in man is known as the HLA region. The complex includes the A, B, and C loci which control cell surface glycoproteins and are detectable serologically, and a D locus which is believed to control cell surface antigens that may be detected in mixed lymphocyte reactions. Other loci in the complex include those controlling various components of complement (Ed. B.M.J., 1976).

The A, B, and C loci are not directly involved in the immune response but are closely linked to other loci within the major histocompatibility complex that are responsible for immune responses (Ed. Lancet, 1976). According to Svejgaard and Ryder (1976), the HLA system controls strong alloantigens, many immune responses, and some components of the complement cascade.

The major histocompatibility system of man is shown on the following page.

Cudworth (1978) suggests that many genes in these chromosomal regions probably control differences in immune response and therefore differential susceptibility to a variety of diseases. The HLA antigens almost certainly act as markers for the existence of immune response genes which are in linkage disequilibrium with the HLA system.



Immune response genes have been found in guinea pigs and mice which are closely linked to the H2 system. Rosenberg and Kidd (1977) state that by analogy with mice, the HLA complex probably contains additional loci which control responses to specific antigens, and immune associated loci which code for surface antigens found on different lymphocyte populations.

There are many mechanisms whereby such genes may operate in association with the major HLA antigens to confer susceptibility to certain diseases. Many reports of associations of various diseases with HLA antigens have been published. A list of some of the positive associations between HLA antigens and disease is shown here (Hobart and McConnell, 1975).

Disease	Antigen
Hodgkin's disease	HLA-B5
	HLA-A1
	HLA-B8
Acute lymphatic leukemia	HLA-A2
	HLA-B12
Ankylosing spondylitis	HLA-BW27
Graves disease	HLA-B8

Coeliac disease	HLA-B8
Myasthenia gravis	HLA-B8
Chronic active hepatitis	HLA-B8
Systemic lupus erythematosis	HLA-B15
Multiple sclerosis	HLA-A3
	HLA-B7

Almost all associations are with the second locus of the HLA system, i.e., the B locus or in Mannick's (1973) terminology the four or second sublocus. Diseases showing an association with B locus antigens tend to be male predominant and sometimes show indirect evidence of bacterial infection, whilst some diseases with a relatively weak association with B locus antigens have now been shown to have a closer association with the D locus and are often female predominant with suspicion of a latent virus infection (Ed. B.M.J., 1976).

Bodmer and Bodmer (1974) propose four mechanisms to account for associations between HLA antigens and disease. Thus:

1. HLA antigens might induce tolerance to foreign antigens with which they cross-react, including antigens which are components of a viral pathogen.

2. HLA antigens may serve as receptors, or interact with receptors, for specific viruses.

3. Pathogenic agents might incorporate into their coat a portion of membrane from the cell which they are attacking that carries HLA antigens.

4. Immune response genes linked to HLA genes can provide the basis for disease associations. Diseases showing the most significant association with the HLA complex are autoimmune and therefore this mechanism seems most likely.

In addition to the mechanisms proposed above, there is the possibility that a structural similarity between certain HLA antigens

and hormone receptors may result in competitive binding with the hormone being absorbed onto the wrong receptors (Svejgaard and Ryder, 1976). This idea is supported by the work of Majsky and Jakoubkova (1976) who found a transient loss of HLA antigens on lymphocytes during menstruation and during ovulation, whilst in the intermittent period the HLA antigens were consistently demonstrated. They concluded that the HLA antigens on the lymphocytes were masked or blocked by hormones.

Tauber (1976), however, states that all recognition is based on a comparison with an appropriate standard and that on a molecular level markers of biological individuality become the very receptors for immunological recognition, serving as a direct standard against which foreigness can be measured and therefore no special mechanism is needed to explain HLA and disease association.

McDevitt and Bodmer (1974) support the idea of immune response genes linked to HLA genes, stating that disease association can be interpreted in terms of the effects of genes in the HLA region which control immune responses that are critical for disease susceptibility. HLA linked specific immune response genes are most probably important genetic factors predisposing to resistance or susceptibility to a variety of neoplastic, autoimmune, and infectious diseases in man. McDevitt and Bodmer (1974) believe that the disease associations are the result of HLA linked immune response genes and explain that the associations are not absolute for the following reasons:

1. If environmental factors are involved, then not all individuals with the right genotype will necessarily be exposed. The effect of the genotype may be "all or none," i.e., not fully penetrant, but may interact with the level of environmental exposure.

2. The disease in question may be heterogeneous and have more than one aetiology.

3. Other genes, not linked to the HLA system, may be involved in disease susceptibility.

4. If the whole effect is due to one or more loci closely linked to those determining HLA antigens, recombination may disrupt the antigen association.

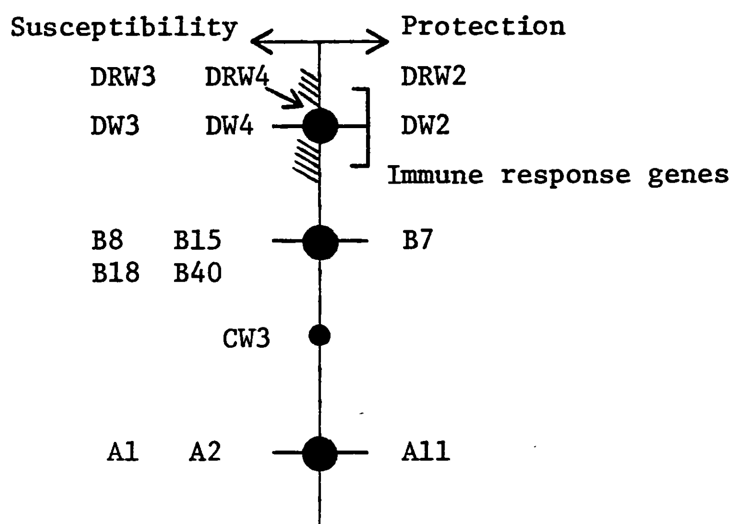
The association of disease with HLA specificities is usually expressed as relative risk which is defined as the ratio of the risk of development of the disease in those with the antigen divided by the risk in those lacking it (Rosenberg and Kidd, 1977).

Diabetes mellitus is believed to be a genetically determined disorder (Singal et al., 1973) in which inherited susceptibility plays an important part. Singal et al. (1973) found that the frequency of HLA-B15 was significantly increased in insulin-dependent patients than in insulin-independent diabetics and control subjects. Nerup et al. (1974) found an increase in HLA-B8 and HLA-B15 in diabetics. HLA-B8 was increased in juvenile diabetics but not in the maturity-onset diabetics, whilst HLA-B15 was increased in both groups. The same workers stated that there is a 2-3 times higher chance of developing insulin-dependent diabetes if a subject is HLA-B8 or HLA-B15 positive compared to an individual lacking these antigens. Cudworth and Woodrow (1974) typed 100 patients and found HLA-B8 was present in 54% of juvenile-onset diabetics compared with 31.8% of maturity-onset diabetics, and HLA-B15 was present in 18% and 12%, respectively. When their data were combined with that of Nerup et al. (1974) and Finkelstein, Zeller and Walford (1972), the relative risk for juvenile-onset diabetes was 2.41 for individuals possessing

HLA-B8 and 2.34 for those with HLA-B15. In a further study, Cudworth and Woodrow (1975) tested 150 diabetic patients with an age of onset less than 30 years and 68 diabetic patients where onset was over 30 years of age and again they found differences in the frequencies of HLA-B8 and HLA-B15 when compared to 300 control subjects. They combined their results with some from Copenhagen and found a relative risk of 2.4 for HLA-B8 and 2.09 for HLA-B15. The relative risk for individuals possessing both antigens was 4.67. The frequencies of these two antigens in maturity-onset diabetics were not significantly different from those found in control subjects. Maturity-onset diabetes is not HLA linked but has a strong hereditary tendency whereby 60% of children of two such diabetics will develop the disease by the age of 60 years (Ed. B.M.J., 1975).

In a study of 288 juvenile-onset diabetics of 30 years or less at diagnosis, Cudworth and Woodrow (1976) found relative risks for HLA-B8, HLA-B15, and HLA-B18 were 2.54, 2.0, and 2.4, respectively. They also showed a negative association with HLA-B7 and low relative risks for individuals possessing HLA-B5 and HLA-BW35. The combined relative risk for patients possessing antigens HLA-B8 and HLA-B15 was 5.4. In addition, they found a significantly increased frequency of HLA-B8 in late-onset diabetics who were insulin-dependent and normal frequencies in insulin-independent diabetics. Further studies have confirmed the increased frequencies of HLA-B8, HLA-B15, and HLA-B18 (Langraf et al., 1976; De Moerloose et al., 1978; Cudworth, 1978) but Rubinstein et al. (1976) stated that HLA antigens, B8 and B15, are doubtful or non-existent in populations other than those studied in England or Denmark, and they suggested that the HLA system is not directly involved in diabetes, but that it maintains linkage

disequilibrium with nearby genes that are more important. Bottazzo and Doniach (1976) concluded that testing for the lymphocyte marker DW3 gives a better correlation of the "autoimmune" gene since it might be closer to the immune response genes, and Rubinstein et al. (1976) stated that the association of diabetes with DW3 is greater than those of HLA-B8 and HLA-B15. Cudworth (1978) reported a study of 323 insulin-dependent diabetics with an age of onset less than 30 years and found an increased relative risk of developing diabetes was observed in patients who were HLA-A1, A2, B8, B18, B15, B40, CW3, Bf^{S}, DW3, DW4, DRW3, and DRW4 positive. A significant association was found in the frequency of CW3 compared to controls, but a stronger association was demonstrated with HLA-DW3 and DW4 specificities which gave a relative risk of 6.4 and 3.7, respectively (Nerup et al., 1976; Cudworth, 1978). A positive association between juvenile-onset diabetes and DRW3 and DRW4 was suggested by Cudworth (1978) and a negative association with DRW2. De Moerloose et al. (1978) found the most significant association of HLA antigens and diabetes was with DRW3 which gave a relative risk of 9.2 compared to 2.9 for HLA-B8. Cudworth (1978) proposed the following pattern of susceptibility and protective HLA factors in type I (juvenile-onset diabetes:



The present study was designed to ascertain whether similar disturbances in HLA antigen frequency could be found in the population of diabetic patients in the Bath area. Family studies were also undertaken to determine whether possession of particular antigens gave an increased disposition to the disease.

Patients and Methods

HLA phenotyping. Ninety-six juvenile-onset type diabetics were screened for their HLA type. Two groups of control subjects were used in this study. These were (a) 417 healthy, non-selected people from the same geographical area tested over the same period of time by the same laboratory and (b) 66 maturity-onset type diabetics who also complied with the above criteria.

HLA genotyping. HLA genotypes were obtained for 28 of the juvenile diabetics by a study of the HLA types of their families. In these cases, family histories were taken and when possible the proband, his siblings, both diabetic and non-diabetic, were HLA typed. This was done to determine whether in the respect of the affected siblings, one, both, or neither of the HLA haplotypes were identical.

HLA typing. HLA typing was performed by the National Tissue Typing Reference Laboratory, Southmead, Bristol. Nine A locus and seventeen B locus antigens were tested. The significance of disburbances in the frequencies of these antigens were assessed by the Fisher Irvin exact chi-square test.

Results

Four HLA antigens, HLA-B8, B15, B18, and BW40 appeared with an increased frequency in the juvenile-onset diabetics as compared with the maturity-onset diabetics and control subjects. The increased frequency and relative risk for HLA-B18 and HLA-BW40 were not significant. The increase in HLA-B8 and B15 was significant with the antigens running either independently or together (Table 6.1).

Table 6.1. Frequencies of HLA antigens in juvenile-onset diabetics, maturity-onset diabetics, and control subjects

HLA Antigens	Control Subjects N = 417	Juvenile Diabetics N = 96	Maturity-onset Diabetics N = 66
B8	108 (26%)	48 (50%)*	18 (27%)
B15	54 (13%)	25 (26%)*	6 (9.1%)
B8, B15	16 (3.8%)	17 (17.7%)**	0 (0%)
B7	117 (28%)	10 (10.4%)*	22 (33%)
A1, B8	92 (22%)	43 (45§)**	16 (24%)

*0.01 > P > 0.001.

**0.001 > P.

The haplotype HLA-A1, B8 was also significantly increased, whilst the antigen HLA-B7 was decreased. The relative risk statistic (Svejgaard et al., 1974) showed that HLA-B8 and HLA-B15 positive individuals carry an increased risk of developing the disease of 2.86 and 2.37 times, respectively, whilst the combined risk for HLA-B8 and B15 positive individuals is 4.4. HLA-B7 positive individuals and those carrying the HLA-A1, B8 haplotype were calculated to carry relative risks of 0.30 and 2.86, respectively.

Over the course of the study more cases were recorded during autumn and winter months than in the spring or summer (Table 6.2). These results, however, present no evidence of clustering of cases associated with any particular HLA phenotype.

When the patients were examined by age categories (Table 6.3), it appeared that school age children were most likely to contract the disease, with the suggestion of a second peak in the early twenties. The possession of HLA-B8 may be conferring an increased susceptibility on this latter group, but no other age group showed any association with a particular phenotype.

HLA and Families. Genotypes of the four families with two or more diabetic siblings are shown in Table 6.4. It showed that in each of the three families with two affected siblings, the diabetic siblings each shared both haplotypes; in the families with a third unaffected sibling, one shared both and the other neither haplotype with their respective affected siblings. In the family with three affected siblings, two had both haplotypes identical and the third shared one with these two and the other haplotype with the fourth, unaffected, sibling.

In 20 families, HLA genotypes of the propositus were also compared with those of his siblings, both diabetic and non-diabetic (Table 6.5). These families included 16 sibships with one diabetic child and four with two or more diabetic children. If the first family member affected is considered as the index case for each sibship and is excluded from the table, the rest of the siblings are assorted as shown.

