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**THE CLONING AND CHARACTERIZATION OF A
HUMAN INSULIN AUTOANTIBODY IN TYPE I DIABETES MELLITUS**

Mimi L. Yurack

Thesis submitted to the Department of Biochemistry in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

University of Ottawa,
Ottawa, Ontario, Canada

January, 1993



Mimi L. Yurack, Ottawa, Canada, 1993



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Abstract

Insulin autoantibodies (IAA) are, by definition, present in an individual who has not received exogenous insulin. The objectives of this project were: (1) to obtain monoclonal IAA-secreting cell lines, (2) to clone and characterize by sequence analysis the variable regions of the light and heavy chains (V_L and V_H) of a human monoclonal IAA, and (3) to determine the degree of restriction of the V_L and the V_H gene usage at the onset of Type I DM.

Type I DM patients seropositive for IAA were identified using a competitive insulin autoantibody assay. Their lymphocytes were isolated, immortalized with Epstein-Barr Virus and cloned. Two monoclonal insulin antibody-secreting cell lines were obtained: (1) 1-0.5G1 was obtained from a Type I DM patient treated with exogenous human insulin, and (2) 3-0.5F10 was obtained from a non-insulin treated Type I DM patient; the monoclonal antibody secreted by 3-0.5F10 is therefore an IAA.

The V_L and V_H cDNAs of the monoclonal IAA secreted by the 3-0.5F10 cell line were cloned and sequenced. The genes were assigned to well defined germ-line elements, the influence of somatic hypermutation in the clonal evolution of IAA was evaluated and the sequences were compared with other antibody sequences.

PCR amplification using IAA V_H and V_L specific primers followed by Southern analysis using IAA specific probes was performed. All of the Type I DM patients studied at the time of diagnosis had B lymphocytes which were producing antibodies with IAA-homologous V_L and V_H . Thus, the usage of IAA-homologous V_L and V_H elements in antibodies was found to be restricted in Type I DM patients at the time of diagnosis.

This thesis is dedicated to my Lord, my Strength and my Song.

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To my husband Paul, your love, faith and wisdom form the backbone of this thesis.

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Introduction

Diabetes mellitus is a heterogeneous group of disorders characterized by hyperglycemia owing to an absolute or relative deficiency of insulin. The major types of primary diabetes are the Type I or insulin-dependent diabetes mellitus and the Type II or non-insulin-dependent diabetes mellitus. Type I DM is an autoimmune disease in which the β cells of the pancreas are destroyed with resultant insulin insufficiency and clinical diabetes.

The human pancreas contains 10^5 – 10^6 islets of Langerhans scattered throughout the exocrine parenchyma (Ogilvie, 1937). These structures contain several hundred endocrine cells arranged around a dense capillary network (Gepts and In't Veld, 1987). Alongside the blood vessels, the islets are penetrated by unmyelinated nerve fibers of the adrenergic, cholinergic or peptidergic type accompanied by Schwann cells (Gepts and In't Veld, 1987). Reliable estimates of the total and relative endocrine mass have been made available (Stefan et al., 1982): when all endocrine cells are taken into account, including those outside the islet, the quantitatively predominant cell type is the insulin-containing β cell (60-70%, v/v), followed by the glucagon-containing α cell (10-20%) and the pancreatic polypeptide-containing PP cell (10-20%), whereas the somatostatin-containing δ cell is least abundant (5-10%). In absolute terms, the mean endocrine cell mass in normal individuals is 1.6% of the total gland weight.

The pathogenesis of Type I DM was linked to the pancreas over one hundred years ago (Mering and Minkowski, 1889). At the beginning of this century investigators reported that diabetic individuals had islet lesions that included inflammatory reactions (Lernmark, 1987). However, the islets of Langerhans had not yet been linked to the site of insulin production, and the etiology and pathogenesis of diabetes

remained unclear. It was not until 1921 that Canadian investigators extracted insulin from the pancreas which proved to ameliorate most of the diabetic symptoms (Banting, 1922). Although this work proved to be worthy of the Nobel Prize, it soon became clear that insulin was not a cure for diabetes.

Following many decades of investigation, Gepts reported that the major morphologic alteration in the pancreas at the onset of Type I DM was a disruption in the architecture of the islets of Langerhans and a loss of β cells associated with infiltration by mononuclear cells or "insulinitis" (Gepts, 1965). Insulinitis was thought to be of pathogenic importance when it was shown to be associated with experimental diabetes mellitus induced by immunization with insulin (Lacy and Wright, 1965), by injections of low dose streptozotocin (Like and Rossini, 1976), or following infection with "diabetogenic" viruses (Rayfield and Ishimura, 1987). Further support for the hypothesis that Type I DM has an autoimmune pathogenesis was obtained from the demonstration of a close association to the HLA locus on chromosome 6 (Platz et al., 1981), of cell-mediated immunological abnormalities including increased activated T-lymphocyte number and cytotoxicity (Drell and Notkins, 1987) as well as the presence of autoantibodies directed against natural components of islet cells (Bottazzo et al., 1974).

Insulin Autoantibodies

The immunological attack on the pancreatic β cells begins several years before the presentation of clinical Type I DM and occurs during an asymptomatic "prediabetic" period (for review see Palmer, 1987). One aspect of this autoimmune process is the occurrence of autoantibodies directed against islet antigens in the blood of newly diagnosed Type I DM patients and in individuals during the pre-diabetic period. These autoantibodies include islet cell antibodies, both cytoplasmic and cell-surface directed

(Ziegler et al., 1990), antibodies to the 64 kD autoantigen which has been putatively identified as the enzyme glutamic acid decarboxylase (Baekkeskov et al., 1990), antibodies to a bovine albumin peptide which cross-react with the p69 antigen at the surface of the β cell (Karjalainen et al., 1992) and insulin autoantibodies (Palmer et al., 1983).

Insulin autoantibodies (IAA), by definition, are present in an individual or animal that has not received exogenous insulin. The association of IAA as part of the autoimmune picture in Type I DM was first recognized by Palmer et al. (1983) with the finding of IAA in 18% of newly diagnosed, untreated, Type I diabetic patients. The percentage of IAA positive individuals at diagnosis of Type I DM obtained by the same investigators increased to 40% with refinements in their insulin antibody assay (Palmer et al., 1987). This incidence is in agreement with the reported frequencies ranging from 20% to 50% found by various other laboratories (Atkinson et al., 1986, Karjalainen et al., 1986, Wilkin et al., 1985, Arslanian et al., 1985, McEvoy et al., 1986). The variability between laboratories relates to the differences in assay technique and patient selection (Wilkin, 1990). Attempts have been made to standardize the measurement of IAA by establishing Annual International Immunology and Diabetes Workshops (Wilkin et al., 1987, Wilkin et al., 1988, Palmer et al., 1990, Kuglin et al., 1990, Greenbaum et al., 1992).

It has been postulated that IAA, alone or in conjunction with other autoantibodies such as islet cell antibodies (ICA), may be a marker of pancreatic β cell destruction in the human (Atkinson et al., 1986, Srikantha et al., 1986, Dean et al., 1986, McEvoy et al., 1986, Betterle et al., 1987, Ludvigsson et al., 1988, Thivolet et al., 1988). Interestingly, it has been found that absolute levels of IAA correlate with rate of progression of the disease (Ziegler et al., 1989), as well as with the magnitude of T lymphocyte alterations

in the pre-diabetic state (Faustman et al, 1991). IAA production may also be human leukocyte antigen (HLA) restricted in newly diagnosed Type I DM patients with a strong positive association of IAA with the DR4 antigen (Ziegler et al., 1991). Further evidence that IAA may be a marker for the autoimmune process was obtained in studies with animal models of Type I DM including the BioBreeding (BB) rat (Wilkin et al., 1986) and the nonobese diabetic (NOD) mouse (Pontessilli et al., 1987, Marayama et al., 1988, Ziegler et al, 1988).

Development of IAA

Although the precise mechanism whereby the immune system reacts against self-antigens in autoimmune diseases has not been elucidated, several hypotheses for the development of IAA may be considered. Firstly, just as some diabetic patients develop insulin antibodies when treated with human insulin, presumably due to aggregation or change in tertiary structure of the manufactured insulin, a patient with Type I DM may develop IAA as a result of an alteration in insulin structure occurring during β cell destruction (Palmer, 1987). Alternatively, proinsulin, or even preproinsulin, instead of insulin, may be the antigen (Kaplan, 1984, Kuglin et al., 1988, Böhmer et al., 1991).

It has been proposed that a completely unrelated antigen that shares one or more epitopes with insulin may be involved (Saegusa et al., 1985). In accordance with this hypothesis, it has also been postulated that autoantibodies arise as a result of an immune response to foreign antigens such as infectious agents that share, by molecular mimicry, a common structure with the host protein (Query and Keene, 1987). One or more of these mechanisms may come into play in the development of IAA in humans or animal models.

The Role of IAA in Type I DM

To date, the role of IAA in the pathophysiology of Type I DM remains undefined. Four major possibilities may be considered (Palmer, 1987). Firstly, IAA may not be involved in the disease but are only markers of the β cell destruction. For instance, one or more of the mechanisms previously discussed in the development of IAA in Type I DM could be activated during the pancreatic β cell attack and result in concomitant IAA production. IAA would thus be considered a result of, rather than a cause of, β cell damage. Another possibility is that IAA are not even necessarily markers for the β cell attack but that they just tend to be present in individuals genetically predisposed to autoimmune disease, while a proportion of these individuals are in turn predisposed to develop Type I DM. In support of this hypothesis is the finding of IAA in individuals with other autoimmune disorders such as Graves disease (an autoimmune disease of the thyroid) and systemic lupus erythematosus (a chronic inflammatory disease which can affect any organ of the body) (Wilkin and Nicholson, 1984, Diaz and Wilkin, 1987), in the insulin autoimmune syndrome (Seino et al., 1986), and in individuals treated with drugs such as penicillamine (Benson et al., 1985). These findings suggest that IAA can occur without being markers of the β cell damage.

A third hypothesis is that IAA are directly involved in the autoimmune β cell destruction in Type I DM. In support of this possibility, insulin immunoreactivity has been localized to the plasma membrane of pancreatic β cells (Kaplan et al., 1983). This provides a mechanism whereby IAA could recognize membrane insulin and lead to the autoimmune destructive process of Type I DM (Kaplan, 1984). Potential mechanisms include complement-dependent-antibody mediated cytotoxicity or antibody-dependent cellular cytotoxicity. However, experimental evidence for either of these mechanisms in IAA action is lacking. Interestingly, anti-insulin receptor antibodies, which are also found in the serum of some recent onset Type I diabetic patients (Maron et al., 1983,

Ludwig et al., 1987), have been shown to be anti-idiotypic to IAA (Schechter et al., 1982, Uchigata et al., 1991), that is they bound to and could be blocked by IAA. If insulin receptor antibodies in Type I DM are anti-idiotypic antibodies and IAA were in part responsible for the immunological attack of the pancreatic β cells, the insulin receptor antibodies might be part of the body's natural defense by binding IAA and thereby down-regulating the IAA-mediated β cell destruction.

In opposition to the hypothesis that IAA either directly or indirectly mediate the β cell destructive process are other reports that fail to find a relationship between residual β cell function and insulin antibody titer (Hoogwerf et al., 1985) as well as the lack of an association between IAA and C-peptide levels at diagnosis of Type I DM (Arslanian et al., 1985, Sochett and Daneman, 1989). However, care must be taken not to equate a poor insulin response to glucose with β cell destruction or diabetes, as the phenomenon may be reversible (Tarn et al., 1988), and the β cell response to intravenous amino acids may remain intact while the glucose response is lost (Ganda et al., 1984).

The fourth hypothesis is that IAA are part of the body's defense against immunologic destruction of the β cells. The finding of autoantibodies in individuals without autoimmune disease suggests that autoantibodies are the norm and part of health rather than always associated with autoimmune disease (Sanz et al., 1989). In fact, about 50% of IgM-secreting cells appearing after both mitogen and immunogen injection into mice produce IgM that is not specific for the immunogen but that does have specificity for self-IgG (Dresser, 1978). Cohen and Cooke suggested that between 10% and 30% of B-lymphocytes in normal healthy individuals were engaged in producing autoantibodies (Cohen and Cooke, 1986). They proposed that autoantibodies may help protect against autoimmune disease by blinding the immune system to

environmental epitopes cross-reactive with self by passive blocking of the antigen (Cohen and Cooke, 1986). The hypothesis that IAA may be protective is supported by the finding of Fineberg and co-workers that the presence of low affinity IAA in nondiabetic individuals is a normal event (Fineberg et al., 1991).

Whether one of the above or another mechanism accounts for the association of IAA with Type I DM is at present unknown. In fact, the mechanisms discussed above may not be mutually exclusive and possibly the mechanism varies with the antibody idotype. Perhaps we may find that some types of IAA are present in normal individuals and serve a protective function while different types of IAA may be associated with Type I DM.

Immunology of IAA

No group has so far successfully established human hybridomas from the plasma cells of IAA positive Type I DM patients who have not received exogenous insulin, so that the clonal diversity of IAA in Type I DM has not been studied directly. Nevertheless, evidence has been obtained from studies which points to their limited clonality (McLaren et al., 1989, Wilkin and Nicholson, 1984). Previously reported data have shown that IAA are frequently of a single light-chain type and, where it has been possible to obtain a saturation binding curve, apparently oligoclonal or monoclonal (Diaz and Wilkin, 1987). Furthermore, binding studies of insulin variants against a panel of IAA containing sera suggest that IAA are restricted in their binding site (Diaz and Wilkin, 1988). Protein data on the clonality of IAA also suggests that IAA are synthesized by a restricted and conserved B lymphocyte repertoire (Trump and Ziegler, 1988).

Several groups have demonstrated the restriction of an IAA response that is human insulin specific and that involves the epitope incorporating B30 threonine, which is the single site of amino acid substitution between human and porcine insulin (Wilkin and

Nicholson, 1984, Sklenar et al., 1987, SODOYEZ-GOFFAUX et al., 1988). It has been demonstrated by the use of mouse monoclonal antibodies raised against human insulin that highly epitope-restricted IAA, such as those that bind exclusively to human insulin, are represented in the normal repertoire of a polyclonal response to insulin (Mirza et al., 1987). Thus, with regard to human insulin-specific IAA, there seems to be an unusual degree of clonal restriction in the autoimmune response of such individuals rather than an aberration in the epitope.

Monoclonal studies have also revealed the existence of individual clones within the immune repertoire that cross-react equally with human, porcine, and bovine insulin (Storch et al., 1985, Mirza et al., 1987, Sanz et al., 1989). Cross-reaction could imply the recognition of a single epitope common to many insulins by a monoclonal serum or it could imply unshared epitopes by a polyclonal serum. Evidence for clonal restriction in cross-reactive IAA sera similar to that of human insulin-specific sera remains circumstantial, although a case report suggests that IAA serum derived from a patient with insulin autoimmune syndrome, which demonstrated cross-reactivity with human, porcine, and bovine insulin, was restricted to the epitope incorporating asparagine B3 (Uchigata et al., 1989). Whether the IAA that occur in the insulin autoimmune syndrome are similar to the IAA that occur as part of the autoimmune process in Type I DM has not been determined. By learning more about the B lymphocyte clonality of IAA (by study of the diversity, conservation and epitope preference of antibody clonotypes), information would be gained which would provide insight into the significance of IAA in Type I DM.