Table 6.2. Month of onset of new cases of juvenile-onset diabetes.

HLA Phenotype	Jan.	Feb.	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Totals
B8 (Without B15)	5	3	7	3	1	1	1	2	1	2		5	31
B15 (Without B8)	2	1	1					2	1		1		8
B8, B15	2	4	1		1	1		1	1	2	2	2	17
Other Phenotypes	4	4	3	4	2	1	1	2	2	5	6	6	40
Totals	13	12	12	7	4	3	2	7	5	9	9	13	96

Table 6.3. Age distribution of new cases of juvenile-onset diabetics.

HLA Phenotype	Age (Years)					
	1-5	6-10	11-15	16-20	>20	Totals
B8 (Without B15)	1	9	8	2	11	31
B15 (Without B8)		1	2	2	3	8
B8, B15	2	6	6		3	17
Other Phenotypes	2	11	10	6	11	40
Totals	5	27	26	10	28	96

Table 6.4. Genotypes of four families with two or more diabetic siblings.

Family	Diabetic sibling		Non-diabetic sibling	
1	JB	A1, B8/A2, B15	UB	A1, B8/A2, B15
	AB	A1, B8/A2, B15		
2	CS	A1, B8/A9, B27		
	NS	A1, B8/A9, B27		
3	KF	A2, B15/A2, B15	SF	A2, B7/A2, B21
	DF	A2, B15/A2, B15		
4	GJ	A9, B15/A1, B8	SJ	A3, B7/A3, B7
	LJ	A9, B15/A1, B8		
	KJ	A9, B15/A3, B7		

Table 6.5. HLA identity and type I diabetes in siblings of propositus

HLA Identity	Diabetic	Non-diabetic	Totals
Identical	4	9	13
Non-identical	1	19	20
Totals	5	28	33

Four of 13 (31%) HLA identical siblings were themselves diabetic, where only 1/20 (5%) of the non-identical siblings were diabetic. This difference did not achieve statistical significance ($0.10 < p < 0.50$).

Discussion

In the present study the previous findings of Nerup et al. (1974), Cudworth and Woodrow (1974 and 1975) were confirmed. An increased frequency of the antigens HLA-B8 and B15 and the haplotype HLA-A1, B8 were found in juvenile-onset diabetics. The protective value conferred by the presence of the antigen HLA-B7 was also confirmed (Cudworth and Woodrow, 1976). The relative risk statistic shows that HLA-B8 and HLA-B15 positive individuals carry a 2.8 and 2.4 increased chance of developing diabetes. This is in agreement with the figures presented by Cudworth and Woodrow (1975). Individuals positive for both HLA-B8 and B15 carry an additive risk.

In agreement with Nerup et al. (1976) there was no disturbance in the frequencies of these antigens in the population of maturity-onset type diabetes studied, thus emphasising the evidence for genetic heterogeneity and the different pathogenic processes which are involved in the two major clinical forms of diabetes.

Thus, HLA studies indicate that there is no simple association between HLA and this disease, as apparently occurs in the case of coeliac disease which shows a strong relationship with HLA-B8. In the case of juvenile-onset insulin-dependent diabetes, the B locus studies, strengthened by the evidence from mixed lymphocyte culture (D locus) typing that DW3 and DW4 both confer on increased risk of the disease, suggest that two HLA linked genes confer susceptibility. Further evidence that more than one HLA linked gene, possibly

operating by a different pathogenetic mechanism in juvenile-onset diabetes, is the finding that the relative risk is additive when both B8 and B15 are present. Cudworth and Woodrow (1976) quoted evidence that the relative risk is higher for those individuals homozygous for B8 than those who are heterozygous, but not as high as that for individuals possessing both HLA-B8 and B15 antigens. This may indicate that the risk of developing juvenile-onset diabetes is not additive when the gene operating via one particular mechanism is inherited on both HLA chromosomes, in contrast to when two diabetogenic genes are inherited. Rubinstein, Suciu-Foca, and Nicholson (1977) disagree that these two genes have different mechanisms as shown by the additive effect. They state that the disease is inherited as a recessive trait with a penetrance of about 50%. However, Neel (1977) points out that whenever incomplete penetrance is introduced, it is often impossible to clearly distinguish between multifactorial, dominant, recessive, simple one-locus additive and overdominant inheritance. Cudworth (1978) quoted several investigators who proposed autosomal recessive inheritance but with the postulation that penetrance is markedly reduced such that only about one-fifth of the subjects with the genotype actually develop the disease. Cudworth, however, believes that it is unlikely diabetes would be controlled by a single gene and that by invoking incomplete penetrance of this magnitude, it is possible to prove almost everything has a genetic basis.

Rubinstein et al. (1976) and Suciu-Foca and Rubinstein (1976) also reported that gene cross-over in parents of diabetic children was 15-fold. They postulated that genes for susceptibility must be in close proximity to the HLA complex for the linkage to be detected. Neel (1977) points out that if there is not an increase in crossing

over in the parents, the strength of the argument for a recessive hypothesis is reduced, and that some type of additive genetic hypothesis involving one or several loci, seems the most likely alternative for the data presented. Shaw, Kansal, and Gatti (1976) support Rubinstein's claim and quote the rare event of a bilateral HLA recombinant in juvenile diabetes mellitus. They suggest that this shows support for an increased frequency of HLA recombinations in families of patients with juvenile diabetes mellitus. The results presented in this study agree with those of Nerup (1976) and Cudworth (1978) that two genes confer susceptibility to diabetes upon the individual.

The finding that when one member of an identical twin pair was diabetic and the other was affected in 50% of the twin pairs (Nelson et al., 1975) suggests that whatever the genetic predisposition to the disease may be, it finds its clinical expression in about half those who are predisposed, i.e., there is a 50% penetrance of the genotype and leads to the likelihood that the relative aetiological importance of genetic and environmental factors is different in the two groups where juvenile diabetes is concerned. Nelson et al. (1975) stated that if no difference was found in the frequencies of HLA-B8 in concordant and discordant twins, then the concept of a single major HLA-linked gene causing susceptibility is supported and development of the disease would depend on an environmental factor in individuals possessing this gene.

There are three potential principal causes of an association of type I diabetes with an HLA antigen. These are as follows:

1. Ethnic stratification, i.e., both the disease and the HLA type are common in the ethnic group under study. This is an unlikely

explanation as the reports of HLA disturbances come from diverse centres.

2. Causation, i.e., the HLA type is in a direct chain of events leading to the disease. The fact that many diabetics present without the suspected antigens militates against this.

3. Association, i.e., the HLA alleles are linked to one or more alleles responsible for a predisposition to the disease.

Family studies are of help in explaining these possibilities. In addition, because they exclude artifacts of population structure and are not affected by linkage disequilibrium, they may provide evidence for simple dominant or recessive inheritance of susceptibility (Rosenberg and Kidd, 1977). If the major susceptibility is dependent on the inheritance of an HLA-linked gene, then it would be expected that affected siblings would always be identical for one or both HLA haplotypes unless a cross-over or recombination had occurred during meiosis. Cudworth and Woodrow (1975) present strong evidence that this is so by demonstrating in HLA A and B haplotype studies that diabetic siblings usually inherit at least one haplotype in common.

Recombination events during meiosis are rare between various HLA loci, so that identification of HLA A and B haplotypes probably indicates inheritance of the maternal and paternal HLA chromosome regions en bloc. In the present analysis of family genotypes, 4/5 pairs of diabetic siblings possess two identical haplotypes. As the chances of two random siblings having two identical phenotypes are only 25%, this figure indicates a trend to autosomal recessive transmission.

A more specific genetic hypothesis advanced by Rubinstein et al. (1977) suggests that type I diabetes is due to a recessive gene closely linked with HLA-D and with 50% penetrance. If this is so, the first affected child with a sibship should be homozygous for a gene close to HLA and the rest of the sibship should fall into two categories from the point of view of the susceptible gene:

1. HLA identical to the first affected and homozygous for the gene closely linked to the susceptibility gene.

2. HLA non-identical, i.e., sharing with him either one or neither of the carrier haplotypes. This category includes children heterozygous for the gene and the children who lack it altogether.

Analysis of the results in this study on the basis of HLA A/B haplotypes (Table 6.5) failed to support this hypothesis.

The mode of transmission of a susceptibility gene remains obscure, but it seems probable that such a gene would mediate interaction with environmental factors such as viruses that have potential cytopathic effects on the β-cells of the pancreas. Despite lack of evidence of viral initiation of the disease obtained by study of viral antibody titres (Gamble et al., 1969 and 1973), the finding of lymphocytic infiltration of the islets of Langerhans' in newly diagnosed type I diabetes (Gepts, 1965) could be in keeping with viral damage.

An alternative hypothesis is that HLA-linked genes control immune responsiveness to viruses which have a potentially cytopathic effect on the β-cells of the pancreas. A role for the immune response can also be invoked if there were any cross-reaction between viral and pancreatic antigens.

CHAPTER VII.

Immunological Features of Juvenile-Onset Diabetic Patients Correlated to HLA Type.

Introduction

Any explanation of the aetiology of juvenile-onset, insulin-dependent diabetes mellitus (type I) must include the direct experimental evidence of organ specific autoimmune phenomena that are associated with this condition. Antipancreatic cell mediated immunity has been reported (Nerup et al., 1971; MacCuish et al., 1974; Richens, Ancill, and Hartog, 1976) in diabetic patients against various pancreatic antigens and liver mitochondria. This has been described more fully in Chapter III. Also a transient lgG antibody against pancreatic islet cells has been demonstrated in the early stages of disease in these patients (Lendrum et al., 1975; Irvine et al., 1977; Bottazzo et al., 1976) and to occur more persistently in diabetic patients with additional endocrinopathies (Bottazzo et al., 1974; MacCuish et al., 1974). Bottazzo et al. (1974) first described persistent ICAs in patients with polyendocrine disease and their results were supported by MacCuish et al. (1974). Subsequently, antibodies to islet cells were detected in 50% of young, recently diagnosed juvenile-onset type diabetics (Lendrum et al., 1975; Irvine et al., 1977). In addition, MacLaren et al. (1975) have reported that a high proportion of sera from patients with juvenile-onset

diabetes have been shown to react with a cell surface antigen in suspensions of viable cells from a human insulinoma maintained in prolonged tissue culture; this probably represents a distinct antigen-antibody system. The significance of these antibody markers in the aetiology of juvenile-onset diabetes remains uncertain.

Additional evidence of autoimmunity in this condition is seen in animal experiments where it is found that immunisation with homogenates of isolated islets of Langerhan's can induce a transient diabetic state (Nerup et al., 1974a) producing decreased glucose tolerance, cell-mediated immunity against the endocrine pancreas, discrete lymphocytic infiltration of the islets of Langerhan's, and β-cell destruction.

Of further interest is the depression of phytohaemagglutinin transformation of lymphocytes in diabetic patients which reflects the state of cell-mediated immunity (MacCuish et al., 1974b). However, MacCuish et al. conclude that the depression of lymphocyte transformation in these diabetics is indicative of poor control of the disease, i.e., a metabolic disorder as opposed to an inherent altered T-cell reactivity.

In addition to these factors the importance of environmental factors in the onset of this type of diabetes must be recognised. Thus, a bimodal age of onset in those cases below sixteen years of age (Gamble and Taylor, 1976) and a seasonal variation in frequency of new cases (Bloom et al., 1975) suggest viral factors, especially Coxsackie B_4 against which high titres of neutralising antibodies have been demonstrated in the sera of juvenile-onset diabetics (Gamble et al., 1969 and 1973). However, there is no evidence for viral initiation of the disease and results of immunising experimental

animals with virus are variable (Ed. Lancet, 1976). These two lines of evidence are reconciled by theories that suggest that in genetically susceptible individuals a defective immunological response against environmental (viral) factors leads to β-cell destruction either directly or through autoimmune processes (Cudworth and Woodrow, 1975). It is possible that such a genetic susceptibility may be bestowed by genes associated with HLA antigens. As previously described in Chapter VI, these patients show an increased frequency of HLA-B8, B15, and HLA-DW3 and DW4 antigens (Nerup et al., 1974; Cudworth and Woodrow, 1975; Thomsen et al., 1975).

It is important to know how these three factors, autoimmunity, environment and genetic susceptibility interact. In this study juvenile diabetics have been HLA typed and the occurrence of these antigens correlated with islet cell and other autoantibodies, and immune phenomena.

Materials, Methods and Subjects

HLA Phenotyping. Ninety-six juvenile-onset type diabetics were screened for their HLA type as described in Chapter VI.

Leucocyte Migration Test. In 49 patients with juvenile diabetes, the incidence of cell-mediated immune reactions were investigated using the leucocyte migration test as described in Chapter III.

PHA Transformation. Ten patients with juvenile diabetes were tested for lymphocyte transformation as described in Chapter IV.

Autoantibody Identification. Fifty-four juvenile-onset type diabetics were studied for the presence of ICAs and of thyrogastric, mitochondrial,

smooth muscle, reticulin, and antinuclear antibodies as described previously in Chapter V.

Results

Sera from 17 of the 54 (32%) juvenile-onset type diabetics showed the presence of ICAs. The mean duration of diabetes for the whole group was 4 years 11 months (range 1 week - 20 years). When the patients were subdivided into groups according to the duration of disease, 12/17 (70%) of those within 1 year of onset, 5/18 (28%) of those between 1 and 5 years' duration, 0/19 of those greater than 5 years' duration were positive for ICAs. With the small numbers available, no alterations in the prevalence of ICAs could be discerned with the age of the patients. Thus, in those patients examined within 1 year of diagnosis, 2/3 between the ages of 5 and 10 years, 4/6 between the ages of 11 and 15 years, 2/3 of those between 15 and 20 years, and 4/5 of those more than 20 years were positive for ICAs. There was no apparent association between HLA type and the presence of ICAs (Table 7.1). Furthermore, examination of the haplotypes of the 17 patients who were ICA positive revealed no advantage in possessing both HLA-B8 and B15 (1/17) or being homozygous for B8 (0/17) and B15 (1/17) compared with B8, x (7/17) or B15, x (2/17) heterozygotes.

The sera of 53 of these patients were examined for the presence of other circulating antibodies; 33 were found to be positive in this respect. Of these 33 sera, 12 were positive for gastric parietal cell antibodies, 12 for thyroid antibodies and 18 for non-organ specific antibodies. The mean age of these patients was 23.8 years and the mean duration of disease was 5 years 2 months, and 11

Table 7.1. Frequencies of HLA-B8 and B 15 antigens in ICA positive and ICA negative patients with juvenile-onset type diabetes.

Duration of Disease	HLA B8		HLA B 15		HLA B and B 15		All Other HLA Types Tested	
	ICA + ve	ICA - ve	ICA + ve	ICA - ve	ICA + ve	ICA - ve	ICA + ve	ICA - ve
0 - 1 year	5/8	3/8	3/4	1/4	1/1	0/1	3/4	1/4
1 - 5 years	2/4	2/4	0/1	1/1	0/3	3/3	3/10	7/10
5 years	0/8	8/8	0/0	0/0	0/5	5/5	0/6	6/6
Total	7/20	13/20	3/5	2/5	1/9	8/9	6/20	14/20

Table 7.2. Frequencies of HLA-B8 and B15 antigens in Type I diabetics with and without circulating autoantibodies, other than ICAs.

HLA Type	Group I: IDDs with Other Autoantibodies			Non-organ Specific Antibodies Mitochondria, ANA. Smooth Muscle Reticulin	Group 2 IDDs Without Other Autoantibodies
	Total N = 33	Gastric Paritetal Cell Ab N = 12	Thyroid Ab N = 12	N = 18	N = 20
HLA-B15	11 (33%)*	3 (25%)	6 (50%)	7 (39%)	2 (10%)*
HLA-B8	16 (50%)	8 (67%)	8 (64%)	9 (50%)	12 (60%)
HLA-B8 + B15	6 (16%)	2 (17%)	4 (33%)	3 (17%)	2 (10%)
HLA-A1, B8	12 (36%)	6 (50%)	5 (89%)	7 (39%)	11 (55%)

*The frequencies for these two groups are significantly different ($0.05 > p > 0.02$).

Table 7.3. Mean migration indices obtained for groups of insulin-dependent juvenile diabetics with Coxsackie B_4, pancreatic and mitrochondrial antigens.

HLA Type	Mean Migration Indices (± S.D.)		
	Coxsackie B_4	Mitochondria	Pancreas
HLA B8	0.99 ± 0.18 (15)	0.78 ± 0.17 (24)	0.85 ± 0.11 (17)
HLA B15	0.91 ± 0.18 (5)	0.79 ± 0.12 (12)	0.74 ± 0.06 (6)*
B8 + B15	0.96 ± 0.08 (2)	0.81 ± 0.12 (7)	0.77 ± 0.08 (4)
Not B8, B15	0.94 ± 0.21 (18)	0.84 ± 0.21 (24)	0.84 ± 0.09 (15)

*Students t values sig. diff. ($0.02 < p < 0.05$).

(33%) were also ICA positive. The remaining 20 patients did not possess other circulating autoantibodies. Their mean age was 23.4 years and their mean duration of disease was 3 years and 10 months and 5 (25%) were ICA positive. Table 7.2 shows the frequencies of HLA-B8 and B15 and of HLA-A1, B8 in patients with and without circulating autoantibodies. ICAs were not studied for a long enough period to determine whether or not there was an association of thyroid and gastric antibodies with persistent ICAs.

Forty-nine of the insulin-dependent diabetics were tested for cell-mediated hypersensitivity reactions against human pancreas preparations, rat liver mitochondria and particles of Coxsackie B_4 virus. The mean migration indices for each antigen were calculated according to HLA antigen status. HLA-B15 patients showed a significant depression of migration index with the pancreatic antigen compared to patients who possessed neither HLA-B8 or B15. No further correlation was found between migration index and HLA phenotype. Neither was there any correlation between a positive cellular hypersensitivity reaction and the presence of ICAs.

No relationship was found between PHA responsiveness and HLA antigen status. Only 10 diabetic patients were tested and of these two showed a depression of lymphocyte transformation. With numbers as small as this, it is impossible to make any associations.

Discussion

The finding of an overall incidence of islet cell antibodies in 32% of juvenile-onset type diabetics when no allowance was made for the duration of disease is similar to the findings of other investigators (Irvine et al., 1977a; Bottazzo et al., 1976; Nerup et

al., 1976). Because of the transient nature of ICAs, it is not surprising that no association was found with HLA antigens. Secondly, if, as is suspected, that all juvenile-onset type diabetics possess ICAs at some stage before the onset or for a short time after the onset of disease, then the HLA association would be of no value because it would merely reflect the number of diabetics possessing a particular HLA type. The findings presented here show that 7/20 ICA positive diabetics possessed HLA-B8, 3/5 possessed HLA-B15 and neither of these figures was significantly different from the incidence of 6/20 ICA positive patients with all other HLA types tested.

Associations between the persistence of ICAs, the presence of other circulating autoantibodies, and HLA-B8 have been previously reported (Christy et al., 1976; Irvine et al., 1977). The data presented here are insufficient to support this. However, there was a significant association between HLA-B15 and the presence of other circulating autoantibodies that occur in type I diabetics. HLA-B15 also appeared to be associated with a positive migration index with pancreatic antigen. Thus, two immunological features showed a connection with HLA-B15.

The findings presented support other evidence (Christy et al., 1976) that circulating ICAs do not appear in conjunction with cellular immune reactions. A similar lack of correlation is seen in other autoimmune endocrine disorders such as idiopathic Addison's disease (Nerup and Bendixen, 1969; Platz et al., 1974) that, like type I diabetes, is associated with HLA-B8 (Thomsen et al., 1975).

CHAPTER VIII.

Investigation of the Role of Cytotoxic Lymphocytes in Insulin-Dependent Diabetes Mellitus.

Introduction

Cell-mediated tissue injury is believed to be an essential component of delayed hypersensitivity, of some experimental and human autoimmunities, of various allograft phenomena, and of some forms of tumour rejection. Sensitised lymphoid cells will destroy tissue culture cells possessing the antigen to which the cells are sensitised. Perlmann and Holm (1969) state that this type of reaction has been found in all the situations mentioned above.

Cytotoxic reactions can be divided into three categories. The first is a specific reaction, the target cells possessing the antigen to which the donor is sensitised. The second type is cellular non-specific, cytotoxicity being induced by humoral antibodies to target cell antigens. The third type is also non-specific where cytotoxicity is induced by a variety of agents known to stimulate lymphocytes to blast formation and DNA synthesis.

Perlmann and Holm (1969) reviewed the subject and suggested some in vivo correlates to the various cytotoxic actions described:

1. Delayed hypersensitivity where (a) sensitised lymphocytes may exert a direct cytotoxic action on antigenic constituents of tissue cells, or (b) sensitised lymphocytes may become activated by

interaction with antigen leading to nondiscriminatory injury of surrounding tissue cells, or (c) free antibody-antigen complexes on the surface of macrophages may be formed if humoral antibodies are present, or locally produced in some delayed reaction. These may stimulate and induce a cytotoxic action in a certain fraction of nonsensitised lymphocytes. Antigen-antibody complexes on tissue cells may lead to direct destruction of tissue cells by activation of nonsensitised lymphocytes through the Fc receptor attachment.

2. Autoimmunity. This is usually recognised by the presence of humoral autoantibodies. However, the pathogenic role of these antibodies in most diseases is unknown. Cell-mediated immune reactions are considered to be instrumental in the production of tissue lesions in certain autoimmune diseases. Cell-mediated destruction of tissue culture cells has been described for several human and experimental autoimmunities. Co-existing humoral antibodies may act synergistically or antagonistically to the cellular mediators. Antibodies may participate in the production of experimental allergic encephalitis by exerting cytolytic reactions in conjunction with complement or by interacting with lymphoid cells in antibody-induced cytotoxicity.

3. Graft-versus-host reactions where interactions between donor and host lymphoid cells may produce activated lymphoid cells which may provoke tissue lesions by the same mechanism that occurs when lymphocytes are stimulated in mixed culture.

4. Allograft rejection by cell-mediated reactions may be induced by small amounts of antibodies to transplantation antigens. Antibody might be produced locally in the graft or elsewhere and may interact with lymphoid cells infiltrating the graft. It may be that

sensitised lymphocytes destroy graft cells by a direct cytotoxic action. In addition, sensitised cells may recruit nonsensitised cells by a reaction with antigen and the release of non-specific soluble factors.

5. Tumour defence where a prerequisite for an immune response is the presence of tumour-specific antigens which have, in general, characteristics of transplantation antigens. The mechanisms described for allograft rejection may all be involved in tumour destruction.

Govaerts (1960) showed the presence of cytotoxic lymphocytes in transplantation reactions and since then cellular immunity to tumour allografts has been described by numerous investigators (Brunner et al., 1968; Brunner et al., 1970; Canty, Wunderlich, and Fletcher, 1971). Non-specific cytotoxicity induced by PHA has been investigated by Holm and Perlmann (1967), whilst antibody-induced cytotoxicity has been described by MacLennan, Loewi, and Howard (1969), MacLennan and Harding (1970), MacLennan, Loewi, and Harding (1970), and Gelfand, Resch, and Prester (1972).

The direct cytotoxic effects observed with lymphocytes from sensitised donors have been shown to be due to T-cells (Golstein et al., 1972; Cerottini et al., 1971), whilst the presence of T-cells is not required for antibody-dependent cell-mediated cytotoxicity (Van Boxel et al., 1972; O'Toole, Saxon, and Bohrer, 1977). However, it has been found that monocytes and macrophages as well as certain lymphocytes bind to certain antibody-coated cells (LoBuglio, Cotran, and Jandl, 1967; Larsson and Perlmann, 1972; Papamichail and Temple, 1975). The antibody-dependent cytotoxic reaction is mediated by Fc receptor bearing lymphocytes which are different from

both T- and B-cells (Froland, Natvig, and Michaelson, 1974). Greenberg et al. (1973) and Brier, Chess, and Schlossman (1975) concluded that antibody-dependent cytotoxic effector activity is a property of "null" cells which have no surface markers.

Antibody-dependent cytotoxicity occurs in xenogeneic, allogeneic, and autoimmune situations (Perlmann, Perlmann, and Biberfeld, 1972). MacLennan and Loewi (1968) pointed out that antibodies are found in a number of disorders for which an autoimmune aetiology has been proposed and that the effect of serum antibody on lymphocytes might be an important facet of the immunopathological processes of these diseases.

Amongst the autoimmune disorders investigated, cytotoxic mechanisms have been demonstrated in Hashimoto's thyroiditis. Podleski (1972) found that human peripheral lymphocytes from some patients were cytotoxic for mouse mastocytoma target cells coated with human thyroglobulin or microsomal antigen from thyrotoxic thyroid glands. Calder et al. (1973), using thyroglobulin-coated chicken red cells as target cells, found that lymphocytes from patients with Hashimoto's thyroiditis showed greater cytotoxicity than those from control subjects. Calder et al. (1976) showed a significant increase in K-cell cytotoxic activity in patients with autoimmune thyroid disease who were newly diagnosed or untreated compared to age and sex matched controls.

In addition to human autoimmune conditions, Ringertz et al. (1971) showed that lymphocytes from guinea pigs immunised with homologous thyroglobulin lysed thyroglobulin-coated chicken red blood cells. Sera from these animals rendered lymphocytes from normal guinea pigs cytotoxic for thyroglobulin-coated chicken red

cells. Calder et al. (1974a) showed a similar action with normal human lymphocytes in the presence of sera from patients with Hashimoto's thyroiditis.

The purpose of this study was to investigate the possibility that cytotoxic lymphocytes might be operative in juvenile-onset, insulin-dependent diabetes mellitus. The presence of both direct cytotoxic activity (sensitised T-cells) and antibody-dependent (K-cell) cytotoxic activity was investigated.

Materials and Methods

Introduction. Some of the problems involved in cytotoxicity assays have been reviewed by Perlmann, 1972.

In addition to lymphocytes, macrophages from sensitised donors or from normal donors in the presence of small amounts of antibodies can attack target cells specifically. The adherent cells are removed by adsorption to glass or nylon columns, or by the feeding of colloidal iron. Effector cells (lymphocytes) are then added in great excess, but the effects of small amounts of nonlymphocytic cells cannot be disregarded.

The choice of target cell requires consideration of their antigenicity. Other complications are the density of antigenic determinants on the target cell surface and the general susceptibility to lysis of various target cell types depending upon non-immunological factors such as membrane properties, growth cycle, and metabolic requirements.

Primary tissue culture cells have the advantage of natural antigenicity but are not well adapted to tissue culture conditions and are therefore fragile. Established cell lines are better but

may have lost some of their original antigens or acquired new ones due to viral transformation.

Isotope release from target cells represents the most direct assessment of target cell lysis whereas inhibition of incorporation may be due to inhibition of active transport of metabolites. This may not reflect lysis. When isotope release is measured, there is always a background count to be corrected for. The choice of relevant control conditions is critical. Thus, release induced by control lymphocytes may not always be specific, and release values obtained from lymphocyte-free controls may be misleading since culture conditions vary. These problems can usually be overcome when the reactions are fast and the initial reaction velocities are measured. When incubation is over several days, in vitro sensitisation of originally non-immune lymphocytes may occur and increase target cell killing in the controls.