Determination of the nucleic acid sequence of the variable (V) region of the light chain (V_L) and of the heavy chain (V_H) of a human monoclonal IAA would provide information of the immune response to endogenous insulin. It would allow the assignment of genes to well defined germ-line elements (V_H and V_L subfamilies), would

allow analysis of D and J segment usage, and would evaluate the influence of somatic hypermutation in the clonal evolution of this response.

Hypothesis

IAA in Type I DM patients are synthesized by a restricted immunoglobulin (Ig) gene pool. The selection and maturation of IAA in Type I DM reflects common epitopes in patients genetically predisposed to this disease and may be important in the pathophysiology of Type I DM.

Objectives

My objectives when undertaking this study were the following: (1) to obtain monoclonal IAA-secreting cell lines, (2) to clone and characterize by sequence analysis the V_L and V_H of human monoclonal IAA derived from Type I DM patients who had not received exogenous insulin, and (3) to determine the degree of restriction of the IAA V_L and V_H gene usage at the onset of Type I DM.

Experimental Approach

The original experimental approach to this project was to generate human monoclonal IAA by fusion of a human myeloma cell line with B lymphocytes of patients seropositive for IAA (Fig. 1). In the course of these studies, a human myeloma cell line (MM-neo) would be developed and characterized as a fusion partner for the generation of human hybridomas. Alternatively, human monoclonal IAA would be generated by cloning Epstein-Barr Virus (EBV) transformed peripheral blood lymphocytes (PBL) of Type I DM patients seropositive for IAA at diagnosis and selecting clones which secreted IAA into the culture medium.

Total cytoplasmic RNA could then be isolated from the monoclonal IAA-secreting EBV transformed peripheral blood lymphocytes (EBV-PBL). Mixed oligonucleotide primers corresponding to the 5' leader sequence and a conserved 3' constant region primer would be used for polymerase chain reaction (PCR) amplification of the monoclonal IAA V_H and V_L . The amplified PCR products would be cloned into plasmid vectors and characterized by sequence analysis.

In order to determine the degree of restriction of the V region gene usage at the onset of Type I DM, specific oligonucleotide primers would be synthesized which correspond to the complementarity determining regions (CDR) of the IAA V_H and V_L . Total cytoplasmic RNA could then be isolated from various EBV-PBL populations established from Type I DM patients at the time of diagnosis. cDNA synthesized from the RNA would be PCR-amplified using the IAA-specific oligonucleotide primers. Southern analysis would then be performed using IAA V-region specific probes. The selection of the V_L and V_H genes would be restricted if PCR fragments of the expected size are detected in other Type I DM patient cDNA.

Figure 1

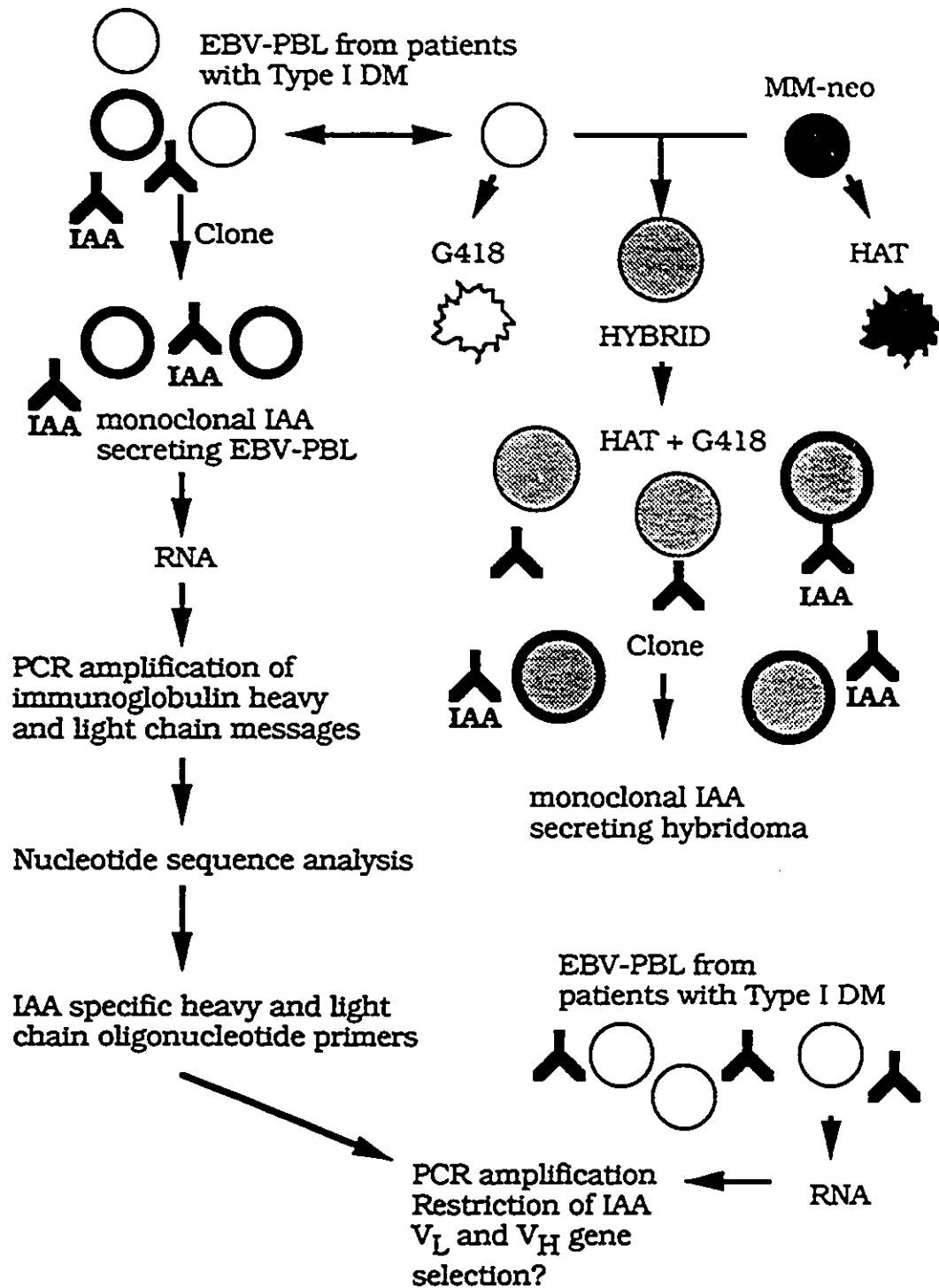
Experimental Approach

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Total cytoplasmic RNA could then be isolated from the monoclonal IAA-secreting EBV-PBL. Mixed oligonucleotide primers corresponding to the 5' leader sequence and a conserved 3' constant region primer would be used for PCR amplification of the monoclonal IAA V_H and V_L . The amplified PCR products would be cloned into plasmid vectors and characterized by sequence analysis.

In order to determine the clonality of the IAA characterized by sequence analysis, specific oligonucleotide primers would be synthesized which correspond to the CDRs of the IAA V_H and V_L . Total cytoplasmic RNA could then be isolated from various EBV-PBL populations established from Type I DM patients at the time of diagnosis and used as the starting material for PCR analysis using these oligonucleotide primers. PCR amplification and Southern analysis of the V_L and V_H cDNA from Type I DM patient EBV-PBL lines at the time of diagnosis could be used to determine the degree of restriction of IAA clonality.

Figure 1



Chapter 1: Measurement of Serum IAA

Introduction

One specific objective was to obtain monoclonal IAA-secreting cell lines from a patient with Type I DM. In order to achieve this objective, it was imperative to identify patients with Type I DM who had circulating levels of IAA. Detectable levels of IAA in the serum of a Type I DM patient ensures that the population of peripheral blood lymphocytes (PBL) from the blood of this same patient contains IAA-secreting B lymphocytes. These lymphocytes could be immortalized by infection with Epstein-Barr Virus (EBV) (Miller et al., 1972) or by fusion with a myeloma cell line (Kozbor and Roder, 1982). The resulting immortalized population of PBL would be likely to include IAA-secreting B lymphocytes which could be cloned.

IAA in the serum of Type I DM patients can be detected either by liquid-phase radioimmunoassay (RIA; Palmer et al., 1983) or by solid-phase enzyme-linked immunosorbent assay (ELISA; Wilkin et al., 1985). Results from the Third International Workshop on the Standardization of IAA and from other studies demonstrated that ELISA and RIA laboratories often obtained different results with the same serum (Palmer et al., 1990, Wilkin et al., 1989, Wilkin et al., 1988). At the Fourth International Workshop on the Standardization of IAA, it was suggested that IAA measured by RIA may be more closely associated with the Type I DM disease process (Levy-Marchal et al., 1991, Greenbaum et al., 1992). The data from the Fifth International Workshop on the Standardization of IAA demonstrated reasonable inter-assay variation for quantitation of IAA by RIA in different laboratories and showed that the use of standards did not improve this variation (Greenbaum et al., 1992).

This chapter describes the establishment of an RIA for the measurement of IAA and the identification of Type I DM patients with detectable levels of serum IAA.

Materials and Methods

Materials

Bovine serum albumin (BSA) , bovine gamma globulin Fractions II and III, dextran and methyl cellulose were purchased from Sigma Chemical Co. The charcoal (decolorizing carbon) was purchased from the J.T. Baker Chemical Co. Human [¹²⁵I] insulin, labeled at tyrosine A14 (2000 Ci/mmol), was purchased from Amersham. Humulin (recombinant human insulin) is a trade mark of the Eli Lilly Co. All the other reagents used in this assay were purchased from Fisher Scientific. The polyclonal guinea pig anti-porcine insulin antibody was kindly provided by Dr. J. Braaten from the Ottawa Civic Hospital (Ottawa, Canada).

Methods

Patient Population

Patients with Type I DM and those at very high risk of developing Type I DM (pre-diabetic) were diagnosed according to the definitions outlined by the National Diabetes Data Group (1979). Serum was obtained from the following categories of patients: **(1) pre-diabetics (before diagnosis of Type I DM)** with normal fasting plasma glucose but with abnormal oral or intravenous glucose tolerance; pre-ketosis prone stage; Type I DM usually diagnosed within 2 years of observing glucose intolerance; **(2) Type I DM patients** at the time of diagnosis, before administration of exogenous insulin, as well as various times after initiation of insulin therapy. Most of these patients were seen at the Pediatric Endocrine Clinics at the Children's Hospital of Eastern Ontario. The collaboration of Drs. Etienne Sochett and Arnold Faight in obtaining the blood samples is gratefully acknowledged. Informed consent was obtained from these patients and/or their parents for blood letting (see Form 1, appendix A). Anonymous control children serum samples were kindly provided by Dr. Hans Heick from the

Medical Biochemistry Laboratory at the Children's Hospital of Eastern Ontario. Of these samples, a number of control sera were randomly selected to establish the baseline IAA level for the control population.

An insulin binding assay for the measurement of IAA in the Type I DM patient and control sera was established. Using a polyclonal guinea pig anti-porcine insulin antibody, the insulin binding assay was characterized. This guinea pig serum was chosen instead of a human anti-insulin serum because it was more abundant. A preliminary experiment was performed to study the specific insulin binding of a guinea pig anti-porcine insulin antibody as a function of serum dilution.

Titration of the Polyclonal Guinea Pig Anti-porcine Insulin Antibody

The competitive insulin autoantibody assay (CIAA) was performed on polyclonal guinea pig anti-porcine insulin antibody of increasing dilutions ranging from 1 in 200 to 1 in 6400 diluted with phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , pH 7.4). Prior to performing the CIAA, the serum was stripped of endogenous insulin by acid charcoal extraction (Srikanta et al., 1986).

The CIAA was performed as described by Vardi et al. (1987): triplicate samples of 150 μl of stripped serum were incubated for 1 h at 4°C with 50 μl reaction buffer (0.04 M phosphate buffer-0.05 % BSA-0.025 % bovine gamma-globulin, pH 7.5) to determine total insulin binding and with 50 μl of the reaction buffer containing humulin at a concentration of 3×10^6 nU/ml serum to determine the nonspecific insulin binding (36 μg humulin per U; Ziegler et al., 1988). [^{125}I] labeled (tyrosine A14) human insulin (specific activity = 2000 Ci/mmol) was added to the tubes to give a final concentration of 10^4 nU/ml stripped serum. The tubes were incubated at 4°C for 7 days. The antibody-bound insulin was precipitated with polyethylene glycol (PEG) and the pellets washed.

The pellets and the supernatants were counted for 9 min and the background counts were subtracted. The triplicate mean total insulin binding (TIB) and nonspecific insulin binding (NIB) were determined:

$$\text{TIB} = \frac{\text{cpm pellet}}{(\text{cpm pellet} + \text{cpm supernatant})} \quad \text{) without unlabeled humulin}$$

$$\text{NIB} = \frac{\text{cpm pellet}}{(\text{cpm pellet} + \text{cpm supernatant})} \quad \text{) with unlabeled humulin}$$

The specific insulin binding (SIB) was then calculated as:

$$\text{SIB} = [\text{mean TIB} - \text{mean NIB}] \times \text{nU tracer/ml serum}$$

Characterization of the CIAA

The CIAA was characterized using the polyclonal guinea pig anti-porcine insulin antibody diluted 1 in 1600 with PBS. Using [^{125}I] labeled human insulin, with humulin as unlabeled competitor, the kinetics of the CIAA were established.

Effect of Stripping Serum on the CIAA

The effect of stripping the polyclonal guinea pig anti-porcine insulin antibody (performed to remove endogenous insulin) prior to performing the CIAA was determined. The polyclonal guinea pig anti-porcine insulin antibody was stripped by acid charcoal extraction (Srikanta et al., 1986) or left unstripped. Triplicate aliquots were incubated for 1 h at 4°C with a constant amount of humulin as cold competitor (3×10^6 nU/ml serum). Increasing concentrations of [^{125}I] labeled insulin were added to the tubes (ranging from $1 - 8 \times 10^4$ nU/ml serum). After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. The insulin binding was determined as previously described. The resulting curves were compared by regression analysis to determine the effect of stripping serum on the outcome of the CIAA.

Binding of [125 I]-labeled Human Insulin as a Function of Unlabeled Competitor

The polyclonal guinea pig anti-porcine insulin antibody was stripped and triplicate aliquots were incubated for 1 h at 4°C with humulin ranging in concentration from 1×10^2 - 12×10^6 nU/ml stripped serum. A constant amount of [125 I] labeled insulin was added to the tubes (10^4 nU/ml stripped serum). After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. Insulin binding was determined as previously described.

Determination of CIAA Intraassay and Interassay Coefficients of Variation (CV)

Polyclonal guinea pig antibody (diluted 1 in 6400 with PBS) and diluted serum from a patient with Type I DM at diagnosis (Patient #10) were used to determine the intraassay coefficient of variation (CV). Patient #10 's serum sample was chosen because it had demonstrated specific insulin binding in preliminary experiments. The Patient #10 serum had been diluted 1 in 3.2 following Ficoll purification of lymphocytes which were used for a related study (chapter 2).