Perlmann and Holm (1969) note that many different methods are used for the assay of cytotoxicity, but these do not measure the same reactions and are therefore difficult to compare. All types of tissue culture cells may serve as target cells. Where target cell antigens are stable in vitro, the culture can be propagated for a long time. Some tissue specific antigens, however, disappear within one week of culture and studies of cytotoxic reactions in autoimmune diseases usually require cultures of freshly explanted tissue.

Chicken erythrocytes have been widely used as target cells and when passively coated with antigen, they are excellent targets for lymphoid cells from donors sensitised to that antigen or after reaction with humoral antibody for lymphoid cells from normal donors.

It is essential to start experiments with effector cell suspensions in which 90-100% of the lymphoid cells are viable. Many workers have stated that the cytotoxic reaction is dependent upon contact between target and effector cell, and that the effector cells must be alive and metabolically viable (Granger and Kolb, 1968; Perlmann and Holm, 1969).

According to Perlmann and Holm (1969), lymphoid cell suspensions used in most experiments consist of mixtures of lymphocytes, monocytes, macrophages, and polymorphonuclear leucocytes and therefore to ascribe effector cell function to a specific cell type, it is necessary to use an homogeneous suspension. However, it is difficult to exclude a small number of non-lymphocytic cells especially when the number of lymphoid cells added is high. Perlmann, Perlmann, and Wigzell (1972) state that previous results indicate that greater than 5% of monocytes or polymorphonuclear leucocytes increase the initial rate of lysis, but give evidence against monocyte involvement where there are less than 0.5% present.

Perlmann and Holm (1969) stated that the best method for measuring cytotoxic reactions is the release of radioisotopes and that ^{51}Cr-chromate is an excellent label for the determination of cell-mediated lysis of tissue culture cells and of chicken erythrocytes. ^{51}Cr is noncovalently bound to proteins and other cell constituents. The chromate is reduced in binding and the isotope is not reutilised. Some of the criteria for an ideal method to determine cell-mediated cytotoxicity are, according to Perlmann and Holm (1969):

1. High sensitivity, to enable detection of cell damage within the shortest possible time of incubation.

2. Low error, allowing measurement of small differences.

3. Quantitative and kinetic measurements of cell damage.

4. Low spontaneous release.

5. That inhibition or promotion of target cell growth by the culture conditions does not affect the results.

They stated the best method to be ^{51}Cr release which fulfills most criteria and can be applied in most experimental conditions and with all types of target cells.

Throughout this work the target cells were chicken red blood cells labelled with ^{51}Cr.

Experimental.

T-cell cytotoxicity. The method used here was based on that described by Calder et al. (1973).

Target Cells. 0.5 ml. of chicken blood was taken from the wing vein of chicks, aged less than 3 months, into preservative-free heparin (Weddell Pharmaceuticals Ltd.) 20 U/ml. The chicken red cells were washed three times in phosphate buffered saline (PBS) and adjusted to a 2% suspension (0.2 ml. packed red cells made up to 10 ml. with PBS). Two ml. of this suspension were incubated for 10 minutes with an equal volume of tannic acid (25 μg/ml.) and the cells then washed in PBS. Two ml. of antigen (200 μg/ml.) were added to the tanned cells and incubated for 10 minutes. The cells were then washed three times in PBS, counted, and adjusted to approximately 10^8/ml. Of this cell suspension, 0.1 ml. was added to 0.1 ml. of sodium ^{51}Cr-chromate, specific activity 100-300 μc/μg chromium (Radiochemical Centre, Amersham, no CJS IP) and incubated for one hour at 37° C. The cells were then washed three times in PBS, counted, and pelletilised until required.

The antigens used in these experiments were pancreatic homogenate, mitochondria or microsomes which were prepared using the method described in Chapter III.

Lymphocytes. 20 ml. of venous blood were taken into preservative-free heparin (20 U/ml.). An equal volume of PBS was added and the mixture layered onto a Ficoll-Triosil column and centrifuged at 400 g. for 20 minutes. The lymphocytes which appear as a band at the Ficoll-Triosil/supernatant interface were removed and washed in PBS followed by two further washes with culture medium. This consists of medium 199 supplemented with antibiotics, glutamine (2 mm. of), and 10% heat inactivated foetal calf serum.

Adherent cells were removed by incubating the suspension in glass bottles for 2 hours at 37° C. The lymphocytes were then counted and adjusted to a final concentration of 5×10^6/ml.

Culture Conditions. 0.5 ml. of the lymphocyte suspension, containing 2.5×10^6 cells, were pipetted in Sterilin tubes (112 mm. x 13 mm.) and 1 ml. of the target cell suspension, containing 10^5 cells was added. This gave a lymphocyte-target cell ratio of 25:1 which is frequently used (Biberfeld and Perlmann, 1970; Perlmann and Perlmann, 1970; Perlmann, Perlmann, and Wigzell, 1972; Calder et al., 1973). In addition, 10^7 sheep red blood cells in 0.1 ml. culture medium were added to prevent spontaneous lysis (Van Boxel et al., 1972; Loewi and Temple, 1972). The cell suspensions were cultured for 18 hours at 37° C. and then centrifuged at 400 g. for 10 minutes. One ml. of the supernatant was removed and counted in a Welltype gamma counter. All cultures were performed in triplicate.

Spontaneous release was estimated by measuring cultures containing unlabelled chicken red cells instead of lymphocytes.

Maximum release was estimated by adding 0.5 ml. of distilled water to 1 ml. of the target cells, and total activity was estimated by measuring the activity of 1 ml. of the target cells.

Results were expressed as a cytotoxic index proposed by Podleski (1972):

$$\frac{\text{Experimental release - spontaneous release}}{\text{Maximum release - spontaneous release}} \times 100$$

where % isotope release = $\frac{\text{mean count x 1.6}}{\text{total activity}} \times 100.$

Patients. Thirteen insulin-dependent diabetics, mean age 28 years 10 months, were compared with 13 control subjects, mean age 26 years 3 months and were age and sex matched where possible.

K-cell Cytotoxicity.

Target Cells. Chicken red cells were washed three times in PBS, counted, and adjusted to approximately 10^8 cells/ml., and labelled with chromium as described.

Lymphocytes. These were prepared as described previously.

Culture Conditions. 0.5 ml. of lymphocytes (2.5 x 10^6) were cultured with 10^5 labelled chicken red cells (1 ml.), 10^7 sheep red cells in a volume of 0.25 ml., and 0.25 ml. of rabbit anti-chicken red cell serum previously diluted to 1/1000 and 1/10000 with culture medium. These dilutions were decided upon after performing a dose-response curve.

Spontaneous release was estimated in tubes where the anti-chicken red cell serum was replaced by medium but with lymphocytes still present. Maximum release and total activity were estimated as previously described.

Results were expressed as specific release: % isotope release in tube with serum - % isotope release in tube with medium.

Patients. Seventeen insulin-dependent diabetics, mean age 24 years 1 month, were compared with 17 control subjects, mean age 25 years 7 months. These were age and sex matched where possible.

Results

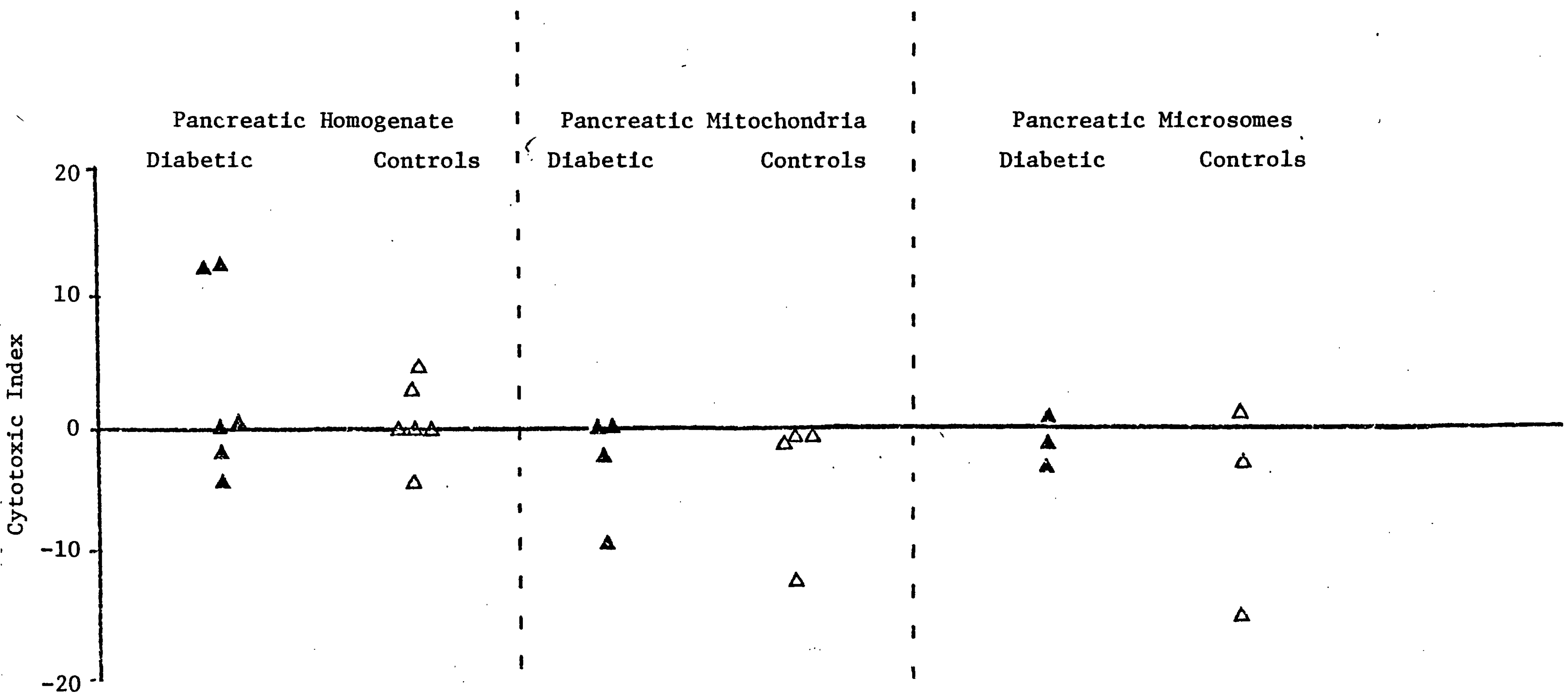
T-cell Cytotoxicity. Table 8.1 shows the cytotoxic indices obtained from thirteen insulin-dependent diabetics and thirteen control subjects. Figure 8.1 shows the cytotoxic indices obtained when lymphocytes were cultured with chicken red cells coated with pancreatic homogenate, mitochondria, or microsomes.

Table 8.1. Cytotoxic indices obtained from 13 insulin-dependent diabetics and 13 control subjects when lymphocytes were cultured with labelled chicken red cells coated with a pancreatic subcellular fraction.

	Cytotoxic Index	
Expt. No.	Diabetic	Control
1	-0.17	-0.17
2	0.00	-2.30
3	0.15	-2.10
4	1.18	2.70
5	12.20	0.00
6	12.30	0.15
7	0.45	4.30
8	1.70	1.40
9	-0.19	-0.76
10	-9.40	-12.00
11	-4.50	-4.00
12	-1.50	-3.20
13	-3.10	-15.00
Mean	0.72	-2.38

The negative values obtained were due to the spontaneous release being higher than the release obtained in either the test or control

Fig. 8.1. Cytotoxic indices obtained when diabetic and control lymphocytes were cultured with labelled chicken red cells coated with various pancreatic antigens.



cultures. Thus, large differences obtained when both values are negative must be considered void.

In only two cases, number 5 and 6, were there any appreciable differences between the cytotoxic indices obtained from using lymphocytes from diabetic patients and control subjects. In these two cases the diabetic cytotoxic indices were 12.20 and 12.30, respectively, compared to control cytotoxic indices of 0.00 and 0.15, respectively. In both cases the target cells were coated with pancreatic homogenate (Fig. 8.1). Patient 5 was a newly diagnosed diabetic whose sera was positive for islet cell autoantibody, whilst patient 6 had become diabetic 6 years previously and was negative for islet cell autoantibody.

In all these experiments, the spontaneous release was rarely above 20% and in the majority of cases was approximately 10% of the total activity. Maximum release corresponded to 70-90% of the total activity.

In addition to these experiments, a further test was performed to verify Calder et al. (1973) results and ensure that the target cells were being adequately coated. Lymphocytes from a patient with Hashimoto's thyroiditis were cultured with target cells coated with thyroglobulin (200 μg/ml.). A cytotoxic index of 22.6 was obtained for the thyroiditis patient compared to 9.7 for the control subject. Calder et al. (1973) found a mean cytotoxic index of 25.46 $\pm$ 3.8 for lymphocytes taken from a patient with Hashimoto's thyroiditis and a mean index of 6.28 $\pm$ 0.80 for control lymphocytes.

K-cell Cytotoxicity. In order to determine the optimal concentration of rabbit anti-chicken red cell serum, an initial experiment was

Fig. 8.2. K-cell cytotoxicity dose-response curve. Specific release of sodium ^{51}Cr-chromate from chicken erythocytes cultured with lymphocytes from a control subject in the presence of varying dilutions of rabbit anti-chicken red cell serum in a volume of 0.25 ml.

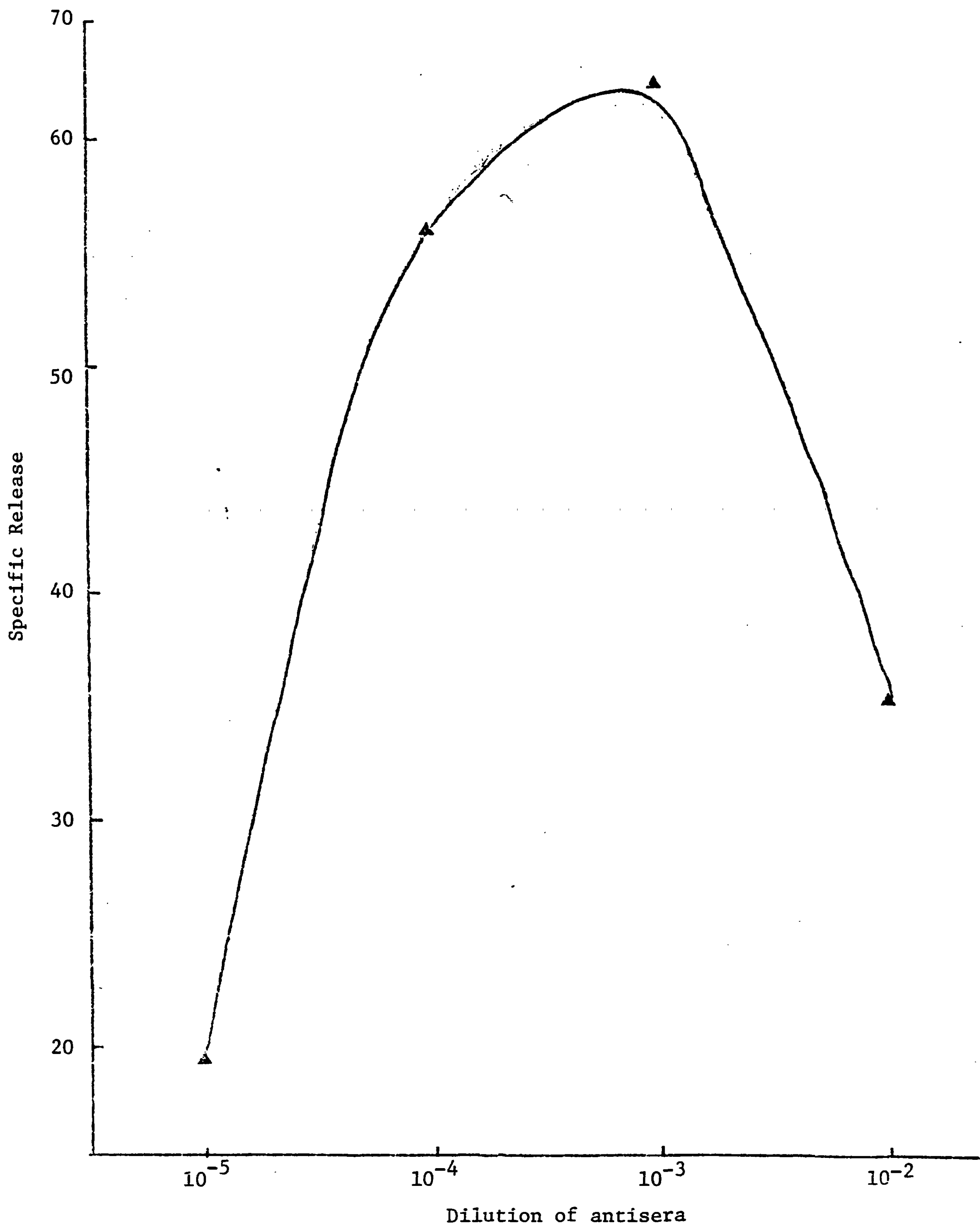


Table 8.2. Specific release of ^{51}Cr from labelled chicken red cells cultured with lymphocytes from insulin-dependent diabetic patients and control subjects in the presence of two dilutions of rabbit anti-chicken red cell serum.

	Specific Release						
	Control			Diabetic			
	Serum dilution			Serum dilution			
	1/1000	1/10000	Mean	1/1000	1/10000	Mean	Islet cell antibody
1	5.35	55.6	54.6	36.0	35.6	35.8	+ ve
2	47.8	51.7	49.8	33.8	38.5	36.2	+ ve
3	49.1	51.1	50.1	48.2	52.1	50.2	- ve
4	58.3	52.8	55.6	50.1	58.8	54.5	+ ve
5	62.5		62.5	51.2	47.0	49.1	- ve
6	54.0	60.4	57.2	58.6	58.6	58.6	+ ve
7	65.0	65.9	65.5	51.0	55.7	53.4	- ve
8	65.0	65.9	65.5	60.1	60.1	60.1	+ ve
9	52.8	51.0	51.9	49.6	60.5	55.1	- ve
10	60.8	53.1	57.0	56.0	64.0	60.0	- ve
11	60.8	53.1	57.0	58.7	60.0	59.4	- ve
12	43.2	44.6	43.9	51.6	54.2	52.9	+ ve
13	52.8	53.8	53.3	44.6	46.3	45.5	- ve
14	45.7	46.3	46.0	38.1	41.2	39.7	- ve
15	62.8	49.3	56.1	64.4	57.9	61.2	- ve
16	61.0	64.7	62.9	58.0	60.4	59.2	- ve
17	45.1	37.9	41.0	40.1	34.6	37.4	- ve
Mean	55.3 ± 7.3	53.6 ± 7.7		50.0 ± 9.0	52.1 ± 9.6		

performed using lymphocytes from a control subject and rabbit anti-chicken red cell serum, in 0.25 ml. aliquots, in the following dilutions, 1/100, 1/1000, 1/10000, 1/100000. Figure 8.2 shows the dose-response curve obtained. The optimum dilutions of rabbit anti-chicken red cell serum for K-cell cytotoxicity are seen to be 1/1000 and 1/10000 and these were used in the experiments described.

Table 8.2 shows the specific release of sodium ^{51}Cr-chromate elicited by lymphocytes from the 17 insulin-dependent diabetics and the 17 control subjects at two dilutions of antiserum. Depression of K-cell cytotoxicity was seen in the diabetic lymphocytes compared to control lymphocytes in experiments 1, 2, 5, 7, 13, and 14. K-cell cytotoxic activity of the diabetic patients was depressed by at least 5% when expressed as specific release of chromium. Four of these patients showed a depression of at least 10%.

When the mean specific release was considered, the diabetic patients' lymphocytes showed a decrease of 5.3% compared to the control subjects' lymphocytes at an antiserum dilution of 1/1000. At a dilution of 1/10000 a decrease of 1.5% was observed.

Discussion

T-cell Cytotoxicity. In this series of experiments the figures for spontaneous release and maximum release corresponded to those reported by other investigators. Calder et al. (1973) found spontaneous release to be between 2% and 15%, whilst maximum release after 20 hours was between 70% and 87%. Podleski (1972) found that coating the cells increased spontaneous release. Thus, he obtained a figure of 20.88% for coated cells and a figure of 17.44% for uncoated cells. Holm and Perlmann (1967) found spontaneous release to be

approximately 25% after 24 hours, although they were using target cells other than chicken red cells which were used here. Perlmann and Holm (1969) stated that under optimal conditions spontaneous release of sodium ^{51}Cr-chromate from chicken erythocytes does not exceed 5% in 24 hours. Perlmann et al. (1970), using chicken erythrocytes as target cells found spontaneous release to be between 10% and 15% usually and never exceeding 20%. They stated that this leakage was due to tannic acid treatment. In the experiments presented here spontaneous release was usually approximately 10% and maximum release was between 70% and 80%.

When the cytotoxic indices obtained with lymphocytes from diabetic patients were compared to those from lymphocytes from control subjects, there were only 2/13 that showed an increase. This is not in keeping with other parameters of T-cell function in diabetes where there is considerable evidence of cell-mediated hypersensitivity using the leucocyte migration inhibition test with the same antigens. To a lesser degree T-cell function, examined by means of lymphocyte transformation using PHA, has also shown a disturbance. In addition, of the two patients showing increased cytotoxic activity, one was newly diagnosed, whilst the other had developed diabetes 6 years previously. It would be expected that greater cell-mediated activity directed against pancreatic antigens would be more apparent during the active stage of the disease process, i.e., prior to or at the onset of diabetes. The results presented here militate against the involvement of T-cell cytotoxic activity in the majority of diabetics. However, only a small number of subjects were tested and these were examined at various times after the diagnosis of diabetes. To get a better idea of cytotoxic T-cell activity patients should be

tested immediately after diagnosis once their condition is stabilised to exclude any metabolic inhibition of lymphocyte activity. Also, the ideal target cell would be cultured islet cells labelled with a radioisotope. Insulinoma cells would also be preferable, but it is possible that their antigenicity may be different than that of the non-malignant islet cell. Alternatively, islet cell homogenates could be used to coat the cells in the manner described here. In the experiments performed here pancreatic homogenate was used which consists of only 1% islet tissue. Consequently, it is possible that not all the target cells were coated with the relevant islet cell antigen.

That this system is reproducible would appear to be borne out by the experiment performed using lymphocytes obtained from a patient with Hashimoto's thyroiditis. The cytotoxic indices obtained closely agreed with the mean indices reported by Calder et al. (1973). They found a cytotoxic index of 25.46 and 6.28 for the thyroiditis patient and control subject, respectively, which compare favourably with the values of 22.6 and 9.7, respectively, which were found here.

<u>K-cell Cytotoxicity</u>. Of the diabetic patients tested, 6/17 showed a depression in cytotoxic lymphocyte activity of at least 5% when compared to control subjects. However, increases in cytotoxic activity were found in some patients, 12 and 15. The largest decreases were found in patients 1, 2, 5, and 7, and this may reflect a depression of K-cell cytotoxic activity in these patients. It could, however, be indicative of a reduced number of K-cells which, in effect, would amount to the same result. In these studies

lymphocytes were counted with no differentiation of the various different types.

To clarify the situation with respect to the two different dilutions of antiserum, mean values for specific release were used. This then takes into account the fact that the optimal activity of one set of lymphocytes might be at a 1/1000 dilution of antiserum, whilst in another set the optimal activity may be at a dilution of 1/10000 or somewhere between the two. Patients showing an overall decrease of at least 5% were 1, 2, 5, 7, 8, 13, 14, whilst those showing an increase were 12 and 15. The mean depression for patients 1, 2, 5, and 7 were 18.8%, 13.6%, 13.4%, and 13.1%, respectively.

Ludwig et al. (1977) found K-cell cytotoxicity depressed in 3 patients from a group of 35 insulin-dependent diabetics. Here 4 diabetics from 17 were depressed by over 10% when compared directly to their own control. Ludwig et al. (1977) concluded that K-cell cytotoxicity did not play a major role in the pathogenesis of diabetes mellitus or that any deficiency was responsible for the lack of immunity found in subjects with this disease. It is known that antibody-dependent cell-mediated cytotoxicity due to non-immune Fc receptor bearing "killer" cells is important in the destruction of bacteria, viruses and tumours and may be of importance in the pathogenesis of autoimmune diseases. From the preliminary results presented here, there is an overall decrease in cytotoxic activity of diabetic lymphocytes as shown by the means. In 4 cases there is a substantial decrease. These results lead to the suggestion that deficiency of this system may well be involved, at least in part, by the lack of defence against infection seen in insulin-dependent diabetics. With respect to the pathogenesis of diabetes mellitus,

conclusions are more difficult. If K-cell cytotoxicity is directly involved, then an increased activity would be anticipated in newly diagnosed diabetes. However, if islet cell damage is the direct result of a viral assault, which later leads to autoimmune manifestations, then depression of K-cell cytotoxicity could be implicated by virtue of the fact that the patient would be more susceptible and less capable of destroying the virus.

Further experiments need to be performed using newly diagnosed type I diabetics, long-term type I diabetics, type II diabetics, and control subjects. In each case a complete dose-response curve for differing dilutions of antiserum needs to be carried out.

CHAPTER IX.

Discussion.

The work presented here, in conjunction with other studies that have been described, does not permit any firm conclusions to be drawn with regard to the aetiology of insulin-dependent diabetes mellitus.

The parameters measured, for the study of immunologic processes that may be involved, confirm the results of other investigators. Cell-mediated hypersensitivity, assessed by the leucocyte migration inhibition test, has been seen to occur against pancreatic fractions, and rat liver mitochondria which is regarded as a general manifestation of autoimmune disease (MacCuish et al., 1974a). The presence of autoantibodies in insulin-dependent diabetes has also been shown and this is in accordance with the results of other investigators (Bottazzo et al., 1974; Nerup et al., 1974; MacCuish et al., 1974). However, the role of islet cell antibody remains to be elucidated as it reacts with other cells of the islets as well as beta cell and results in strictly intracytoplasmic staining. Consequently, their involvement in the pathogenesis of diabetes is difficult to conceive (Kaldany, 1979). Antibody production is more likely to be the result of islet cell damage or, perhaps, as Irvine et al. (1976) suggest, a marker for asymptomatic, latent diabetes. In contrast, MacLaren et al. (1975) have found a cell surface binding antibody to cultured human insulinoma cells in the majority of insulin-dependent diabetics, and recently Lernmark et al. (1978) have demonstrated an

islet cell membrane antibody in the serum of patients with newly diagnosed disease. A possible role of islet cell surface binding antibodies in diabetes may be in initiating antibody-dependent cell-mediated cytotoxic reactions leading to islet cell damage. The relationship between the antibodies discovered by Bottazzo et al. (1974), MacLaren et al. (1975), and Lernmark et al. (1978) is not known.

Further disturbances in immune processes were not found to any great extent. Depression of lymphocyte transformation due to PHA was found in only 2/10 patients tested and this effect may have been the result of metabolic disturbances as suggested by MacCuish et al. (1974b) as opposed to an inherent immune defect implied by Brody and Merlie (1970). In addition, depression of K-cell cytotoxicity was observed in some insulin-dependent diabetics studied in this project, and this may, in part, be responsible for the lack of resistance against infection found in these patients.