Seven samples of the diluted polyclonal guinea pig antibody and the diluted patient serum were assayed concurrently with and without humulin at a concentration of 3×10^6 nU/ml stripped serum. A constant amount of [125 I] labeled human insulin was added to the tubes (10^4 nU/ml stripped serum). After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. The specific insulin binding was determined as previously described.

The intraassay CV was calculated as follows:

$$\text{Intraassay CV} = (\text{Standard deviation (SD) of the Mean SIB}) / \text{Mean SIB} \times 100 \quad (n=7)$$

The CIAA interassay CV was determined using the polyclonal guinea pig antibody diluted 1 in 6400 or 1 in 3200 with PBS as well as the serum from Patient #10 taken at diagnosis of Type I DM (diluted 1 in 3.2 with PBS following lymphocyte purification). Also included in this determination was the serum from Patient #10 obtained after 10 months of treatment with exogenous insulin (diluted 1 in 3.6 with PBS following lymphocyte purification). The CIAA was performed on these stripped serum samples on seven separate assay days.

The interassay CV was calculated as follows:

$$\text{Interassay CV} = (\text{SD of the Mean SIB}) / \text{Mean SIB} \times 100 \quad (n=7)$$

Determination of the IAA levels in Type I DM patients

CIAA was performed on undiluted serum from Type I DM patients (pre-diagnosis, at diagnosis, and at specified times after initiation of insulin therapy) and controls. The serum samples were stripped and the specific insulin binding was determined as previously described. The polyclonal guinea pig antibody diluted 1 in 6400 and 1 in 3200 with PBS was routinely included in each assay as positive control.

In accordance with the Third International Workshop on the Standardization of Insulin Autoantibody Measurement, the cut-off for IAA positivity was determined to be 3 standard deviations (SD) above the mean specific insulin binding of the control population (Palmer et al., 1990). The CIAA interassay CV was low (approximately 10%), and the specific insulin binding of 74 control subjects, determined on separate assay days, were pooled in order to establish the mean and standard deviation of the specific insulin binding of the control population. Statistical analysis was performed to compare specific insulin binding between groups (Student's t-test). Analysis of variance was performed for the determination of the correlation between age and IAA levels.

Results

Titration of Polyclonal Guinea Pig Anti-porcine Insulin Antibody

Fig. 2 shows the titration curve of the polyclonal guinea pig anti-porcine insulin antibody. The dilution of 1 in 1600 was used for the characterization of the CIAA.

Effect of Stripping Serum on the CIAA

The effect of stripping the serum on the outcome of the CIAA was investigated and the results are shown in Fig. 3. Stripping serum with acid charcoal prior to performing the CIAA significantly increased the sensitivity of the assay (higher total binding in stripped serum compared with unstripped serum; $p < 0.05$, Fig. 3A). The nonspecific binding in the serum was unaffected by acid charcoal stripping (Fig. 3B). The increase in total binding was due to a significantly higher specific insulin binding in the stripped serum compared with the unstripped serum ($p < 0.05$, Fig. 3C).

Binding of [125 I] Labeled Human Insulin as a Function of Unlabeled Competitor

Using stripped guinea pig serum at a dilution of 1 in 1600, binding of the [125 I] labeled human insulin was assessed as a function of unlabeled competitor (humulin) added to the reaction mixture (Fig. 4). Maximal displacement was achieved with 3×10^6 nU humulin/ml stripped serum and thus this quantity of competitor was used in all subsequent assays.

Figure 2

Titration of Polyclonal Guinea Pig Anti-porcine Insulin Antibody

The CIAA was performed on stripped polyclonal guinea pig anti-porcine insulin antibody of increasing dilutions (1 in 200 to 1 in 6400 in PBS). Triplicate samples were incubated for 1 h at 4°C with and without humulin at a concentration of 3×10^6 nU/ml stripped serum. A constant amount of [125 I]-labeled insulin was added to the tubes (10^4 nU/ml stripped serum). After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. The specific insulin binding was determined as described in the Methods. Results are expressed as the mean nU [125 I] bound/ml stripped serum $\pm$ SEM.

Figure 2

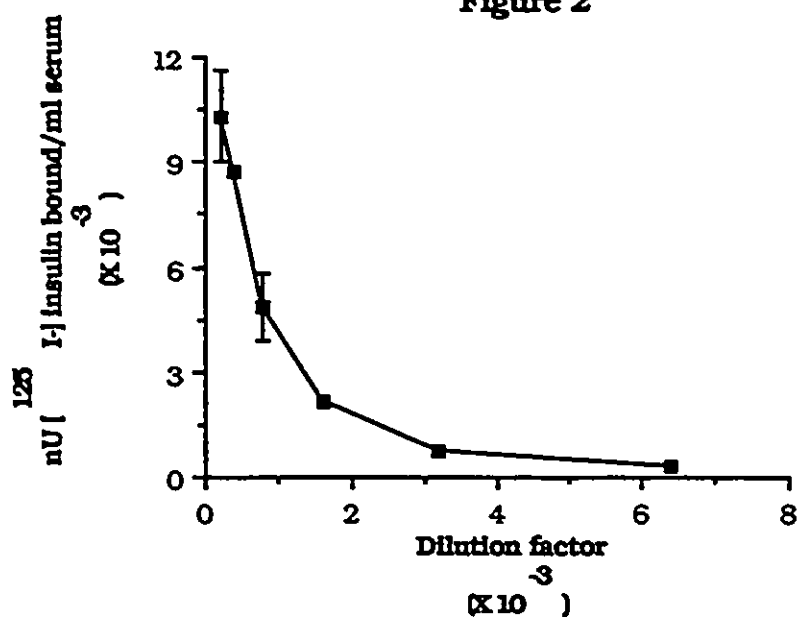


Figure 3

Effect of Acid Charcoal Extraction of Serum on the Insulin Binding in the CIAA

In order to determine total insulin binding, triplicate samples of stripped and unstripped serum were incubated for 1 h at 4°C with reaction buffer to determine total insulin binding and with reaction buffer containing humulin at a concentration of 3×10^6 nU/ml serum to determine nonspecific insulin binding. [125 I] labeled human insulin was added to the tubes to give final concentrations ranging from 1 - 8×10^4 nU/ml serum. After 7 days incubation at 4°C the antibody-bound insulin was precipitated with PEG and the pellets washed.

The pellets and the supernatants were counted for 9 min each. The background counts were subtracted, and the triplicate mean total insulin binding (Fig. 3A), nonspecific insulin binding (Fig. 3B) and specific insulin binding (Fig. 3C) were determined as described in the Methods. Results are expressed as the mean nU [125 I] bound/ml serum $\pm$ SEM. Binding of stripped serum is indicated by the dashed line; binding of unstripped serum is indicated by the solid line. The resulting curves were compared by regression analysis.

*= regression lines were significantly different ($p < 0.05$)

Figure 3A

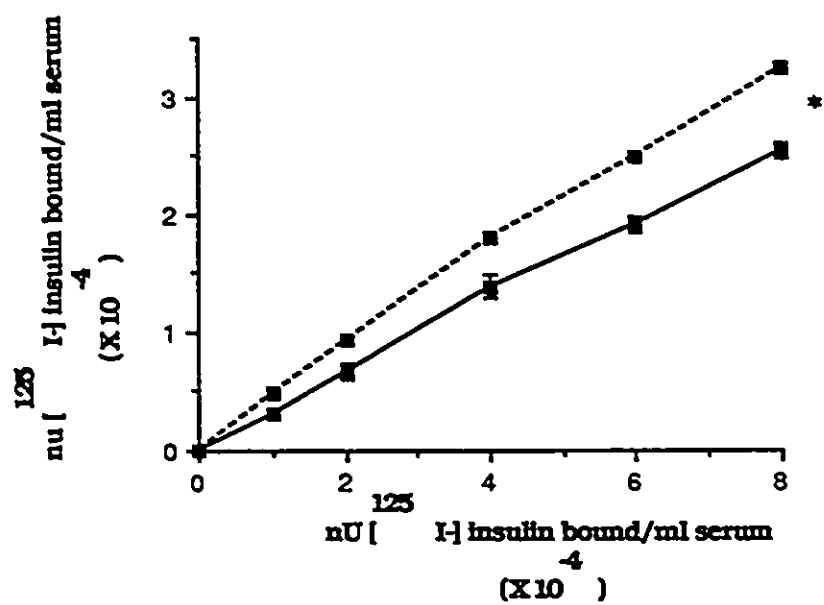


Figure 3B

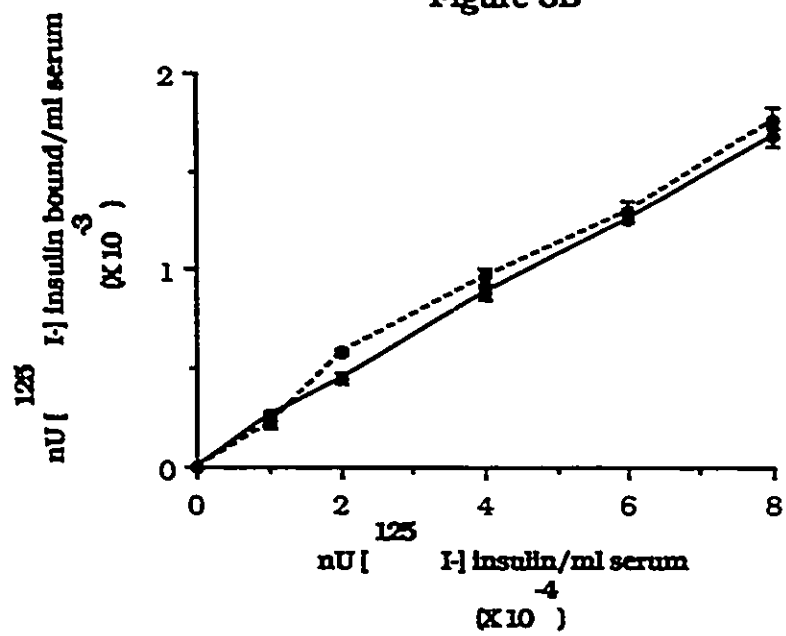


Figure 3C

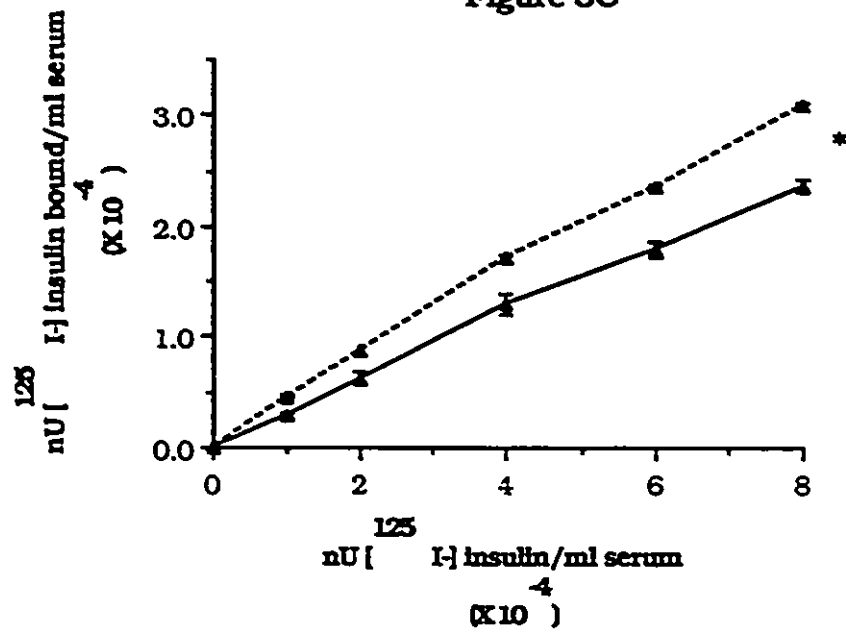
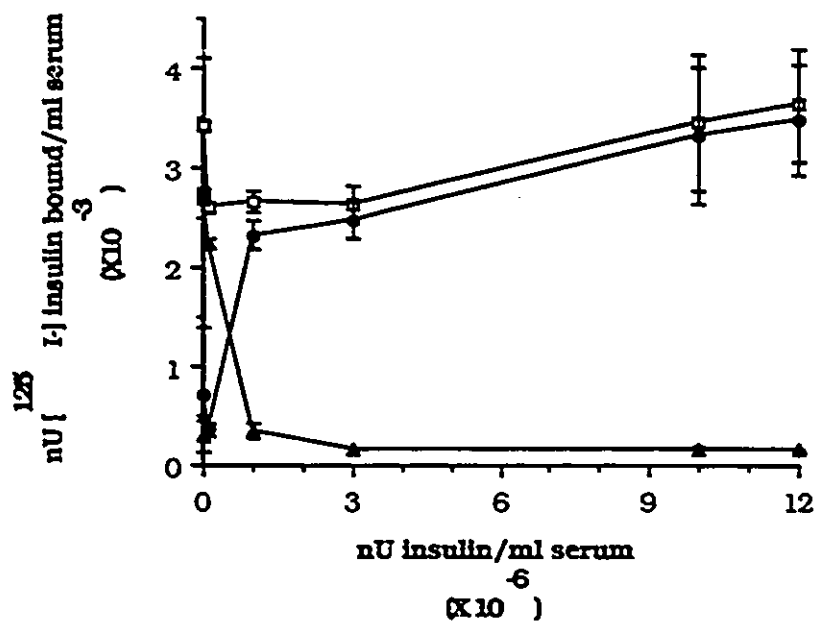


Figure 4

Binding of [125 I]- Labeled Human Insulin as a Function of Unlabeled Competitor

The CIAA was performed on the polyclonal guinea pig anti-porcine insulin antibody diluted 1 in 1600 with PBS and stripped by acid charcoal extraction. Triplicate samples of serum were incubated for 1 h at 4°C with reaction buffer containing humulin ranging in concentration from 1×10^2 to 12×10^6 nU /ml stripped serum. A constant amount of [125 I] labeled human insulin was added to the tubes to give a final concentration of 10^4 nU/ml stripped serum. After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. The total (squares), nonspecific (circles) and specific (triangles) insulin binding were determined as described in the Methods. Results are expressed as the mean nU [125 I] bound/ml stripped serum $\pm$ SEM.

Figure 4



Determination of CIAA Intraassay and Interassay Coefficients of Variation

Diluted serum from a patient with Type I DM at diagnosis as well as the diluted guinea pig anti-porcine insulin antibody were used to determine the intraassay CV (Table 1A). The diluted guinea pig serum had a mean specific insulin binding of 205 nU/ml stripped serum, with an intraassay CV of 5% (n=7). The diluted serum of the patient with Type I DM at diagnosis had a mean specific insulin binding of 34 nU/ml stripped serum with a intraassay CV of 12% (n=7).

The interassay CV was determined for the guinea pig serum at two dilutions and for the diluted serum of Patient #10 at diagnosis as well as after insulin treatment (Table 1B). As determined, the interassay CV for all of the samples ranged from 9 —12%.

Determination of the IAA levels in Type I DM patients

The baseline for IAA levels in our control population was established by measuring the specific insulin binding in 74 serum samples of children seen at CHEO for reasons unrelated to Type I DM. The mean specific insulin binding of this population plus 3 SD was found to be 99 nU/ml stripped serum; this value was set as the cut-off for positivity (Fig. 5A). Fifty percent (11 out of 22) of Type I DM patient serum samples taken at the time of diagnosis demonstrated specific insulin binding levels greater than 99 nU/ml stripped serum (Fig. 5B). The mean specific insulin binding in the Type I DM patient population at diagnosis was significantly higher than in the control population ($p < 0.01$, Table 2).