If these effects are the result of metabolic disturbance rather than an immune defect, they provide an explanation for the lack of resistance to infection. If, however, this susceptibility to infection is due to an inherent immune defect, then there is a greater possibility of pancreotropic viral agents mediating islet cell damage and leading to diabetes. Had K-cell cytoxic activity been increased in diabetes, a role could be found for surface binding autoantibody of the lgG class in mediating antibody-dependent cell-mediated cytotoxicity. Viruses could still be implicated by postulating that damage to the islets or altered surface antigenic determinants may lead to the production of an autoantibody which, in turn, leads to K-cell destruction of the cells.

From most of the studies performed a cell-mediated effect seems to be the most likely to be involved in the pathogenesis of diabetes, but the results of the T-cell cytotoxicity experiments performed here revealed no further information. This is not in accordance with previous tests of leucocyte migration where a cell-mediated reaction was seen using the same pancreatic fractions. Possible reasons for this have been discussed in Chapter VIII.

Evidence for the involvement of viruses in the aetiology of diabetes mellitus have not been confirmed in the study using mice. Many investigators have shown that viruses can induce diabetes in mice (From et al., 1968; Craighead and McLane, 1968; Craighead and Steinke, 1971; Coleman et al., 1973). However, the use of an outbred strain of CD_1 mice may be responsible for the lack of susceptibility to Coxsackie B_4 virus. As discussed previously, it is possible that viruses may initiate the disease in man through an autoimmune response. Had the mice developed diabetes mellitus, the involvement of the immune system could have been assessed by selectively depressing cell-mediated and humoral components of this system. In addition, similar tests of immune function could have been carried out such as migration inhibition and cytotoxicity tests.

In the clinical studies performed there is evidence for viral involvement. It has been shown that a greater percentage of insulin-dependent diabetic patients possess cell-mediated hypersensitivity to Coxsackie B_4 virus than either insulin-indepedent diabetics or control subjects. Further support for viral involvement has been provided by the results of absorption studies with islet cell antibody positive sera from diabetic patients where the intensity of fluorescence was reduced. Additional evidence, albeit circumstantial, is

provided by the finding of a seasonal incidence which has been shown previously (Gamble and Taylor, 1969).

The studies on the histocompatibility system have confirmed the increased frequency of HLA-B8 and B15 in insulin-dependent diabetic subjects and also the protective effect of HLA-B7. Other studies of the HLA system by various investigators have revealed the existence of genetic heterogeneity reviewed by Cudworth (1978). Herein lies a major difficulty in investigating a disease such as diabetes mellitus in that, in its clinically recognised form, it probably represents several similar conditions having different causes and pathogenetic mechanisms (Rotter and Rimoin, 1978). Most investigations have used insulin-dependent diabetics as one group, whereas various subgroups which are aetiologically distinct may have been included. For example, according to Irvine's (1977) classification, types I and II are both insulin-dependent and type I is further subdivided according to pathogenesis whether it be of a viral or autoimmune nature or both. If all the various possible subgroups are included in one group when investigative procedures are carried out, then it would be difficult to determine the environmental precipating factors. According to Cudworth (1978) any investigation into the aetiology or pathogenesis of diabetes should first consider the role of genetic determination and then elucidate how this susceptibility is exploited by environmental factors.

That the disease is associated with the HLA system is evidence for immunopathologic mechanisms occurring as an isolated incident or more probably as initiated by a sensitising effect by viral or chemical agents (Craighead, 1978). Also, that the disease is associated with HLA-B8, which is found in a number of other autoimmune diseases,

is evidence for an immune component in many patients. It is likely that genes on the sixth chromosome affect immunologic responsiveness, and it has been suggested that HLA antigens on the plasma membrane of beta cells may serve as receptors for viruses and possibly chemicals. Like and Rossine (1976) have shown that mice develop diabetes which is associated with insulinitis and β-cell degranulation when repeatedly given subdiabetogenic doses of streptozotocin. This suggests that subtle chemical injury may result in immunologic sensitisation.

In conclusion, it is attractive to speculate that immune response genes associated with the HLA system confer susceptibility or protectiveness to certain viruses which have a potential affinity for the islet cells, particularly the β-cells. The damage effected by these viruses may lead to an immunologic response due to the release of sequestered antigens or by changing cell surface antigen determinants. Therefore, it is possible that the immune system results in damage after the initiating viral attack. Alternatively, susceptibility to viruses conferred by immune response genes may lead to direct and irreversible effects on the islet cells leading to the clinical manifestations of insulin-dependent diabetes mellitus.

Bibliography.

Adams, S. F. (1926) The seasonal variation in the onset of acute diabetes. Arch. Int. Med. 37 861-864.

Allison, A. C., Beveridge, W. I. B., Cockburn, W. C., East, J., Goodman, H. C., Koprowski, H., Lambert, P. H., van Loghem, J. J., Miescher, P. A., Mimms, C. A., Notkins, A. L., Torrigani, G. (1972) Virus associated immunopathology. Bull. Wld. Hlth. Org. 265-272.

Allison, A. C. (1974) Self tolerance and autoimmunity. New Scientist 725-726.

Allison, A. C., Denman, A. M. (1976) Self tolerance and autoimmunity. Br. Med. Bull. 32 124-129.

Andersen, O. O., Arnung, K., Buschard, K., Cristau, B., Christy, M., Kromenn, H., Nerup, J., Platz, P., Ryder, L. P., Svejgaard, A., Thomsen, M., Vestergaard, B. F. (1977) One year prospective study of juvenile-onset insulin-dependent diabetes in the Copenhagen area. Diabetologia 13 337.

Asherson, G. L., Zembata, M. (1976) Suppressor T-cells in cell-mediated immunity. Br. Med. Bull. 32 158-163.

Baba, S., Morita, S., Nizuno, N., Okada, K. (1976) Autoimmunity to glucagon in a diabetic not on insulin. Lancet 585.

Baird, J. D., Strong, J. A. (1975) Davidson's Principles and Practice of Medicine. 11th edition. Edited by J. Macleod.

Barnes, E. W., MacCuish, A. C., London, N. B., Jordan, J., Irvine, W. J. (1974) Phytohaemagglutinin-induced lymphocyte transformation and circulating autoantibodies in women taking oral contraceptives. Lancet 898-900.

Bennett, P. H., Burch, T. A., Miller, M. (1971) Diabetes mellitus in American (Pima) indians. Lancet II 125-28.

Bendixen, G., Soborg, M. (1969) A leucocyte migration technique for in vitro detection of cellular (delayed type) hypersensitivity in man. Dan. Med. Bull. 16 No. 1 1-6.

Biberfeld, P., Perlmann, P. (1970) Morphological observation on the cytotoxicity of human blood lymphocytes for antibody coated chicken erythocrytes. Exp. Cell. Res. 62 433-440.

Blomgren, H. (1976) Evidence for the release of mitogenic factors by both T- and B-lymphocytes in the human. Scand. J. Immunol. 5 1173-1178.

Bloom, A., Hayes, T. M., Gamble, D. R. (1975) Register of newly diagnosed diabetic children. Br. Med. J. 580-583.

Bodmer, W. F., Bodmer, J. G. (1974) The HLA histocompatibility antigens and disease. 10th Symposium in Advanced Medicine 157-174.

Bottazzo, G. F., Florin-Christensen, A., Doniach, D. (1974) Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet II 1279.

Bottazzo, G. F., Doniach, D. (1976) Pancreatic autoimmunity of HLA antigens. Lancet II 800.

Bottazzo, G. F., Doniach, D., Pouplard, A. (1976) Humoral autoimmunity in diabetes mellitus. Acta Endocrin. [Supp.] 205-208 P. 55-64.

Bottazzo, G. F., Lendrum, R. (1976) Separate autoantibodies to human pancreatic glucagon and somatostatin cells. Lancet 873-876.

Bottazzo, G. F., Mann, J. I., Thorogood, G., Baum, J. D., Doniach, D. (1978) Autoimmunity in juvenile diabetics and their families. Br. Med. J. II 165-168.

Boucher, D. W., Notkins, A. L. (1973) Virus-induced diabetes mellitus. I. Hyperglycaemia and hypoinsulinaemia in mice infected with Encephalomyocarditis virus. J. Exp. Med. 37 1226-1239.

Brier, A. M., Chess, L., Schlossman, S. F. (1975) Human antibody-dependent cellular cytotoxicity. Isolation and identification of a subpopulation of peripheral blood lymphocytes which kill antibody-coated autologous target cells. J. Clin. Invest. 56 1580-86.

Brody, J. I., Merlie, K. (1970) Metabolic and biosynthetic features of lymphocytes from patients with diabetes mellitus: Similarities to lymphocytes in chronic lymphocytic leukaemia. Br. J. Haem. 19 193-201.

Brostoff, J. (1970) Migration inhibition studies in human disease. Proc. Roy. Soc. Med. 63 905-906.

Brown, E. E. (1956) Infectious origin of juvenile diabetes. Arch. Pediatrics 73 No. 6 191-198.

Brunner, K. T., Mauel, J., Cerottini, J. C., Chapuis, B. (1968) Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells in vitro; Inhibition by isoantibody and by drugs. Immunology 14 181.

Brunner, K.T., Mauel, J., Rudolf, H., Chapuis, B. (1970) Studies of allograft immunity in mice. I. Induction, development, and in vitro assay of cellular immunity. Immunology 18 501-515.

Bryceson. Personal Communication.

Burch, G. E., Tsui, C. Y., Harb, J. M., Colcolough, H. L. (1971) Pathologic findings in the pancreas of mice infected with Coxsackie B_4 virus. Arch. Int. Med. 128 40-47.

Burch, G. E., Tsui, C. Y., Harb, J. M. (1972) Pancreatic islet cell damage in mice produced by Coxsackie B_1 and Encephalomyocarditis virus. Experientia 28/3 310-311.

Burnet, M. (1959) The clonal selection theory of acquired immunity. Cambridge University Press.

Calder, E. A. (1974) In vitro tests for cell-mediated immunity in thyroid disease. Proc. Roy Soc. Med. 67 502-506.

Calder, E. A., Irvine, W. J., Davidson, N. McD., Wu, F. (1976) T, B, and K cells in autoimmune thyroid disease. Clin. Exp. Immunol. 25 17-22.

Calder, E. A., McLeman, D., Barnes, E. W., Irvine, W. J. (1972) The effect of thyroid antigens on the in vitro migration of leucocytes from patients with Hashimoto thyroiditis. Clin. Exp. Immunol. 12 429-438.

Calder, E. A., Penhale, W. J., Barnes, E. W., Irvine, W. J. (1973) Cytotoxic lymphocytes in Hashimoto thyroiditis. Clin. Exp. Immunol. 14 19-23.

Calder, E. A., Penhale, W. J., McLeman, D., Barnes, E. W., Irvine, W. J. (1973a) Lymphocyte-dependent antibody-mediated cytotoxicity in Hashimoto thyroiditis. Clin. Exp. Immunol. 14 153-158.

Canty, T. G., Wunderlich, J. R., Fletcher, F. (1971) Qualitative and quantitative studies of cytotoxic immune cells. J. Immunol. 106 No. 1 200-208.

Capner, P., Lendrum, R., Jeffries, D. J., Walker, G. (1975) Viral antibody studies in pancreatic disease. Aut. 16 866-870.

Cattaneo, R., Saibene, V., Pozza, G. (1976) Peripheral T-lymphocytes in juvenile-onset diabetes (JOD) and maturity-onset diabetes (MOD). Diabetes 25 No. 3 223-226.

Cerottini, J. C., Nordin, A. A., Brunner, K. T. (1971) Cellular and humoral response to transplantation antigens. I. Development of alloantibody-forming cells and cytotoxic lymphocytes in graft-versus-host reaction. J. Exp. Med. 134 553.

Christy, M., Bottazzo, G. F., Doniach, D., Nerup, J., Platz, P., Thomsen, M., Ryder, L. P., Svejgaard, A. (1976) Association between HLA-B8 and autoimmunity in juvenile diabetes mellitus. Lancet 2 142.

Coleman, T. J., Gamble, D. R., Taylor, K. W. (1973) Diabetes in mice after Coxsackie B_4 virus infection. Brit. Med. J. III 25.

Coleman, T. J., Taylor, K. W., Gamble, D. R. (1973) The development of diabetes following Coxsackie B virus infection in mice. Diabetologia 10 755-759.

Craighead, J. E. (1975) The role of viruses in the pathogenesis of pancreatic disease and diabetes mellitus. Prog. Med. Virol. 19 161-214.

Craighead, J. E. (1978) Current views on the etiology of diabetes mellitus. N. Engl. J. Med. 299 1439-1445.

Craighead, J. E., McLane, M. F. (1968) Diabetes mellitus: Induction in mice by Encephalomyocarditis virus. Science 162 913-914.

Craighead, J. E., Steinke, J. (1971) Diabetes mellitus-like syndrome in mice infected with Encephalomyocarditis virus. Amer. J. Path. 63 No. 1 119-134.

Cristau, B., Kromann, H., Andersen, O. O., Christy, M., Buschard, K., Arnung, K., Hojland Kristensen, K., Peitersen, B., Steinrud, J., Nerup, J. (1977) Incidence, seasonal and geographical patterns of juvenile-onset, insulin-dependent diabetes mellitus in Denmark. Diabetologia 13 281-284.

Cudworth, A. G. (1978) Type I diabetes mellitus. Diabetologia 14 281-291.

Cudworth, A. G., Woodrow, J. C. (1974) HLA antigens and diabetes mellitus. Lancet 1153.

Cudworth, A. G., Woodrow, J. C. (1975) Evidence for HLA-linked genes in juvenile diabetes mellitus. Br. Med. J. 3 133-135.

Cudworth, A. G., Woodrow, J. C. (1976) Genetic susceptibility in diabetes mellitus: Analysis of the HLA association. Br. Med. J. 2 846-848.

Cudworth, A. G., Woodrow, J. C. (1977) Classification of diabetes. Lancet 949-950.

Danowski, T. S. (1957) Diabetes mellitus with emphasis on children and young adults. Publ. Williams and Wilkins. P. 129.

De Moerloose, Ph., Jeannet, M., Bally, C., Raffoux, C., Pointel, J. P., Sizonenk, P. (1978) HLA and DRW antigens in insulin-dependent diabetes. Br. Med. J. 1 823.

Dippe, S. E., Bennett, P. H., Miller, M., Maynard, J. E., Berquist, K. R. (1975). Lack of causal association between Coxsackie B4 virus infection and diabetes. Lancet 1314-1317.

Doniach, I., Morgan, A. G. (1973) Islets of Langerhan's in juvenile diabetes mellitus. Clin. Endrocrin. 2 233-248.

Drash, A. (1975) Nelson Textbook of Pediatrics. 10th edition. Ed. Vaughan and McKay.

Dudgeon, J. A. (1970) Teratogenic effects of Rubella virus. Proc. Roy. Soc. Med. 63 1254-1259.

Dumonde, D. C., Wolstencroft, R. A., Panayi, G. S., Matthew, M., Morley, J., Howson, W. T. (1969) "Lymphokines": Non-antibody mediators of cellular immunity generated by lymphocyte activation. Nature 224 38-42.

Editorial. (1975) Heritability of diabetes. Br. Med. J. 127-128.

Editorial. (1976) HLA and disease: A conundrum. Br. Med. J. 547-548.

Editorial. (1976) Diabetes mellitus and autoimmunity. Lancet 1124-1125.

Editorial. (1976) B lymphocyte alloantigens in man. Lancet 240-242.

Editorial. (1976) Inheritance of virus-induced diabetes mellitus. Lancet 28.

Eskola, J., Frey, H., Molnar, G., Soppi, E. (1976) Biological rhythm of cell-mediated immunity in man. Clin. Exp. Immunol. 26 253-257.

Farquhar, J. W. (1973) In Textbook of Pediatrics. Ed. Forfar and Arneil.

Finkelstein, S., Zeller, E., Walford, R. L. (1972) No relation between HLA and juvenile diabetes. Tissue Antigens 2 74-77.

Forrest, J. M., Menser, M. A., Burgess, J. A. (1971) High frequency of diabetes mellitus in young adults with congenital rubella. Lancet II 332-334.

Froland, S. S., Natvig, J. B., Michaelson, T. E. (1974) Binding of aggregated lgG by human B-lymphocytes independent of Fc receptors. Scand. J. Immunol. 3 375-380.

From, G. L. A., Craighead, J. E., McLane, M. F., Steinke, J. (1968) Virus-induced diabetes in mice. Metabolism 17 1154-1158.

Gamble, D. R., Kinsley, M. L., Fitzgerald, M. G., Bolton, R., Taylor, K. W. (1969) Viral antibodies in diabetes mellitus. Br. Med. J. 3 627-630.

Gamble, D. R., Taylor, K. W. (1969) Seasonal incidence of diabetes mellitus. Br. Med. J. 3 631-633.

Gamble, D. R., Taylor, K. W. (1976) Epidemiological background to diabetes. Acta Endocrinol. [Suppl.] 205 161.

Gamble, D. R., Taylor, K. W., Cumming, H. (1973) Coxsackie virus and diabetes mellitus. Br. Med. J. 4 260-262.

Gepts, W. (1965). Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes 14 619.

Gelfand, E. W., Resch, K., Prester, M. (1972) Antibody-mediated target cell lysis by non-immune cells. Characterisation of the antibody and effector cell populations. Eur. J. Immunol. 2 419-424.

Girard, J. P., Paychere, M., Cuevas, M., Fernandes, B. (1977) Cell-mediated immunity in an ageing population. Clin. Exp. Immunol. 27 85-91.

Goldstone, A. H., Calder, E. A., Barnes, E. W., Irvine, W. J. (1973) The effect of gastric antigen on the in vitro migration of leucocytes from patients with atrophic gastritis and pernicious anaemia. Clin. Exp. Immunol. 14 501-508.

Golstein, P., Wigzell, H., Blomgren, H., Svedmyr, E. A. J. (1972) Cells mediating specific in vitro cytotoxicity. II. Probable autonomy of thymus processed lymphocytes (T-cells) for the killing of allogeneic target cells. J. Exp. Med. 135 890-906.

Govaerts, A. J. (1960) Cellular antibodies in kidney homotransplantation. J. Immunol. 85 516.

Granger, G. A., Kolb, W. P. (1968) Lymphocyte in vitro cytotoxicity: Mechanisms of immune and non-immune small lymphocyte-mediated target cell destruction. J. Immunol. 101 111-120.

Greaves, M., Janossy, G. (1972) Elicitation of selective T- and B-lymphocyte responses by cell surface binding liganols. Transplant. Rev. 11 87-130.

Greenberg, A. H., Hudson, L., Shen, L., Roitt, I. M. (1973) Antibody-dependent cell-mediated cytotoxicity due to a "null" lymphoid cell. Nature New Biol. 242 111-113.

Hadden, D. R., Connolly, J. H., Montgomery, D. A. D., Weaver, J. A. (1972) Coxsackie B virus and diabetes. Br. Med. J. 729.

Hann, S., Kaye, R., Falkner, B. (1976) Subpopulations of peripheral lymphocytes in juvenile diabetes. Diabetes 25 No. 2 101-103.

Hierholzer, J. C., Farris, W. A. (1974) Follow-up of children infected in a Coxsackie virus B_3 and B_4 outbreak: No evidence of diabetes mellitus. J. Infect. Dis. 129 No. 6 741-746.

Hobart, M.S., McConnell, D. (1975) The Immune System. (Blackwell)

Holm, G., Perlmann, P. (1967) Quantitative studies on phytohaemagglutinin-induced cytotoxicity by human lymphocytes against homologous cells in tissue culture. Immunology 12 525-536.

Irvine, W. J. (1974) Immunological aspects of endocrine disease. Proc. Roy. Soc. Med. 67 499-516.

Irvine, W. J. (1977) Classification of idiopathic diabetes. Lancet I 638-42.

Irvine, W. J., Clarke, B. F., Scarth, L., Cullen, D. R., Duncan, L. J. P. (1970) Thyroid and gastric autoimmunity in patients with diabetes mellitus. Lancet 163-168.

Irvine, W. J., Gray, R. S. McCallum, C. J. (1976) Pancreatic islet cell antibody as a marker for asymptomatic and latent diabetes and prediabetes. Lancet 1097-1102.

Irvine, W. J., McCallum, C. J., Gray, R. S., Duncan, L. J. P., Farquar, J. W., Vaughn, H., Morris, P. J. (1977) Pancreatic islet cell antibodies in diabetes mellitus correlated with the duration and type of diabetes, coexistent autoimmune disease and HLA type. Diabetes 26 No. 2 138.

Irvine, W. J., MacCuish, A. C., Campbell, C. J., Duncan, L. J. P. (1976) Organ-specific cell-mediated autoimmunity in diabetes mellitus. Acta Endocrin. [Suppl.] 205-208 P. 65-76.

Isacson, P. (1967) Myxovirus and autoimmunity. Prog. Allergy 10 256-292.

Jacobsson, H., Blomgren, H. (1975) Characterisation of mouse cells releasing or responding to mitogenic factor induced by phytomitogens in vitro. J. Immunol. 114 No. 5 1631-1637.

Junker, K., Egeberg, J., Kromann, H., Nerup, J. (1977). An autopsy study of the islets of Langerhans in acute-onset juvenile diabetes mellitus. Acta. Path. Microbiol. Scand. 85 699-706.

Kaldany, A. (1979) Autoantibodies to islet cells in diabetes mellitus. Diabetes 28 102-105.

Kanich, R. E., Craighead, J. E., Kessler, J. B. (1973) Lesions of renal glomeruli in mice after virus-induced diabetes mellitus-like disease. Diabetologia 9 203-209.

Keen, H., Jarrett, R. J. (1976) Epidemiology of diabetes. In Diabetes Mellitus, Diagnosis and Treatment. Vol. 4. Ed. K. E. Sussman and R. J. Metz.

Koch, C., Nielsen, H. E. (1975) Fractionation of PHA by crossed immunoelectrophoresis and specific immunosorption. Scand. J. Immunol. 4 121-124.

Kreeftenberg, J. G., Leerling, M. F., Loggen, H. G. (1975) B and T cell markers on human lymphoblasts after stimulation with mitogen or antigen. Clin. Exp. Immunol. 22 121-125.

Landgraf, R., Landgraf-Leurs, M. M. C., Lander, T., Scholz, S., Kuntz, B., Albert, E. D. (1976) HLA haplotypes and glucose tolerance in families of patients with juvenile-onset diabetes mellitus. Lancet II 1084-85.

Larsson, A., Perlmann, P. (1972) Study of Fab and F(ab')$_2$ from rabbit lgG for capacity to induce lymphocyte-mediated target cell destruction in vitro. Int. Arch. Allergy 43 80-88.

LeCompte, P. M., Legg, M. A. (1972) Insulinitis (Lymphocytic infiltration of pancreatic islets) in late-onset diabetes. Diabetes 21 762-769.

Lendrum, R., Nelson, P. G., Pyke, D. A., Walker, G., Gamble, D. R. (1976) Islet cell, thyroid, and gastric autoantibodies in diabetic identical twins. Br. Med. J. 1 553-555.

Lendrum, R., Walker, G. (1975) Serum antibodies in human pancreatic disease. Gut. 16 365-371.

Lendrum, R., Walker, G., Cudworth, A. G., Woodrow, J. C., Gamble, D. R. (1976) HLA-linked genes and islet cell antibodies in diabetes mellitus. Br. Med. J. 1 1565-1567.

Lendrum, R., Walker, G., Gamble, D. R. (1975) Islet cell antibodies in juvenile diabetes of recent onset. Lancet 880-883.

Lernmark, A., Freedman, Z. R., Hofmann, C., Rubenstein, A. H., Steiner, D. F., Jackson, R. L., Winter, R. J., Traisman, H. S. (1978) Islet cell surface antibodies in juvenile diabetes mellitus. N. Engl. J. Med. 299 375-380.

Levy, N. L., Notkins, A. L. (1971) Viral infections and diseases of the endocrine system. J. Infect. Dis. 124 94.

Like, A. A., Rossini, A. A. (1976) Streptozotocin-induced pancreatic insulinitis: New model of diabetes mellitus. Science 193 415-417.

Lindenmann, J., Klein, P. A. (1967) Viral oncolysis: Increased immunogenicity of host cell antigens associated with influenza virus. J. Exp. Med. 126 93-108.

LoBuglio, A. F., Cotran, R. S., Jandl, J. H. (1967) Red cells coated with immunoglobulin G: Binding and sphering by mononuclear cells in man. Science 158 1582-1585.

Loewi, G., Temple, A. (1972) Cytotoxicity of immune guinea pig cells. I. Investigation of a correlation with delayed hypersensitivity and a comparison of cytotoxicity of spleen, lymph node, and peritoneal exudate cells. Immunology 23 559-567.

Lomnitzer, R., Rabson, A. A., Koornhof, H. J. (1975) Production of leucocytes inhibitory factor (L.I.F.) and macrophage inhibitory factor (M.I.F.) by PHA-stimulated lymphocytes. Clin. Exp. Immunol. 22 522-527.

Lowry, O. H., Rosebrough, N. J., Farr, L., Randall, R. J. (1951) Protein measurement with Folin-phenol reagent. J. Biol. Chem. 193 265-75.

Ludwig, H., Kraft, D., Schernthaner, G., Scheiner, O., Mayr, W. R. (1977) Antibody-dependent cell-mediated cytotoxicity in insulin-dependent diabetes. Diabetologia (Abstracts) 13 415.

MacCuish, A. C., Barnes, E. W., Irvine, W. J., Duncan, L. J. P. (1974) Antibodies to pancreatic islet cells in insulin-dependent diabetics with coexistent autoimmune disease. Lancet 1529-1531.

MacCuish, A. C., Jordan, J., Campbell, C. J., Duncan, L. J. P., Irvine, W. J. (1974a) Cell-mediated immunity to human pancreas in diabetes mellitus. Diabetes 23 693-697.

MacCuish, A. C., Urbaniak, S. J., Campbell, C. J., Duncan, L. P. J., Irvine, W. J. (1974b) Phytohaemagglutinin transformation and circulating lymphocyte subpopulations in insulin-dependent diabetic patients. Diabetes 23 708-712.

MacLaren, N. K., Huang, S., Fogh, J. (1975) Antibody to cultured human insulinoma cells in insulin-dependent diabetes. Lancet 997-999.

Maclean, N., Ogilvie, R. F. (1959) Observations on the pancreatic tissue of young diabetic subjects. Diabetes 8 83-91.

MacLennan, I. C. M., Hardind, B. (1970) The role of immunoglobulins in lymphocyte-mediated cell damage in vitro. II. The mechanism of target cell damage by lymphoid cells from immunised rats. Immunology 18 405-412.

MacLennan, I. C. M., Loewi, G. (1968) Effects of specific antibody to target cells on specific and non-specific interaction with lymphocytes. Nature 219 1069-1070.

MacLennan, I. C. M., Loewi, G., Harding, B. (1970) The role of immunoglobulins in lymphocyte-mediated cell damage in vitro. I. Comparison of the effects of target cell specific antibody and normal serum factors on cellular damage by immune and non-immune lymphocytes. Immunology 18 397-404.