Of the three patients whose serum IAA levels were assayed in the pre-diabetic period, one was found to be IAA positive 6 months before diagnosis (Patient # 17, Fig. 5C). The specific insulin binding measured in the serum of Patient # 17 at the time of diagnosis had decreased to within normal range.

The serum samples of Type I DM patients who had been injected with human insulin all had specific insulin binding greater than 99 nU/ml (Fig. 5D). The specific insulin binding of the Type I DM patient population treated with human insulin was significantly higher than that of the control population and of the Type I DM patient population at diagnosis ($p < 0.01$, Table 2).

In the small sample studied, no correlation was observed between age and IAA levels ($p = 0.740$, $r = 0.075$) as determined by regression analysis (Fig. 6).

Table 1A

Determination of the Intrassay Coefficient of Variation in the CIAA

The CIAA was performed concurrently on seven samples of: (1) polyclonal guinea pig antibody diluted 1 in 6400 with PBS and, (2) serum from a patient with Type I DM at diagnosis (Patient #10; diluted 1 in 3.2 with PBS). The serum samples were stripped by acid charcoal extraction. Triplicate stripped serum samples were incubated for 1 h at 4°C with and without humulin at a concentration of 3×10^6 nU/ml stripped serum. A constant amount of [125 I] labeled insulin was added to the tubes (10^4 nU/ml stripped serum). After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. The specific insulin binding and the intraassay coefficient of variation (CV) were determined as described in the Methods.

Table 1B

Determination of the Interassay Coefficient of Variation in the CIAA

To determine the interassay CV, the CIAA was performed seven times on separate days on the following samples: (1) polyclonal guinea pig antibody (diluted 1 in 6400 and 1 in 3200 with PBS), (2) serum from Patient #10 at diagnosis with Type I DM (diluted 1 in 3.2 with PBS) and (3) serum from Patient #10 after 10 months of treatment with exogenous insulin (diluted 1 in 3.6 with PBS). Prior to performing the CIAA, the serum samples were stripped by acid charcoal extraction. Triplicate samples were incubated for 1 h at 4°C with and without humulin at a concentration of 3×10^6 nU/ml stripped serum. A constant amount of [125 I-] labeled insulin was added to the tubes (10^4 nU/ml stripped serum). After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. The specific insulin binding and the coefficient of variation were determined as described in the Methods.

Table 1A

Trial #	nU insulin bound/ml serum	
	#10 at diagnosis	GP 1/6400
1	26	196
2	32	192
3	40	212
4	38	220
5	34	202
6	36	198
7	30	216
Mean	34	205
SD	4	10
CV	12%	5%

Table 1B

Trial #	nU insulin bound/ml serum			
	GP 1/6400	GP 1/3200	#10 at diagnosis	#10 insulin-treated
1	210	891	33	471
2	168	1052	38	597
3	208	878	33	558
4	207	1088	41	585
5	199	866	41	623
6	188	855	33	543
7	216	780	38	554
Mean	199	916	37	562
SD	17	112	4	49
CV	9%	12%	11%	9%

Figure 5

Specific Insulin Binding in Controls and Type I DM Patients

Anonymous control children serum samples from the Medical Biochemistry Department at the Children's Hospital of Eastern Ontario (Ottawa, Canada) were randomly selected to establish the baseline IAA level for the control population. Type I DM patients were seen at the Pediatric Endocrine Clinics at the Children's Hospital of Eastern Ontario. The CIAA was performed on serum samples from the following groups:

Figure 5A

Control children

Figure 5B

Type I DM patients at the time of diagnosis

Figure 5C

Patients seen prior to diagnosis of Type I DM

(triangles, Patient #17; squares, Patient #14; circles, Patient #21)

Figure 5D

Type I DM patients treated with human insulin after diagnosis

(triangles, Patient #3; squares, Patient #1, circles, Patient #10)

Triplicate stripped serum samples were incubated for 1 h at 4°C with and without humulin at a concentration of 3×10^6 nU/ml stripped serum. A constant amount of [¹²⁵I] labeled insulin was added to the tubes (10^4 nU/ml stripped serum). After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. The specific insulin binding was determined as described in the Methods.

Results are expressed as the mean nU [¹²⁵I] bound /ml stripped serum. The cut-off for IAA positivity was determined to be 3 standard deviations (SD) above the mean specific insulin binding of the control population (99 nU [¹²⁵I] insulin bound/ml stripped serum; n=74).

Figure 5A

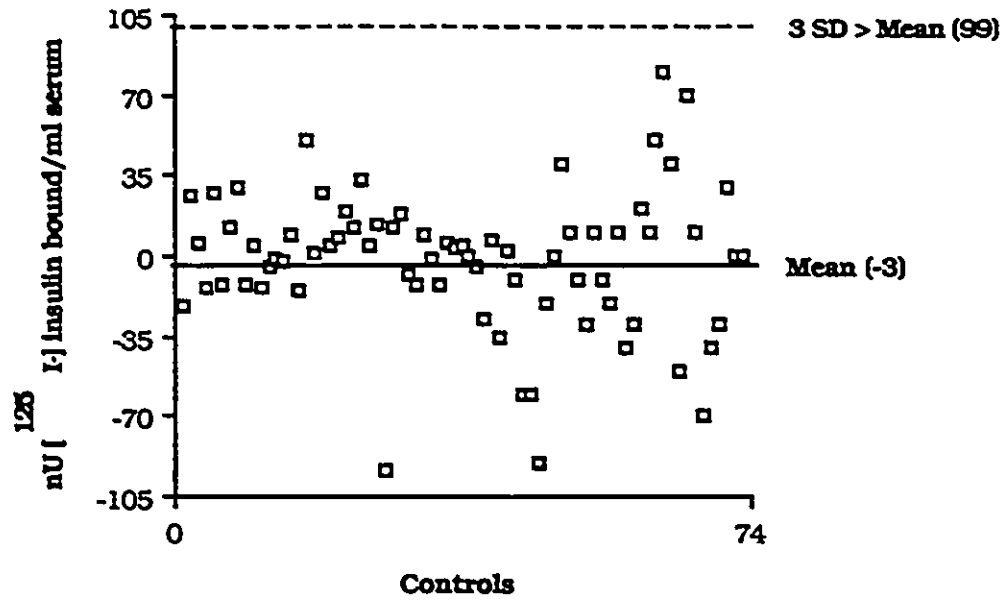


Figure 5B

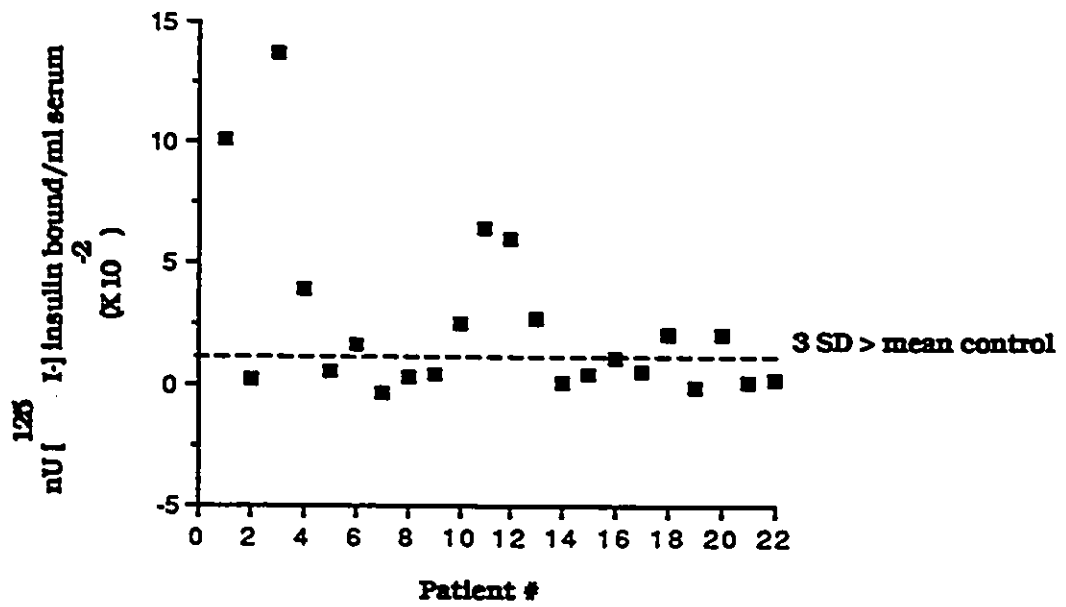


Figure 5C

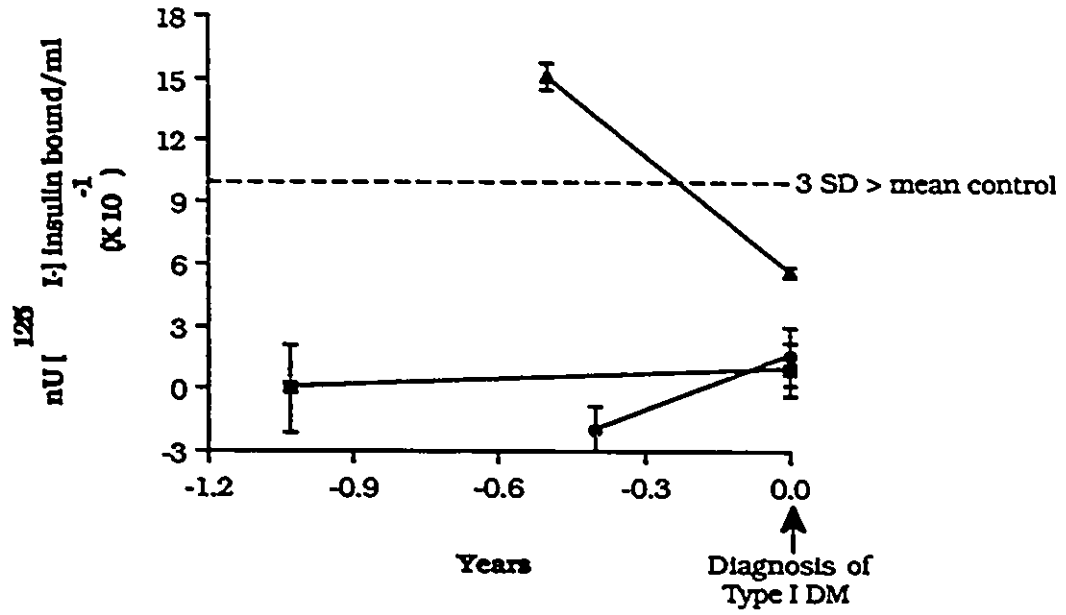


Figure 5D

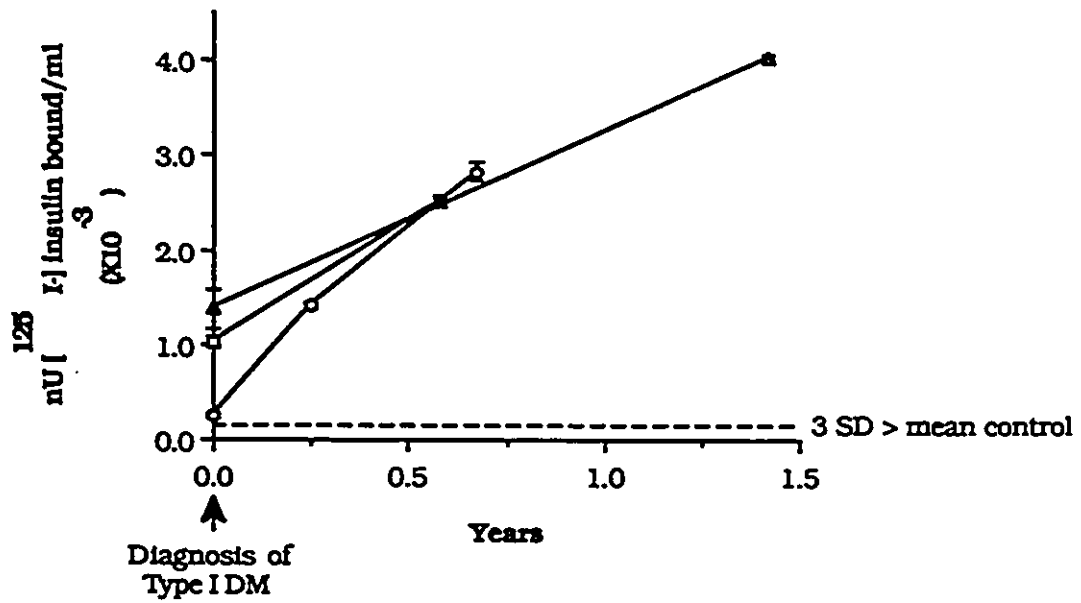


Table 2

**Summary Information of the Results of the CIAA
Performed on the Various Groups Studied**

Triplicate stripped serum samples were incubated for 1 h at 4°C with and without humulin at a concentration of 3×10^6 nU/ml stripped serum. A constant amount of [¹²⁵I] labeled insulin was added to the tubes (10^4 nU/ml stripped serum). After 7 days at 4°C, the insulin bound antibody was PEG-precipitated and the pellets washed. The specific insulin binding was determined as described in the Methods. The cut-off for IAA positivity was determined to be 3 standard deviations (SD) above the mean specific insulin binding of the control population (99 nU [¹²⁵I] insulin bound/ml stripped serum; n=74). Student's t-test was used to compare results (Fastat software).

- a p < 0.01 compared with control
- b p < 0.05 compared with control
- c p < 0.01 compared with Type I DM at diagnosis

Table 2

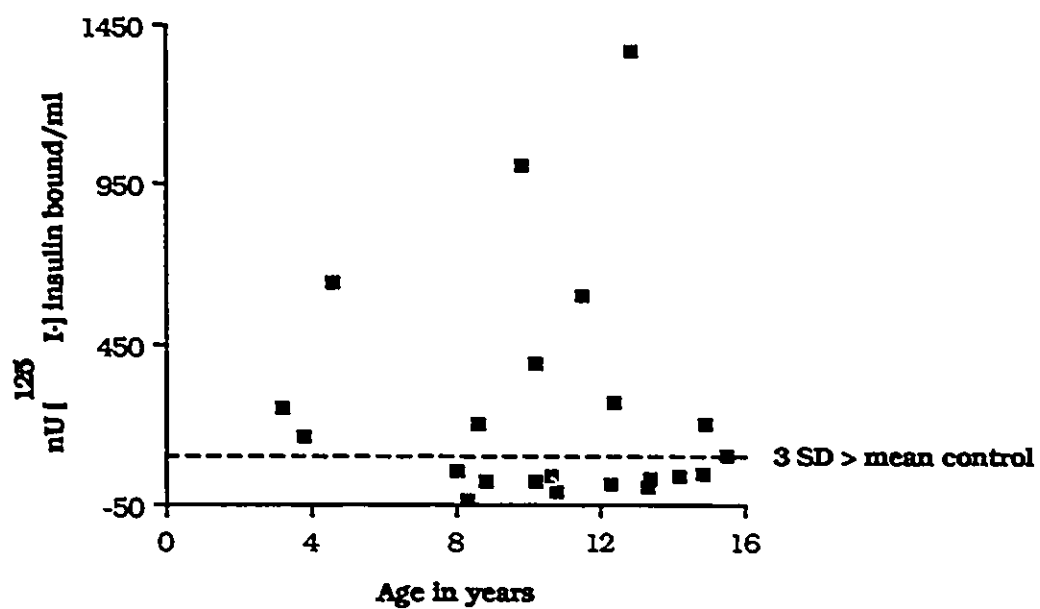
Group	n	Range (nU/ml)	Mean ± SEM (nU/ml)	Percent positive (>99 nU/ml)
Controls	74	-110 — 80	-3 ± 4	0
Type I DM at Diagnosis	22	-30 — 1370	248 ± 77 a	50
Pre-diagnosis	3	-20 — 151	44 ± 54 b	33
After Insulin Injection	4	1420 — 4039	2700 ± 539 a, c	100

Figure 6

**Specific Insulin Binding in Relation to Age of Onset
in Newly Diagnosed Type I DM Patients**

Results are expressed as the mean μ [125 I] bound/ml stripped serum. Dashed line indicates the upper limit of the normal range. Regression analysis was performed (Fastat software). No correlation between age of onset and IAA levels was seen ($p = 0.740$, $r = 0.075$).

Figure 6



Discussion

International Workshops on the Standardization of Insulin Autoantibody Measurement have been held to assess the various assays employed for the detection of IAA (Wilkin et al., 1987, Wilkin et al., 1988, Palmer et al., 1990, Kuglin et al., 1990). During the course of the First Workshop, it became evident that the fluid phase (RIA) and the solid phase (ELISA) antibody detection assays were discordant (Wilkin et al., 1987). The Second Workshop demonstrated improved concordance among laboratories with both the RIA and ELISA when measurements were based on signals displaceable by preincubation with excess unlabeled insulin (Wilkin et al., 1988). A recommendation arising from the Second Workshop was the use of human instead of bovine or porcine insulin (both labeled and unlabeled) which was accepted by most of the laboratories participating in the Third Workshop (Palmer et al., 1990). Despite the use of human insulin, the Third Workshop demonstrated that the RIA and the ELISA laboratories often obtained different results with the same serum. Major differences have been found to exist between these two assay systems (Koch et al., 1989): the RIA is in particular very sensitive to the avidity or affinity of the antibody: high avidity antibodies can be detected, while very low avidity antibodies may not be detected at all. In contrast, ELISA is sensitive to only a very limited degree to the affinity or avidity of the antibody; thus, low avidity antibodies can be detected more readily than with RIA. (Affinity is a measure of the binding strength between an epitope and an antibody combining site. Avidity is the functional combining strength of an antibody with its antigen which is related to both the affinity of the reaction between epitopes and antigen combining sites and the valencies of the antibody and antigen.) Results arising from the Fourth Workshop, as well as other studies, indicated that fluid phase assays may be superior to solid phase assays for identifying disease associated IAA signals

(Kuglin et al., 1990, Greenbaum et al., 1992, Levy-Marchal et al., 1991).

It was necessary to establish an assay for the detection of disease related IAA in our Type I DM patient serum samples which would include the recommendations from the International Workshops. The competitive insulin autoantibody assay (CIAA) developed by Vardi et al. (1987) included all the necessary features: (1) fluid-phase assay RIA, (2) use of a physiologic amount of [^{125}I -] labeled human insulin, and (3) parallel incubations with excess unlabeled human insulin.

The serum samples used in our study were taken from Type I DM patients (pre-diagnosis, at diagnosis and following months of insulin therapy) who had not been fasting at the time of blood collection. It was postulated that endogenous insulin may compete with tracer for binding to IAA in this assay and thereby result in falsely low values. For this reason, it was necessary to evaluate whether stripping away the endogenous insulin from the antibody would significantly increase the sensitivity of the CIAA. Therefore the effect of acid charcoal extraction (Srikanta et al., 1986) on the specific insulin binding levels in the CIAA was evaluated.

Using a polyclonal guinea pig anti-porcine insulin antibody, the insulin binding assay was characterized. Stripping the serum prior to performing the CIAA resulted in a significant increase in the specificity of the insulin binding. Therefore, it was decided that acid charcoal extraction be performed on the serum samples prior to performing the CIAA.

Using the guinea pig serum at a 1 in 1600 dilution, binding of [^{125}I -] labeled human insulin was assessed as a function of unlabeled humulin added to the reaction mixture. Maximal displacement was achieved with 3×10^6 nU humulin/ml stripped serum and this quantity of competitor was used in all subsequent assays.

Diluted serum from Patient #10 at the time of diagnosis with Type I DM was used to determine the intraassay CV. Dilution of the Patient #10 serum resulted in a mean

specific insulin binding that fell within the normal range of the control patient population in our CIAA ($\sim 110 - 80$ nU/ml stripped serum, Table 1A). The diluted guinea pig serum specific insulin binding level was comparable to that of a Type I DM patient positive for IAA (> 99 nU insulin/ml stripped serum, Table 1A). The intraassay CV for the patient serum was 5% while that of the guinea pig anti-porcine insulin antibody was 12% (Table 1A). These CVs compare with that obtained by Vardi et al (1987) at 10% for a low-positive IAA value.

The CIAA interassay CV was determined using the guinea pig antibody as well as diluted Patient #10 serum at the time of diagnosis and after 10 months of insulin treatment. The interassay CV of the samples ranged from 9 to 12% (Table 1B). This low interassay CV validated pooling the IAA values of serum samples from various control subjects assayed on separate days to obtain a mean specific insulin binding for the control population. A total of 74 control serum samples were stripped by acid charcoal extraction and then assayed for IAA levels by the CIAA. None of the control serum samples tested exceeded the cut-off for positivity (99 nU/ml stripped serum) which was the mean plus 3 SD of the control population.

The Type I DM patient population at diagnosis had significantly higher specific insulin binding than the control population. Fifty percent of newly diagnosed Type I DM patients were found to be IAA positive which is in agreement with Vardi et al (1987) in which CIAA was performed without stripping the serum samples. Therefore, although the studies with the guinea pig anti-porcine antibody showed that stripping serum samples increased the binding capacity of IAA, the inclusion of the acid charcoal extraction step in our CIAA did not change the incidence of IAA positivity in the Type I DM patient population at diagnosis. This suggests that despite more antibody being free to bind [125 I] labeled insulin, new populations of binding sites have not been exposed

Another problem with PCR is that it misreads template DNA, thereby introducing point mutations. The observed error rate of Taq polymerase was one substitution in 2500 bases of sequenced DNA (Larrick et al., 1989). It is for this reason that two clones from each of the IAA regions were sequenced. The mutations distinguishing the cDNAs from previously published sequences were observed in both clones.

or generated by acid charcoal extraction. This finding is in agreement with a study in which stripping by acid charcoal extraction was found to increase the insulin binding capacity of serum from a patient with newly diagnosed Type I DM but not the diluted serum that gave half maximal binding (Rayner et al., 1987).

No relationship was seen between the level of IAA and the age of onset of Type I DM. Although this seems to contradict previous observations of a higher prevalence and binding of IAA in younger individuals (McEvoy et al., 1986, Arslanian et al., 1985, Srikantha et al., 1986, Ziegler et al., 1989, Vardi et al., 1988, Sochett and Daneman, 1989), it might readily be explained by the fact that only three children under the age of 8 years, who usually exhibit high levels of IAA, were available for this investigation. Similarly, at the Medical Department of the Schwabing City Hospital (Munich, Germany), no significant correlation was found between IAA and age in their population of newly diagnosed Type I DM patients which included no children under the age of 8 years old (Ziegler et al., 1991). At the Joslin Clinic (Boston, MA), no significant difference was found between IAA and age in newly diagnosed Type I DM patients with onset after 15 yr of age (Ziegler et al., 1990).

Patient # 3 at the time of diagnosis with Type I DM had the highest level of IAA in our Type I DM patient population at diagnosis (1370 nU/ml stripped serum). The lymphocytes purified from blood collected at the time of diagnosis from Patient # 3 (as well as from others with high levels of IAA) could be screened for insulin binding activity in order to obtain monoclonal IAA producing cell lines.

Out of the three pre-diabetic patients studied, one was positive for IAA (Patient # 17). However, the IAA levels in this patient returned to within normal range at the time of diagnosis. One possible explanation for this drop in the level of IAA at the time of diagnosis is that this patient may no longer have had a residual β -cell mass and thus no circulating insulin which could stimulate the production of IAA.

The patients who had undergone insulin therapy and who were available for our study were all IAA positive at the time of diagnosis. The immunogenicity of exogenous insulin may play a role in insulin antibody production as evidenced by the dramatic increase in specific insulin binding within a few months of treatment with exogenous human insulin in all the Type I DM patients studied. In agreement with this observation, Ludvigsson and co-workers found that all the Type I DM patients studied developed insulin antibodies (IA; antibodies directed towards the exogenous insulin) within 3 months of treatment with exogenous insulin and that IA production was more pronounced among patients with autoantibodies if they had both ICA and IAA (Ludvigsson et al., 1988). In another study, IAA positive diabetic patients treated with human insulin had significantly higher titers of IA than the IAA negative patients 3 months after the diagnosis of the disease (Karjalainen et al., 1988). Thus, it is possible that patients with an active autoimmunity towards endogenous insulin produce insulin antibodies more easily than those patients who are IAA negative at diagnosis. Unfortunately, this present study did not provide data on Type I DM patients who had undergone insulin therapy and who were initially IAA negative at diagnosis.

The patients whose serum samples were measured for IAA levels also donated a sufficient volume of blood to be used for the purification of lymphocytes (described in chapter 2). Lymphocytes isolated from those patients with Type I DM who were found to have circulating levels of IAA (highest levels were in Patient #3) were immortalized by infection with EBV. The resulting immortalized population of PBL would be likely to include IAA-secreting B lymphocytes which could be cloned and screened for their ability to secrete insulin antibodies in the supernatant. In this way, a monoclonal IAA-secreting cell line could be established.

Chapter 2: Characterization of A Novel Human Myeloma Fusion Partner

Introduction

The principal reason for wanting to generate monoclonal IAA-secreting cell lines is to capture and immortalize the autoimmune response to insulin in Type I DM patients. One experimental approach to achieve this objective is to immortalize human B lymphocytes, derived from patients with Type I DM at diagnosis, using Epstein-Barr virus (EBV) derived from the EBV-secreting cell line B95-8 (Miller et al., 1972). EBV infection leads to the polyclonal activation of the infected lymphocytes (Rosen et al., 1977) and permanent stimulation of cell growth, a phenomenon termed "immortalization". Those cells that grow are termed lymphoblastoid cell lines or EBV-PBL. This immortalization preserves the characteristics of the original B lymphocytes, including EBV receptors, complement receptors, surface immunoglobulin, and secretory immunoglobulin. Thus, lymphoblastoid cell lines would be established that would continuously secrete immunoglobulin (Ig). Detection of cells secreting IAA would lead to cloning and selection of IAA-producing clones, which would be subcloned until a stable line was established. The disadvantages of this experimental approach are that (1) only a very small number of B lymphocytes in the initial polyclonal population can be cloned and will be good stable secretors of antibody and (2) some of the lines die out after some weeks or months in culture (Edwards and O'Hare, 1986). The EBV infection method to immortalize B lymphocytes has the other disadvantages of the requirement for careful and repeated cloning and the problem of handling the virus. On the other hand, the absence of irrelevant Ig chains belonging to a fusion partner is an advantage.

It is possible to stabilize EBV-PBL and enhance their secretion of Ig by hybridizing them with a stock myeloma cell line (Kozbor and Roder, 1982). Hybridoma cell lines producing human autoantibodies have previously been made (Valente et al., 1982, Shoenfeld and Schwartz, 1982, Eisenbarth et al., 1982). Human cell lines producing human monoclonal antibodies are far more difficult to make than rodent cell lines producing rodent monoclonal antibodies. An important reason for the success of rodent fusions is that an animal is hyperimmunised and boosted for fusion, and hybridization is remarkably selective for B lymphocytes responsive to, and hence stimulated by, antigen boost. Conversely, for human fusions, antigen hyperimmunisation is usually not possible.

Insulin specific human monoclonal antibodies have been previously generated using human-human hybridoma technology (Livneh et al., 1986, Sanz et al., 1989). These monoclonal insulin antibodies were derived from patients who had been injected with heterologous insulin. Because of this "antigen boost", it is impossible to distinguish whether these antibodies were directed against the injected insulin or against endogenous insulin. No group has thus far been successful in generating a human monoclonal IAA derived from a person with Type I DM who has not received insulin therapy.

The main hurdle to overcome for successful human hybridoma formation remains the lack of a suitable myeloma partner. In general, human myelomas do not grow well in culture and tend to hybridize at low frequency. Furthermore, myelomas (human or animal) usually synthesize and secrete immunoglobulins which can interfere with detection of the antibody of interest.

The human fusion partner which was widely used at the time of undertaking of this project was the IgG positive GM4672 human lymphoblastoid cell line available from the Human Genetic Cell Repository (National Institute for General Medical Sciences).

It was of interest however to characterize a human myeloma cell line MM-BA as a potential fusion partner for the development of human IAA-secreting hybridomas. The MM-BA cell line was kindly made available to us by Dr. C. Izaguirre (Virus Laboratory, Health and Welfare Canada). In Dr. Izaguirre's laboratory, the MM-BA cell line was mutagenized and selected for 6-thioguanine resistance and HAT (hypoxanthine, aminopterin, thymidine) sensitivity. Thus, although MM-BA cells were as yet an undefined system in human hybridoma technology, it was expected to be a better fusion partner than GM4672 since MM-BA was a true myeloma.

In fusions with animal spleenocytes, the fused spleen cells are selected by virtue of their acquired immortality upon fusing with the myeloma cell. For the purpose of this present study, previously immortalized EBV-PBL would be fused with human myeloma cells in order to obtain IAA-secreting hybridomas. A selectable marker must therefore be conferred to the MM-BA cells prior to fusion which would permit the survival of the fused cells under conditions which would kill the unfused EBV-PBL. One such selectable marker is geneticin sulfate (G418) resistance.

This chapter deals with the establishment of EBV-PBL lines from Type I DM patients as well as with the development and characterization of a G418 resistant human myeloma cell line, MM-neo, as a potential fusion partner for EBV-PBL. This chapter also outlines the attempts at generating human hybridomas secreting monoclonal IAA by fusion of EBV-PBL from a patient with Type I DM at diagnosis with the MM-neo cell line.

Materials and Methods

Materials

Isolation of Peripheral Blood Lymphocytes (PBL)

Buffers and solutions were made with reagent grade chemicals. The Ficoll-Paque was obtained from Pharmacia. The sterile centrifuge tubes used throughout this study were manufactured by Falcon and supplied by Fisher Scientific. The freezing medium used was fetal bovine serum (FBS; Gibco/Bethesda Research Laboratories, BRL) containing 10% dimethylsulfoxide (DMSO; Fisher Scientific).