MacLennan, I. C. M., Loewi, G., Howard, A. (1969) A human serum immunoglobulin with specificity for certain homologous target cells which induces target cell damage by normal human lymphocytes. Immunology 17 897-910.

Mahmoud, A. D. F., Rodman, H. M., Mandel, M. A., Warren, K. S. (1976) Induced and spontaneous diabetes mellitus and suppression of cell-mediated immunologic responses. J. Clin. Invest. 52 362-367.

Majsky, A., Jakoubkova, J. (1976) Loss of HLA antigens associated with hormonal state. Lancet 859.

Mann, J. I., Thorogood, M., Smith, P. G. (1978) Space clustering of juvenile-onset diabetics. Lancet 1 1369-1370.

Mannick, J. A. (1973) New look at HLA typing--importance of the four antigens. N. Engl. J. Med. 288 No. 14 735-736.

Marx, J. L. (1975) Suppressor T-cells: Role in immune regulation. Science 188 245-247.

Maugh, T. H. (1975) Diabetes: Epidemiology suggests a viral connection. Science 188 347-351.

Maugh, T. H. (1975a) Diabetes: Model systems indicate viruses as a cause. Science 188 436-438.

McCrae, W. M. (1963) Diabetes mellitus following mumps. Lancet 1 1300-1301.

McDevitt, H. O., Bodmer, W. F. (1974). HLA, Immune response genes and disease. Lancet 1269-1275.

McKay, D. G., Margaretten, W. (1967) Disseminated intravascular coagulation in virus diseases. Arch. Int. Med. 120 129-152.

Murray, R. K., Suss, R., Pitot, H. C. (1967) Isolation and characterisation of cytoplasmic components of cancer cells. Methods in Cancer Research. II 244.

Neel, J. V. (1977) The genetics of juvenile-onset type diabetes mellitus. [Editorial] N. Engl. J. Med. 297 1062-63.

Nelson, P. G., Arthur, L. J. H., Gurling, K. J., Gamble, D. R., Taylor, K. W. (1977) Familial juvenile-onset diabetes. Br. Med. J. 2 1126.

Nelson, P. G., Pyke, D. A., Cudworth, A. G., Woodrow, J. C., Batchelor, J. R. (1975) Histocompatibility antigens in diabetic identical twins. Lancet 2 193-194.

Nelson, P. G., Pyke, D. A., Gamble, D. R. (1975) Viruses and the aetiology of diabetes: A study in identical twins. Br. Med. J. 4 249-51.

Nerup, J., Bendixen, G. (1969) Anti-adrenal cellular hypersensitivity in Addison's disease. II. Correlation with clinical and genological findings. Clin. Exp. Immunol. 5 341.

Nerup, J., Binder C. (1973) Thyroid, gastric, and adrenal autoimmunity in diabetes mellitus. Acta endocrin. 72 279-286.

Nerup, J., Andersen, O. O., Bendixen, G., Egeberg, J., Poulsen, J. E. (1971) Antipancreatic cellular hypersensitivity in diabetes mellitus. Diabetes 20 424-427.

Nerup, J., Andersen, O. O., Bendixen, G., Egeberg, J., Poulsen, J. E. (1973) Antipancreatic hypersensitivity in diabetes mellitus. Antigenic activity of foetal calf pancrea and correlation with clinical type of diabetes. Acta Allerg. 28 223.

Nerup, J., Andersen, O. O., Bendixen, G., Egeberg, J., Poulsen, J. E., Vivien, M., Westrup, M. (1973a) Antipancreatic, cellular hypersensitivity in diabetes mellitus. Experimental induction of antipancreatic, cellular hypersensitivity and associated morphological β-cell changes in the rat. Acta Allerg. 28 231-249.

Nerup, J., Platz, P., Andersen, O. O., Christy, M., Lyngsoe, J., Poulsen, J. E., Ryder, L. P., Thomsen, M., Nielsen, L. S., Svejgaard, A. (1974) HLA antigens and diabetes mellitus. Lancet 864-866.

Nerup, J., Andersen, O. O., Bendixen, G., Egeberg, J., Poulsen, J. E., Vivien, M., Westrup, M. (1974a) Glucose intolerance and islet damage in mice immunised with homologous endocrine pancreas. Horm. Metab. Res. 6 173.

Nerup, J., Platz, P., Andersen, O. O., Christy, M., Egeberg, J., Lyngsoe, J., Poulsen, J. E., Ryder, L. P., Thomsen, M., Svejgaard, A. (1976) HLA, autoimmunity and insulin-dependent diabetes mellitus. In Creutzfeldt, W., Kobberling, J., Neel, J., Eds. The Genetics of Diabetes Mellitus. Springer-Verlag, Berlin, Heidelberg, New York.

Notkins, A. L. (1977) Virus-induced diabetes mellitus. Arch. Virol. 54 1.

Notkins, A. L., Mahar, S., Scheele, C., Goffman, J. (1966) Infectious virus-antibody complex of chronically infected mice. J. Exp. Med. 124 81-97.

Notkins, A. L., Mergenhagen, S. E., Howard, R. J. (1970) Effects of virus infections on the function of the immune system. Ann. Rev. Microbiol. 24 525-538.

O'Toole, C., Saxon, A., Bohrer, R. (1977) Human lymph node lymphocytes fail to effect lysis of antibody-coated target cells. Clin. Exp. Immunol. 27 165-171.

Papamichail, M., Temple, A. (1975) Characterisation of the human effector cell causing antibody-mediated cytotoxicity. Clin. Exp. Immunol. 20 459-67.

Perlmann, P. (1972) Lymphocyte cytotoxicity. Technical aspects. Trans. Proc. IV 295.

Perlmann, P., Holm, G. (1969) Cytotoxic effects of lymphoid cells in vitro. Adv. Immunol. 11 117-193.

Perlmann, P., Perlmann, H. (1970) Contactual lysis of antibody-coated chicken erythrocytes by purified lymphocytes. Cell Immunol. 1 300-315.

Perlmann, P., Perlmann, H., Biberfeld, P. (1972) Specifically cytotoxic lymphocytes produced by preincubation with antibody-complexed target cells. J. Immunol. 108 558-561.

Perlmann, P., Perlmann, H., Wasserman, J., Packalen, T. (1970) Lysis of chicken erythrocytes sensitised with PPD by lymphoid cells from guinea pigs immunised with Tubercle Bacilli. Int. Arch. Allergy 38 204-216.

Perlmann, P., Perlmann, H., Wigzell, H. (1972) Lymphocyte mediated cytotoxicity in vitro. Induction and inhibition by humoral antibody and nature of effector cells. Transplant. Rev. 13 91-114.

Platz, P., Ryder, L. P., Staub-Nielsen, L., Svejgaard, A., Thomsen, M., Nerup, J., Christy, M. (1974) HLA and idiopathic Addison's disease. Lancet 289.

Podleski, W. K. (1972) Cytotoxic lymphocytes in Hashimoto's thyroiditis. Clin. Exp. Immunol. 11 543-548.

Potter, M. R., Moore, M. (1977) The effect of adherent and phagocytic cells on human lymphocyte PHA responsiveness. Clin. Exp. Immunol. 27 159-164.

Ragab, A. H., Hazlett, B., Cowan, D. H. (1972) Response of peripheral blood lymphocytes from patients with diabetes mellitus to phytohaemagglutinin and candida albicans antigen. Diabetes 21 No. 8 906-907.

Richens, E. R., Ancill, R. J., Gough, K. R., Hartog, M. (1973) Cellular hypersensitivity to mitochondrial antigens in diabetes mellitus. Clin. Exp. Immunol. 13 1-7.

Richens, E. R., Irvine, W. J., Williams, M. J., Hartog, M., Ancill, R. J. (1974) Cellular hypersensitivity to mitochondrial antigens in diabetes mellitus and its relationship to the presence of circulating autoantibodies. Clin. Exp. Immunol. 17 71-75.

Richens, E. R., Hartog, M., Ancill, R. J. (1976) Autoimmunity and viral infection in diabetes mellitus. Clin. Exp. Immunol. 23 40-46.

Ringertz, B., Wasserman, J., Packalen, T., Perlmann, P. (1971) Cellular and humoral immune responses in experimental autoimmune thyroiditis. Int. Arch. Allergy 40 917-927.

Roitt, I. M. (1971) Essential Immunology. Blackwell Publication, P. 152.

Roitt, I. M. (1974) Essential Immunology. Blackwell Scientific Publication.

Roitt, I. M., Doniach, D. (1965) Methods used in the investigation of autoimmune diseases. In Symposium of the 5th Congress of Int. Acad. Path. 1-5. Blackwell Scientific Publication.

Roitt, I. M., Torrigiani, G., Greaves, M. F., Brostoff, J., Playfair, J. H. L. (1969) Cellular basis of immunological responses. Lancet 367-371.

Rosenberg, L. E., Kidd, K. K. (1977) HLA and disease susceptibility: A primer. N. Engl. J. Med. 297 No. 19 1060-1063.

Ross, M. E., Hayashi, K., Notkins, A. L. (1974) Virus-induced pancreatic disease: Alterations in concentration of glucose and amylase in blood. J. Infect. Dis. 129 No. 6 669-674.

Rotter, J. I., Rimoin, D. L. (1978) Heterogeneity in diabetes mellitus--update 1978. Diabetes 27 No. 5 599-605.

Rubinstein, P., Suciu-Foca, N., Nicholson, J. F., Fotino, M., Molinard, A., Harisiadis, L., Hardy, M. A., Reemtsma, K., Allen, F. H. (1976) The HLA system in the families of patients with juvenile diabetes mellitus. J. Exp. Med. 143 1277-1282.

Rubinstein, P., Suciu-Foca, N., Nicholson, J. F. (1977) A recessive gene closely linked to HLA D and with 50 percent penetrance. N. Engl. J. Med. 297 1036-1040.

Scott, E. M., Griffiths, I. V. (1957) Diabetes mellitus in Eskimos. Metabolism 6 320-325.

Shaw, J. F., Kansal, P. C., Gatti, R. A. (1976) Diabetes mellitus, chromosomal aberration, and malignancy. Lancet 315.

Singal, D. P., Blajchman, M. A. (1973) Histocompatibility (HLA) antigens, lymphocytotixic antibodies and tissue antibodies in patients with diabetes mellitus. Diabetes 22 No. 6 429-432.

Smithells, R. W., Sheppard, S., Marshall, W. C., Peckham, C. (1978) Congenital rubella and diabetes mellitus. Lancet 1 439.

Sorborg, M. (1967) In vitro detected of cellular hypersensitivity in man. Acta. Med. Scand. 182 167-174.

Sonksen, P. H. (1972) Aetiology and epidemiology of diabetes. Br. J. Hosp. Med. 151-156.

Sorenson, R. L., Shank, R. D., Elde, R. P. (1975) Immunoperoxidase demonstration of human serum globulin binding to islet tissue. Diabetes 24 No. 2 230-237.

Steinke, J., Soeldner, S. J. (1977) In Harrison's Principles of Internal Medicine. Thorn, G. W., Adams, R. D., Braunwald, E., Isselbacker, K. J., Petersdorf, R. C., Eds. Blakiston Publication.

Suciu-Foca, N., Rubinstein, P. (1976) Recombinations in juvenile diabetes mellitus. Lancet 1 371-372.

Sultz, H. A., Hart, B. A., Zielezny, M. (1975) Is mumps an etiologic factor in juvenile diabetes mellitus? J. Pediatric. 86 654-656.

Svejgaard, A., Ryder, L. P. (1976) Interaction of HLA molecules with non-immunological ligands as an explanation of HLA and disease associations. Lancet 2 547-549.

Tauber, J. W. (1976) "Self": Standard of comparison for immunological recognition of foreigness. Lancet 2 291-293.

Thomsen, M., Nerup, J., Platz, P., Christy, M., Andersen, O. O., Ryder, L. P., Staub-Nielsen, L., Ramussen, K., Svejgaard, A. (1975) MLC typing in juvenile diabetes mellitus and idiopathic Addison's disease. Transplant. Rev. 22 125.

Trowell, H. (1974) Incidence of diabetes in children. (Letter). Lancet 1510.

Turk, J. L., Polak, L., Parker, D. (1976) Control mechanisms in delayed-type hypersensitivity. Br. Med. Bull. 32 No. 2 165-169.

van Boxel, J. A., Stobo, J. D., Paul, W. E., Green, I. (1972) Antibody-dependent lymphoid cell-mediated cytotoxicity: No requirement for thymus-derived lymphocytes. Science 175 194-196.

van Loghem. (1965) Viral infection and idiopathic autoimmune disease. Vox. Sang. 10 1-5.

Wales, J. K., Hambling, M. H. (1973) Coxsackie B virus and diabetes. (Letter) Br. Med. J. 120.

Wellman, K. F., Amsterdam, D., Brancato, P., Volk, B. W. (1972) Fine structure of pancreatic islets of mice infected with the M variant of the Encephalomyocarditis virus. Diabetologia 8 349-357.

Wiktor, T. J., Kuwert, E., Koprowski, H. (1968) Immune lysis of rabies virus-infected cells. J. Immunol. 101 No. 6 1271-1282.

Williams, R. C., Messner, R. P. (1975) Alterations in T and B cells in human disease states. Annual. Rev. Med. 26 181-192.

Wright, P. H., Gingerich, R. L., King, S., Lacy, P. E. (1976) Pancreatic lesions induced in rabbits and guinea pigs with pancreatic antigens. Clin. Exp. Immunol. 25 328-337.

Zimmet, P., Taft, P., Guinea, A., Guthrie, W., Thoma, K. (1977) The high prevalence of diabetes mellitus on a central Pacific island. Diabetologia 13 111-115.