Monocyte Conditioned Medium

Human blood from normal adult volunteers was collected in heparinized tubes (Beckton Dickinson Vacutainer Systems). The petri dishes for tissue culture were manufactured by Nunc and supplied by Gibco/BRL. The Iscove's Modified Dulbecco's Medium (IMDM), fungizone (amphotericin), penicillin and streptomycin were all purchased from Gibco/BRL. All media were filter sterilized with 0.2 µm bell filters purchased from Gelman Sciences. Lipopolysaccharide (LPS) from *E. coli* serotype 0111:B4 was obtained from Sigma Chemical Company.

Immortalization of PBL with EBV

The EBV secreting marmoset leukocytes B95-8 (Miller et al., 1972) were purchased from the American Type Culture Collection (ATCC # CRL 1612) (Hay et al., 1988). B95-8 cells were maintained in RPMI 1640 (Gibco/BRL). Cyclosporin-A (CSA) was a generous gift from Sandoz Canada.

Tissue Culture

The tissue culture flasks and 96-well plates were manufactured by Falcon and supplied by Fisher Scientific. The 24-well tissue culture plates were manufactured by Nunc and supplied by Gibco/BRL. Hypoxanthine 6-hydroxypurine, aminopterin, thymidine and geneticin sulfate (G418) were all purchased from Sigma Chemical Co. The electroporator and electroporation chambers were purchased from BRL.

Plasmid Purification

All buffers and salts used for the amplification and purification of the plasmid pSV2neo were reagent grade. Bacto-agar, bacto-tryptone and yeast extract were manufactured by Difco Laboratories and supplied by BDH. The ampicillin used for the selection of resistant cells was purchased from Gibco/BRL. The restriction enzymes and the kilobase (kb) marker λ DNA/ *Hind*III were purchased from BRL.

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot Analysis

All buffers and solutions were of reagent grade. The Nonidet p-40 (NP-40) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. The human lymphoblastoid cell line IM-9 (van Boxel and Buell, 1974) was purchased from the American Type Culture Collection (ATCC # CCL 159) (Hay et al., 1988). Gels for electrophoresis were prepared with acrylamide, N,N'-methylene bisacrylamide and ammonium persulphate, all purchased from Fisher Scientific. The N,N,N',N'-tetramethylethylenediamine (TEMED), sodium dodecylsulfate (SDS) and reagents for staining were purchased from Sigma Chemical Co. Buffers and solvents for staining were either of electrophoretic or reagent grade. The rainbow protein molecular weight marker was purchased from Amersham Canada Ltd.

Transblotting of the proteins was performed using the Bio-Rad Transblot Unit (Bio-Rad Laboratories). The nitrocellulose trans-blot transfer membrane was also obtained from Bio-Rad Laboratories. Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. All of the antibodies used for Western analysis were obtained from Sigma Chemical Co except for the horseradish peroxidase (HRPO) conjugated goat anti-rabbit IgG which was obtained from Bio-Rad Laboratories. The 4-chloro-1-naphthol was purchased from Bio-Rad Laboratories.

Nunc 96-well plates employed for the ELISA were purchased from Gibco/BRL. The antibodies employed were the same as those employed for the Western blot analysis. All the chemicals used for the buffers and solutions were of reagent grade. Tween 20 was purchased from Fisher Scientific. The o-phenylenediamine dihydrochloride (OPD) was purchased from Sigma Chemical Co and the hydrogen peroxide was purchased from Fisher Scientific. The dual wavelength ELISA microplate reader was manufactured by Biotek and purchased from Mandel Scientific.

Fusions

The polyethylene glycol (PEG) 1500 was purchased from BDH. Human recombinant interleukin-6 (IL-6) was purchased from Gibco/BRL.

Screening of hybridomas for IAA secretion

The humulin (Human recombinant insulin) used to coat the ELISA plates was purchased from Eli Lilly and Company.

Methods

Isolation of Peripheral Blood Lymphocytes (PBL)

PBL were obtained from prediabetic patients, patients with Type I DM at diagnosis (before treatment with exogenous insulin) and from Type I DM patients treated with exogenous insulin. Most of these patients were seen at the Pediatric Endocrine Clinics at the Children's Hospital of Eastern Ontario. PBL were also isolated from normal adult volunteer blood donors seen at the Ottawa Civic Hospital as well as at the Ottawa branch of the Red Cross.

The PBL were isolated from the blood samples no longer than 4 h after the time of bloodletting. PBL were separated by centrifugation through a Ficoll gradient as follows: between 8 and 15 ml of heparinized blood were dispensed in a 50 ml tube and diluted to 35 ml with PBS at room T. This diluted blood was layered very gently onto 12 ml of Ficoll-Paque and the tube was centrifuged at 1800 rpm in a Beckman GPKR centrifuge for 30 min at room T with the brake off. (All centrifugation of cells in this study was performed using the Beckman GPKR centrifuge).

The diluted top plasma layer was removed to a fresh tube and kept at -80°C until measured for IAA. The cloudy interface was removed with a sterile plugged pasteur pipet, transferred to a clean 50 ml tube and washed twice with PBS. The cells were resuspended in freezing medium and stored in liquid nitrogen or used immediately for immortalization with EBV-containing B95-8 supernatant.

Preparation of Monocyte Conditioned Medium (MoCM)

MoCM is a rich source of IL-6 (Tosato et al., 1988) which is a B lymphocyte growth factor that promotes the proliferation of human B lymphocytes infected with EBV.

Approximately 100 ml of whole human blood was used for each preparation of batch MoCM. This blood was taken from underweight blood bags which were donated by the Red Cross and distributed by the Ottawa Civic Hospital Blood Bank. The bags of blood were no older than 24 h from the time of blood letting.

The PBL were isolated and resuspended in IMDM-20% FBS at a concentration of 5×10^6 cells per ml. (For the purpose of the studies presented throughout this thesis, the medium termed IMDM or RPMI always includes 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone; only the % FBS varies). Aliquots of 10 ml of the cell suspension were dispensed into tissue culture dishes and incubated at 37°C in a 5% CO₂ atmosphere for 2 h. The non adherent cells were removed by gentle rinsing of the dishes with IMDM. Subsequently, LPS was added to the dishes to a final concentration of 1 µg LPS/ml IMDM-20% FBS. The cells were incubated for 48 h, at which time the culture supernatant was harvested, sterilized through a 0.2 µm filter and dispensed into 1 ml aliquots which were frozen at -20 °C.

Harvest of Supernatant Containing EBV

The EBV secreting B95-8 marmoset cells (Miller et al., 1972) were grown in RPMI-10% FBS and incubated at 37°C in 5% CO₂ in T150 tissue culture flasks. When the adherent cells were confluent, the supernatant was removed and centrifuged at 1400 rpm for 10 min at 4°C. The supernatant was dispensed into 5 ml aliquots and frozen at -80°C until used in the immortalization of PBL. Each batch of supernatant was tested for the ability to transform PBL by infecting PBL purified from the blood of normal donors at the Ottawa Civic Hospital.

Immortalization of PBL with EBV Supernatant

PBL were resuspended in EBV supernatant at a concentration of 2×10^6 cells/ml, transferred to a T25 flask and incubated at 37°C in 5% CO₂ atmosphere for 3 h. At the end of the incubation, the cells were transferred to a 50 ml tube and centrifuged at 1200 rpm for 10 min at room T with the brake on the lowest setting. The cells were then washed with IMDM and resuspended at a concentration of 10^6 cells/ml in IMDM-10% FBS, 10% MoCM and 1 µg/ml CsA. The cells were dispensed into 24 well tissue culture plates (1 ml/well). The plates were incubated for 5 days after which time one half of the medium was removed and replaced with fresh IMDM-10% FBS and 1 µg/ml CsA. After 10 days, the cells were expanded and frozen in liquid nitrogen.

Determination of G418 Sensitivity

The time course of EBV-PBL and MM-BA cell death in various concentrations of G418 was determined using PBL isolated from the blood of a Ottawa Civic Hospital control donor. PBL were transformed with EBV as described above. MM-BA and control EBV-PBL (6×10^4 cells/well) were plated in triplicate in a 24-well plate in IMDM, 10% FBS alone or containing 0, 0.75, 1.0 and 1.5 mg G418/ml. The cells were fed fresh medium every 4-5 days and examined under the microscope for cell death by morphological appearance every 2-3 days. Live cells were typically bright under the light microscope and had intact plasma membranes while the dead cells contained dark granules dispersed throughout the cytoplasm and were much smaller than the live cells. The percentage of viable cells in each of the triplicate wells was determined every 2-3 days.

Preparation of the pSV2neo Plasmid for Electroporation

The plasmid pSV2neo (5729 bp) provides the dominant selectable marker for resistance to G418 in mammalian cells (Colbère-Garapin et al., 1981). For amplification of this plasmid, L-Broth medium containing 100 µg ampicillin/ml was inoculated with a single colony of the *E. coli* strain of HB101 harbouring the pSV2neo plasmid. After an overnight incubation with vigorous shaking at 37°C, the plasmid was isolated by the alkaline lysis method (Maniatis et al., 1982).

The amplified pSV2neo was linearized with *EcoRI* overnight at 37°C, phenol/chloroform extracted, ethanol precipitated, washed with 70% ethanol, dried under vacuum and resuspended in TE (10 mM Tris-Cl and 1mM EDTA pH 8.0). Quantification of the linearized plasmid was performed by reading the absorbance (Abs) of the sample at 260 nm. The linearized plasmid was run on a 0.7% agarose gel along with λ DNA/ *HindIII* as marker and stained with ethidium bromide.

Electroporation of MM-BA Cells

The MM-BA cells were grown in IMDM-10% FBS. The cells were pelleted, washed with PBS and transferred to electroporation chambers such that each chamber contained 4.7×10^6 cells in 1 ml PBS. An aliquot of 34 µg of linearized pSV2neo plasmid in 60 µl TE pH 8.0 was added to each chamber. The electroporation chambers containing the cells with the DNA were placed in the chamber safe and left on ice for at least 10 min. The chamber safe was then connected to the pulse source and each sample was pulsed after setting the desired capacitance and voltage. Various conditions for the electroporation were applied to the cells. The voltage applied to the electroporation chambers varied between 100 and 250 V. The field strength varied between 250 and 625 V/cm. The capacitance varied between 800 and 1180 µF. The resistance was set to the low position and the charge rate set to medium as recommended by the manufacturer.

After the pulse was administered, the electroporation chambers containing the cells and the DNA were left on ice for 20 min. The contents of the chamber were then transferred to a 50 ml tube and made up to volume with IMDM-10% FBS. After 1 h at 37°C, the diluted cells were plated out in 24-well plates at a density of 2×10^5 cells/well and incubated at 37°C in a 5% CO₂ atmosphere.

After 48 h, two thirds of the supernatant of the wells containing the electroporated cells was removed and replaced with fresh medium containing G418 to give a final concentration of 0.75 mg/ml G418. The cells were then fed with IMDM-10% FBS-0.75 mg/ml G418 every 4-5 days.

As control, MM-BA cells electroporated without exogenous DNA were plated out in IMDM-10% FBS-0.75 mg/ml G418 to verify complete cell death in the absence of immortalization with pSV2neo.

Cloning of MM-BA G418 Resistant Cells (MM-neo)

The wells containing the G418 resistant cells were expanded and the cells subcloned by limiting dilution in IMDM-10% FBS-0.75 mg/ml G418 at theoretical concentrations of 1 and 0.5 cell/well. The cells were then incubated at 37°C in a 5% CO₂ atmosphere and fed every 4-5 days.

The wells were examined for growth and wells which contained single clones were expanded. These cells were subcloned in IMDM-10% FBS-0.75 mg/ml G418 in order to ensure clonal stability.

Characterization of MM-neo Light (L) and Heavy (H) Ig Chains

Since the parental MM-BA cell line was derived from a patient with an IgA producing myeloma, it was necessary to determine whether this cell line as well as the derived MM-neo cell line produced Ig κ or λ L chain and/or the Ig α H chain.

MM-BA, MM-neo and the human B lymphoblastoid cell line IM-9, which produces IgG κ (van Boxel and Buell, 1974), were grown in IMDM-10% FBS for one week. The cells were pelleted and washed with PBS. Aliquots of 1.5×10^6 cells were lysed in 2 ml of PBS containing 1% NP-40 and 1mM PMSF. Aliquots of 50 μ l of the cell lysates and of human serum diluted 1 in 500 in PBS, along with 15 μ l of the stock protein marker (1mg/ml), were made up to 100 μ l in sample buffer and were run on a 10% SDS-polyacrylamide gel (Laemmli, 1970). The gel was either stained with Coomassie blue or transferred onto a nitrocellulose blot (Towbin et al., 1979).

For immunoblot analysis, the nitrocellulose blots were air dried and blocked with PBS-0.5% Tween 20 for 1 h at room T followed by 1 h incubation at room T with PBS-1% BSA. The primary antibodies were diluted in PBS-1% BSA as recommended by the manufacturer: for κ L chain determination, rabbit anti-human κ L chain was diluted 1 in 200; for λ L chain determination, rabbit anti-human λ L chain was diluted 1 in 200; and for α H chain determination, rabbit anti-human IgA was diluted 1 in 16. The primary antibodies were incubated with the nitrocellulose blots for 1 h at room T. The blots were then washed with PBS-0.05% Tween 20 and the secondary antibody, a HRPO conjugated goat anti-rabbit IgG, was added at a dilution of 1 in 3000 in PBS-1% BSA. The blots were incubated further for 1 h at room T and washed with PBS-0.05% Tween 20. Colour development was performed by the addition of 4-chloro-1-naphthol and the reaction was terminated with the addition of 0.1% sodium azide.

ELISAs were performed in order to determine whether the MM-BA and MM-neo cell lines secreted the Ig α H and/or the λ L chain which they were found to produce by Western blot analysis. Triplicate wells were coated with 100 μ l aliquots of supernatant from confluent MM-BA and MM-neo cells which had been grown in IMDM-15% FBS. Wells coated with IMDM-15% FBS or with the supernatant of confluent IM-9 cells,

which are known to produce IgG with a κ L chain (van Boxel and Buell, 1974) and which had also been grown in IMDM-15% FBS, served as negative controls. Human serum diluted 1 in 50 with PBS was the positive control. Triplicate uncoated wells were also included to account for nonspecific binding to the ELISA microassay plate.

Triplicate wells containing the test solution were incubated at room T for 2 h and washed twice with PBS-0.05% Tween 20 and once with PBS. The wells were blocked with PBS-1% BSA for 2 h at 37°C and washed again. Aliquots of 100 μ l of the same primary antibodies described above for the Western blot analysis were added to each of the triplicate wells. The plates were then incubated at room T for 2 h and washed. Aliquots of 100 μ l goat anti-rabbit IgG HRPO conjugated antibody diluted 1 in 3000 in PBS-1% BSA were then added to the wells and left to incubate at room T for 2 h, after which time they were washed once again.

The substrate for the colour reaction was prepared by dissolving o-phenylenediamine dihydrochloride (OPD) in McIlvaine's buffer (18 mM citric acid, 64 mM Na_2HPO_4 , pH6.0) at room T. Immediately prior to the addition of substrate to the wells, H_2O_2 was added to the substrate solution to a final concentration of 0.01%. Aliquots of 100 μ l substrate solution were added to the wells and incubated for 30 min at room T, in the dark to prevent degradation of OPD. The reaction was terminated by the addition of 100 μ l of 12% H_2SO_4 . The specific absorbance (Abs) of the colorigenic product generated was obtained by reading the Abs at a wavelength of 490 nm and correcting for non-specific absorbance readings at a wavelength of 450 nm with the use of a dual wavelength ELISA microplate reader. The Abs readings of the test solutions were blanked against the mean of the triplicate uncoated wells.

The Abs readings of the supernatant of the MM-BA, MM-neo and IM-9 confluent cells were compared with the IMDM-15% FBS using the unpaired student's t-test.

Human Leukocyte Antigen (HLA) Typing of MM-neo Cells

HLA-typing was kindly performed by Dr. D.P.S. Sengar at the Tissue Typing lab of the Ottawa General Hospital.

Determination of the Parameters of Fusion of EBV-PBL with MM-neo

The EBV-PBL used in this study were derived from a patient with Type I DM at diagnosis (Patient #18, chapter 1). The cells were plated out in triplicate in 24-well plates at a concentration of 5×10^4 MM-BA cells/well in IMDM-15% FBS alone or containing: (1) 0.75 mg G418/ml; (2) HAT (100 μ M hypoxanthine, 1.6 μ M thymidine and 0.02 μ M aminopterin; Olsson et al., 1983) or (3) HAT-0.75 mg G418/ml. The cells were fed every 4-5 days with fresh medium and examined for viability under the microscope.

Fusions of EBV-PBL with MM-neo

The MM-neo cells as well as the EBV-PBL obtained from a patient with Type I DM at diagnosis (Patient #18, chapter 1) were grown in IMDM-15% FBS and maintained in exponential growth prior to fusion. The cells were expanded every 24-48 h and were not allowed to reach greater than $1-2 \times 10^5$ cells/ml. An aliquot of the MM-neo cells was grown in IMDM-15% FBS, 30 μ g/ml 6-thioguanine for 1 week prior to fusion to verify HAT sensitivity of the MM-neo cells.

The basic fusion protocol was essentially as described by Fountoulakis et al. (1985) with some modifications. EBV-PBL (5×10^7) and MM-neo cells (10^8) were separately pelleted and washed twice with IMDM in order to remove the FBS. The EBV-PBL were added to the MM-neo cells in a 50 ml centrifuge tube and the cells were centrifuged at 1300 rpm. The supernatant was aspirated and the cell pellet was gently dispersed and warmed in a 40°C water bath. One ml PEG 1500 (45% in IMDM, pH 8.0) was added dropwise with a

volume pipet over 1 min with continuous swirling and stirring of the cells. The cells were resuspended gently for 30 seconds (s) and allowed to sit for a further 90 s. One ml IMDM was added over 30 s followed by the addition of another 5 ml IMDM over another 30 s. Finally, 5 ml IMDM was added all at once. The tube was allowed to sit for 90 s after which time the volume was made up to 40 ml with IMDM. The cells were centrifuged at 1300 rpm at room T for 5 min. The supernatant was aspirated and the cells were resuspended in IMDM-15% FBS containing HT (100 μ M hypoxanthine, 1.6 μ M thymidine) and plated out into 24 well plates at a concentration of 4.5×10^5 cells/well. After 24 h, one half of the medium was replaced with IMDM-15% FBS containing HAT and 0.75 mg/ml G418. As controls, unfused MM-neo cells and EBV-PBL were plated out at the same concentration in IMDM-15% FBS alone or containing either: (1) HAT; (2) HT-0.75 mg/ml G418 (without aminopterin); or (3) HAT-0.75 mg/ml G418. The fused cells were fed every 4-5 days with IMDM-15% FBS-HAT-0.75 mg/ml G418 while the control cells were fed with their respective medium. The cells were examined every 2-3 days for cell death.

After 22 days in culture, when all the control unfused MM-neo cells in HAT had died, the fused cells were fed with IMDM-15% FBS-HT-0.75 mg/ml G418 (no aminopterin). After 30 days in culture, when all the control unfused EBV-PBL in IMDM-15% FBS-HAT-0.75 mg/ml G418 had died, the fused cells were fed with IMDM-20% FBS containing HT (no G418). At 40 days post fusion, the cells were dispersed and were maintained in IMDM-20% FBS containing 100 pg/ml IL-6 (to enhance hybridoma growth; Tosato et al., 1988) until the appearance of hybridomas.

Testing of Hybridomas for IAA Secretion

ELISA microassay plates were coated with humulin in coating buffer (50 mM NaHCO_3 pH 9.6, 50 mM Na_2CO_3) at a concentration of 2 μ g/ well and incubated at 4°C

overnight. The following day, the plates were washed twice with PBS to remove unbound insulin. The wells were blocked for 2-3 h at 37°C with PBS-1% BSA and then washed twice with PBS-0.05% Tween 20 and once with PBS.

After fusion of the EBV-PBL with MM-neo cells, two wells were found to contain hybridomas after selection. The cells in these wells were allowed to grow to confluence in IMDM-20% FBS-100 pg/ml IL-6. After seven days in the same medium, quadruplicate 100 µl aliquots of the supernatant of these two wells were transferred to a 96-well ELISA microassay plate which had been coated with human insulin and blocked. Also included in this assay were quadruplicate humulin coated wells containing either: (1) 100 µl aliquots of PBS, 1% BSA (no primary antibody); (2) 100 µl aliquots of 100 pg IL-6/ml IMDM, 20% FBS, (3) 100 µl aliquots of supernatant from confluent MM-neo cells which had also been grown in IMDM-20% FBS-100 pg/ml IL-6 and (4) 100 µl aliquots of the serum from a patient with Type I DM at diagnosis which had been found to be IAA positive by a competitive radioimmunoassay (Patient #18, chapter 1) were included as positive controls. The quadruplicate samples were incubated in the insulin coated wells for 1-2 h at room T after which time the plates were washed twice with PBS-0.05% Tween 20 and once with PBS to remove unbound antibody.

The secondary antibody employed in the screening assay was a goat anti-human polyvalent antibody horseredish peroxidase conjugate, diluted 1 in 3000 in PBS-1% BSA. Aliquots of 100 µl of this antibody were added to each well and incubated at room T for 1-2 h after which time the plates were rewashed. Colour development was then performed as previously described.

The Abs readings of the supernatant of the two wells containing the hybridomas and the MM-neo confluent cells were compared with those containing IMDM-20% FBS-100 pg/ml IL-6 using the unpaired student's t-test.

Cloning of the Hybridomas

The hybridomas were grown in IMDM-20% FBS-100 pg/ml IL-6 and subcloned by limiting dilution in 96-well plates at theoretical concentrations of 1 and 0.5 cell/well in IMDM-20% FBS-100 pg/ml IL-6. The supernatant of the resulting subclones were retested for IAA secretion by ELISA as previously described.

Karyotyping of Cloned Hybridomas

Karyotyping of MM-neo and the subcloned hybridoma (12C1-C12) was kindly performed by Dr. H. C. Wang at the Children's Hospital of Eastern Ontario. Standard procedures were used for cell harvesting and chromosome preparation. When the cells reached confluency, colcemid (0.04 µg/ml) was added to the cell culture for 30 min prior to harvest. Cells were then resuspended in 75 mM KCl for 10 min and fixed in methanol: acetic acid (3:1). Chromosomes were prepared by the air dry method, G-banded and analysed.

Results

Isolation of PBL

A decrease in the yield of purified lymphocytes was observed when the blood was allowed to sit in the dark at room T overnight before being processed. In view of the fact that only 8-15 ml of blood was obtained from very young children and that it was important to isolate a representative population, all blood samples were processed as quickly as possible (usually within 4 h of bloodletting). In order to obtain lymphocytes that secreted IAA and not IA directed against the exogenous insulin, it was imperative that the blood be taken from patients with Type I DM at diagnosis before the administration of insulin.

Immortalization of PBL with EBV

Although the isolated PBL were to be stored in liquid nitrogen and transformed only if the cells originated from individuals seropositive for IAA, the frequency of immortalization was found to be reduced when the cells were frozen for even short periods of time. To ensure maximal yield and lymphocyte representation in the immortalized population, the lymphocytes were routinely transformed with EBV supernatant the same day as the bloodletting.

Determination of EBV-PBL and MM-BA Sensitivity to G418

In fusions of untransformed cells with myeloma cells, a fused cell is selected by virtue of its acquired immortality resulting from the fusion with the myeloma cell. In this case, cells immortalized by EBV infection were being fused. Thus, a selectable resistance had to be conferred to the MM-BA myeloma cells. We chose resistance to the antibiotic G418. MM-BA cells were very stable in culture and had a doubling time of

37 h in IMDM-10% FBS. It was anticipated that a G418-resistant variant of MM-BA would be equally stable and would confer this stability and antibiotic resistance to a hybridoma upon fusion with EBV-PBL. G418 resistance can be conferred to a cell by transfecting the plasmid pSV2neo. The plasmid pSV2neo encodes an aminoglycoside phosphotransferase (neomycin phosphotransferase II) which inactivates the aminoglycoside antibiotic G418 by catalyzing the transfer of the terminal phosphate of ATP to the drug (Bryan, 1984).

Before fusion of a G418 variant of MM-BA, it was necessary to determine which concentration and length of exposure to G418 would result in the death of all the EBV-PBL. EBV-PBL were completely dead after 20 days in 1.0 and 0.75 mg/ml G418 (in IMDM-10% FBS) and after 13 days in 1.5 mg/ml G418 (Fig. 7A). Thus, for the purpose of fusions with EBV-PBL, MM-BA cells which were also sensitive to G418 (Fig. 7B) had to survive in 1.5 mg/ml G418 for 13 days or in 0.75 mg/ml G418 for at least 20 days (the preferred choice cost-wise).

Development of MM-neo

The linearized plasmid pSV2neo was transfected into the MM-BA cells by electroporation under conditions which yielded transfectants: 34 μ g of EcoRI linearized pSV2neo plasmid was added to 5×10^6 MM-BA cells in a total of 1 ml PBS. The field strength was 625 V/cm, the displayed voltage was 250 V, the capacitance was 800 μ F and the resistance was set at the low position. Control MM-BA cells which were electroporated under identical conditions in the absence of pSV2neo were plated out in culture medium containing IMDM-10% FBS-0.75 mg/ml G418.

After 19 days in medium containing 0.75 mg/ml G418, the MM-BA cells which were electroporated in the absence of pSV2neo had all died. However, live cells were observed in the wells containing MM-BA cells electroporated in the presence of pSV2neo.

Figure 7

Survival of EBV-PBL and MM-BA in G418

Triplicate samples of EBV-PBL (6×10^4 cells/well; Fig. 7A) and MM-BA cells (6×10^4 cells/well; Fig. 7B) were incubated in IMDM-10% FBS containing various concentrations of G418. The cells were fed every 4-5 days and the percentage of viable cells in each well was determined every 2-3 days. The results are expressed as the mean $\pm$ SEM % survival of control (the triplicate mean of viable cells in G418 as a percentage of the triplicate mean of viable cells in medium without G418).

Circles. 0.75 mg G418/ml

Triangles. 1.0 mg G418/ml

Squares. 1.5 mg G418/ml

Figure 7A

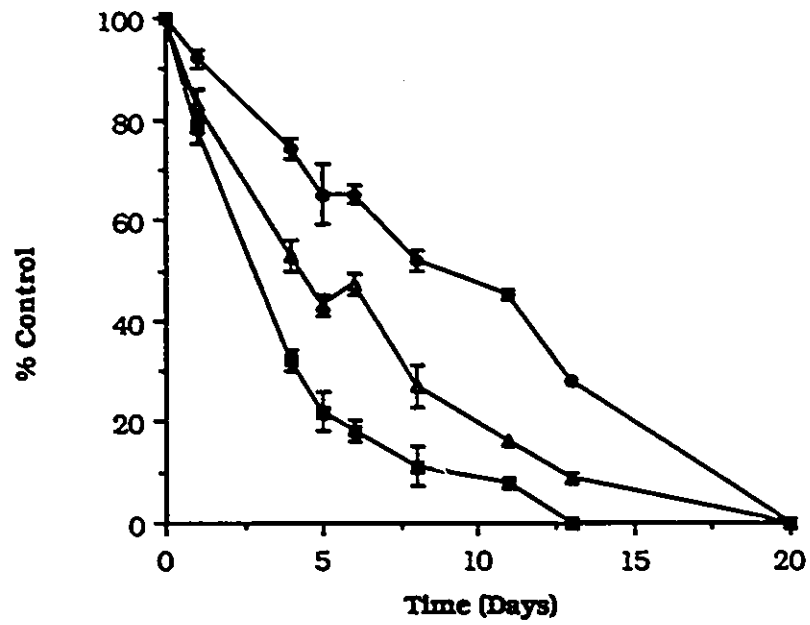
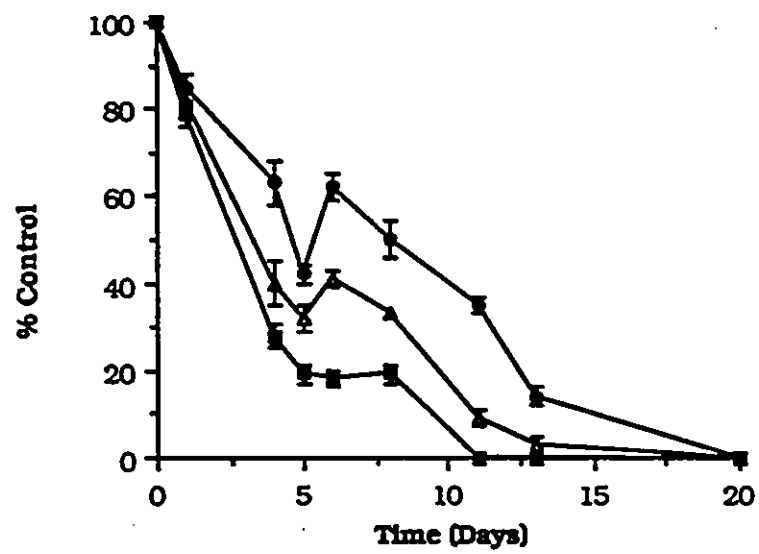


Figure 7B



The wells which contained healthy cells were graded for the fastest proliferating clones in order to determine which wells to expand and subclone. Thus, the cells from five wells were subcloned by limiting dilution and were maintained in IMDM-15% FBS-0.75 mg/ml G418. These cells were examined for growth and finally eighteen healthy, rapidly proliferating clones were obtained. The four fastest proliferating cell lines were subcloned once again. The resulting cell line D3-1C2-A9 (MM-neo) was the fastest proliferating of the cell lines which had been subcloned twice. These MM-neo cells had a doubling time of 36 h in IMDM-10% FBS-0.75 mg/ml G418.

Characterization of MM-neo L and H chain

A. Production of Ig H and/or L chain

Since the MM-BA cell line was derived from an IgA producing myeloma, it was necessary to establish whether MM-BA and MM-neo produced the α H chain and/or the κ or λ L chain. SDS-PAGE was therefore performed on cell lysates followed by either Coomassie blue staining or by double antibody Western blot analysis. The human B lymphoblastoid cell line IM-9, which produces an IgG κ antibody (van Boxel and Buell, 1974), as well as human serum at a dilution of 1 in 500, were included as controls.

Double antibody Western blot analysis using the anti-human κ L chain antibody (Fig. 8, panel A) failed to reveal κ L chain production in the cell lysates of MM-BA (lane 1) and MM-neo (lane 2). κ L chain production was detected with the anti- κ antibody in the positive controls, IM-9 cell lysate (lane 3) and human serum at a dilution of 1 in 500 (lane 4). The protein band corresponding to the κ L chain was approximately 24 kDa which compares with the κ L chain theoretical molecular weight of approximately 23 kDa (Dayhoff, 1972).

Figure 8

Characterization of H and L Chain Isotypes by Western Blot Analysis

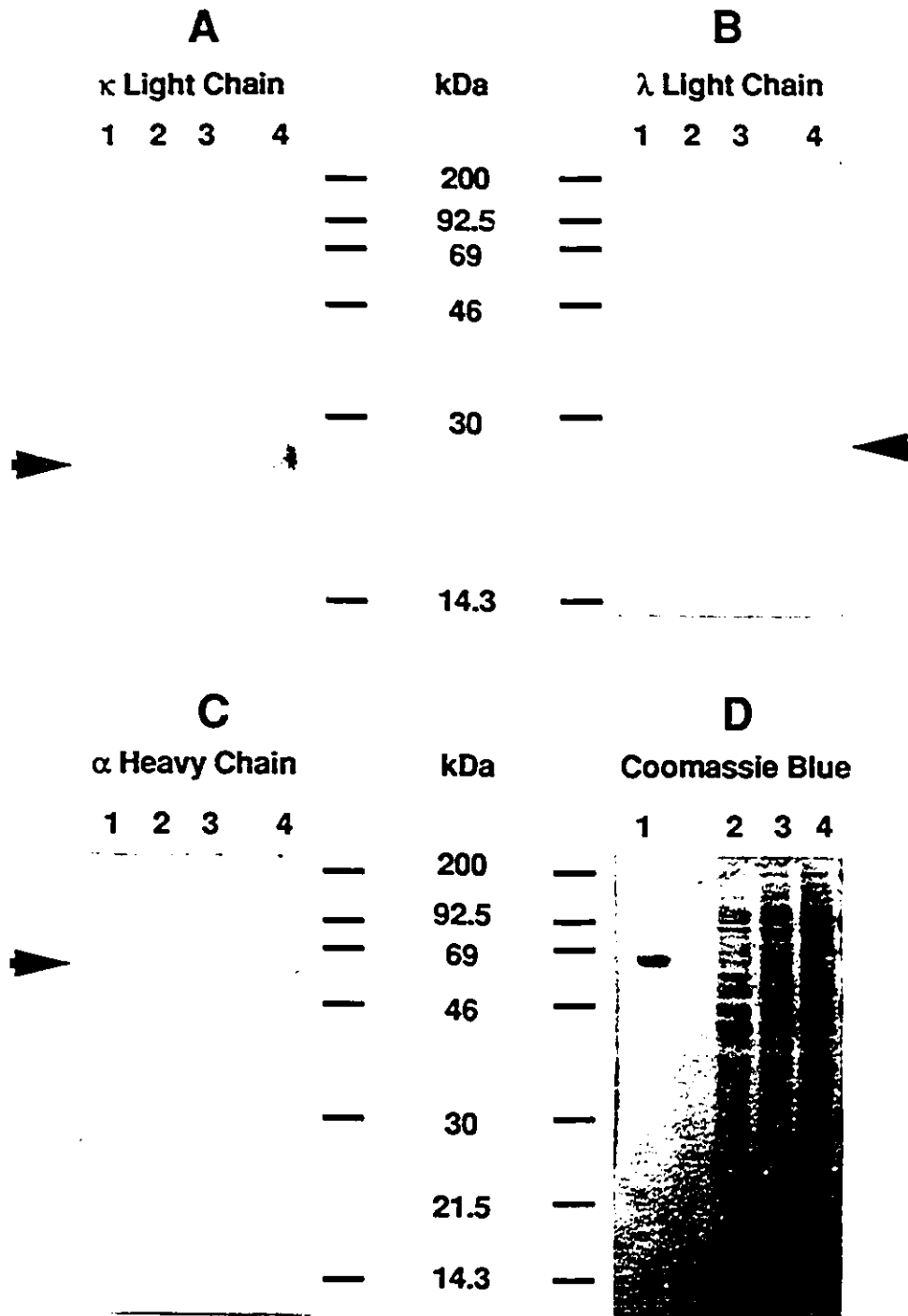
Aliquots of 1.5×10^6 MM-BA, MM-neo and IM-9 cells were lysed in 2 ml of PBS containing 1% NP-40 and 1mM PMSF. Aliquots of 50 μ l of the cell lysates and of human serum diluted 1 in 500 in PBS along with 15 μ l (1mg/ml stock) of the protein marker were made up to 100 μ l with sample buffer and were run on a 10% SDS-polyacrylamide gel.

After electrophoresis, the samples were transferred unto a nitrocellulose membrane for immunoblot analysis (**panel A, B and C**) or were stained with Coomassie blue (**panel D**). The lanes in **panels A, B and C** contain: lane 1, MM-BA lysate; lane 2, MM-neo lysate; lane 3, IM-9 lysate; and lane 4, human serum diluted 1 in 500. The lanes in **panel D** contain: lane 1, human serum diluted 1 in 500; lane 2, IM-9 lysate; lane 3, MM-neo lysate; and lane 4, MM-BA lysate.

For immunoblot analysis, the primary antibodies were diluted in PBS, 1% BSA as follows: for κ L chain determination (**panel A**), rabbit anti-human κ L chain was diluted 1 in 200; for λ L chain determination (**panel B**), rabbit anti-human λ L chain was diluted 1 in 200; and for α H chain determination (**panel C**), rabbit anti-human IgA was diluted 1 in 16. The secondary antibody employed for all of the Western blot analyses was an HRPO conjugated goat anti-rabbit IgG at a dilution of 1 in 3000 in PBS-1% BSA.

Colour development was performed by the addition of 4-chloro-1-naphthol and the reaction was terminated with the addition of 0.1% sodium azide.

Figure 8



λ L chain production was detected with the anti-human λ antibody (Fig. 8, panel B) in the cell lysate of MM-BA (lane 1) and of MM-neo (lane 2) as well as in the positive control, human serum at a dilution of 1 in 500 (lane 3). The protein band corresponding to the λ L chain was approximately 27 kDa which compares with the λ L chain theoretical molecular weight of approximately 23 kDa (Dayhoff, 1972). No λ L chain production was detected in the negative control, IM-9 cell lysate (lane 4). Western blot analysis revealed α H chain production (Fig. 8, panel C) in the MM-BA (lane 1) and the MM-neo (lane 2) cell lysates as well as in the positive control, human serum at a dilution of 1 in 500 (lane 4). The protein band corresponding to the α H chain was approximately 58 kDa which compares with the α H chain theoretical molecular weight of approximately 50 kDa (Dayhoff, 1972). The α H chain was not detected in the negative control, the cell lysate from IM-9 (lane 3).

The Coomassie blue staining of the SDS-PAGE gel (Fig. 8, panel D) shows that the negative results in the Western blot analyses are not simply due to insufficient amounts of protein loaded onto the gel. The cell lysates of IM-9 (lane 2), MM-neo (lane 3) and MM-BA (lane 4) contained approximately the same amount of protein which was greater than the protein present in the human serum at a dilution of 1 in 500 (lane 1) which served as positive control in all Western blot analyses.

B. Secretion of Ig H and/or L chain

Since the Western blot analysis revealed that the MM-BA and MM-neo cells produced Ig λ L chain and α H chain, it was of interest to determine whether these same chains were also secreted by the cells. ELISAs were performed on the supernatant of the confluent MM-BA and MM-neo cells which had been grown in IMDM-15% FBS. Triplicate wells which were coated with: (1) PBS-1% BSA; (2) IMDM-15% FBS; (3) the

supernatant of confluent IM-9 cells which had been grown in IMDM-15% FBS and (4) triplicate uncoated wells which served as negative controls. Human serum diluted 1 in 50 with PBS served as positive control.

The results of the ELISA revealed that both the MM-BA and MM-neo cells secrete Ig λ L chain and α H chain: the Abs readings of the supernatant of the confluent MM-BA and MM-neo cell lines were higher than IMDM-15% FBS alone ($p < 0.015$; Fig. 9).

Tissue Typing of MM-neo

Tissue typing of MM-neo myeloma cells was performed for the HLA-A, B and C class I antigens. The HLA typing gave the following haplotypes : A3, A24, Cw7, Bw4 and Bw6. Although the HLA-A and-C specific antigens were picked up , HLA-B specific alleles were not detected. However, the cell line was found to be clearly positive for the two broad or supertypic specificities (Bw4 and Bw6) of the HLA-B locus. These results demonstrate that the MM-neo myeloma cell line is indeed of human origin.

Determination of the Parameters of Fusion of EBV-PBL with MM-neo

It has been established that no viable EBV-PBL remained after 20 days in IMDM-10% FBS-0.75 mg/ml G418. However, to provide a richer medium for hybridoma growth, it had been decided that the fusions of MM-neo with EBV-PBL would be performed in IMDM-15% FBS. It was therefore necessary to determine the length of exposure to IMDM-15% FBS-0.75 mg/ml G418 which was sufficient to kill EBV-PBL while allowing MM-neo cells to proliferate. It was also of interest to determine the length of exposure to HAT-IMDM 15% FBS which killed off the MM-neo cells but not the EBV-PBL.

Figure 9

Measurement of Myeloma H and L Chain Secretion

Double antibody ELISA was performed to measure secretion of λ L chain (**Fig. 9A**) and α H chain (**Fig. 9B**) by MM-BA and MM-neo cells. Triplicate 100 μ l samples of the supernatant of MM-BA and MM-neo confluent cells grown in IMDM-15% FBS were assayed. Triplicate 100 μ l samples of 1) IMDM-15% FBS, 2) the supernatant of IM-9 confluent cells grown in IMDM-15% FBS and 3) triplicate uncoated wells served as negative controls. Triplicate 100 μ l samples of human serum diluted 1 in 50 in PBS served as positive controls.

The Abs readings of the test solutions were blanked against the mean of the triplicate uncoated wells. The results are expressed as mean Abs readings $\pm$ SEM.

Statistical analysis was performed using the unpaired student's t-test.

* $p < 0.015$ when compared with the medium (IMDM-15% FBS)

Figure 9A

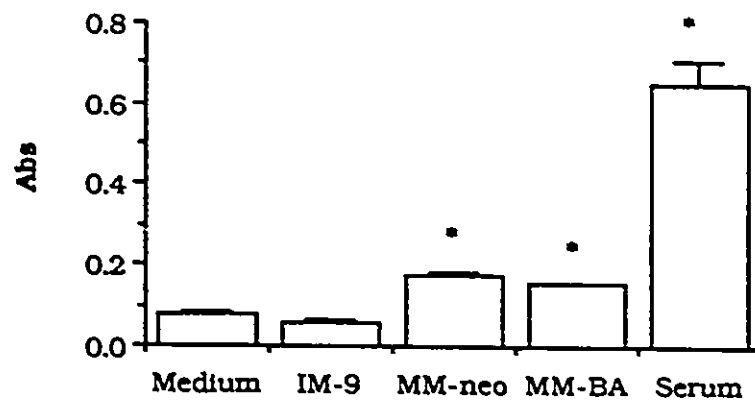
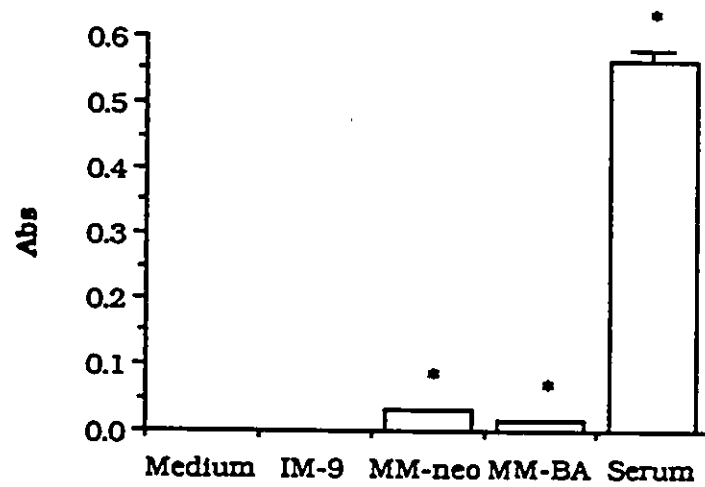


Figure 9B



The EBV-PBL used in this study were derived from a patient with Type I DM at diagnosis whose serum had previously been shown to be seropositive for IAA by competitive radioimmunoassay (Patient #18; chapter 1). These EBV-PBL as well as MM-BA and MM-neo cells were plated out in triplicate in 24-well plates at a concentration of 5×10^4 cells/well in IMDM-15% FBS alone or containing: (1) 0.75 mg/ml G418, (2) HAT or (3) HAT-0.75 mg/ml G418.

The MM-neo cells continued to proliferate in IMDM-15% FBS-0.75 mg/ml G418 throughout the 30 day period of testing (Fig. 10A) and in fact were maintained in G418 for several months without loss of G418 resistance. All the MM-BA cells, however, had died in IMDM-15% FBS-0.75 mg/ml G418 after 29 days in culture (Fig. 10A). The EBV-PBL had died in IMDM-15% FBS-0.75 mg/ml G418 after 27 days in culture (Fig. 10A).

Thus, exposure to IMDM-15% FBS-0.75 mg/ml G418 for at least 27 days would provide an adequate selectable marker for fusions in which the unfused EBV-PBL die but not the hybridomas which would have acquired the G418 resistance from the MM-neo myeloma fusion partner.

The MM-neo cells retained the HAT sensitivity of the parental MM-BA cells as shown in Fig. 10B. All the MM-neo and the MM-BA cells had died in IMDM-15% FBS-HAT by 17 days in culture. The EBV-PBL were also sensitive to IMDM-15% FBS-HAT, although to a lesser degree than the MM-neo cells. After 17 days in HAT, all the MM-neo cells as well as the MM-BA cells were dead while $36 \pm 1\%$ of the EBV-PBL survived. The differential HAT sensitivity of the MM-neo cells and the EBV-PBL could serve as a means for selecting unfused MM-neo cells: after fusion of MM-neo with EBV-PBL, the cells will be kept in IMDM-15% FBS-HAT until all the unfused myeloma cells are dead (approximately 17 days) and then aminopterin will be removed from the medium.

Figure 10

Survival of MM-neo, MM-BA and EBV-PBL in Selection Medium

The MM-neo, MM-BA and EBV-PBL cells were plated out in triplicate in 24-well plates at a concentration of 5×10^4 MM-BA cells/well in IMDM-15% FBS alone or containing 0.75 mg/ml G418 (**Fig. 10A**), HAT (**Fig. 10B**) or HAT-0.75 mg/ml G418 (**Fig. 10C**).

The cells were fed every 4-5 days and the percentage of viable cells in each of the wells was determined every 2-3 days. The results are expressed as the mean $\pm$ SEM % survival of control (the triplicate mean of viable cells in G418 and/or HAT as a percentage of the triplicate mean of viable cells in medium alone).

Circles, EBV-PBL

Triangles, MM-neo

Squares, MM-BA

Figure 10A

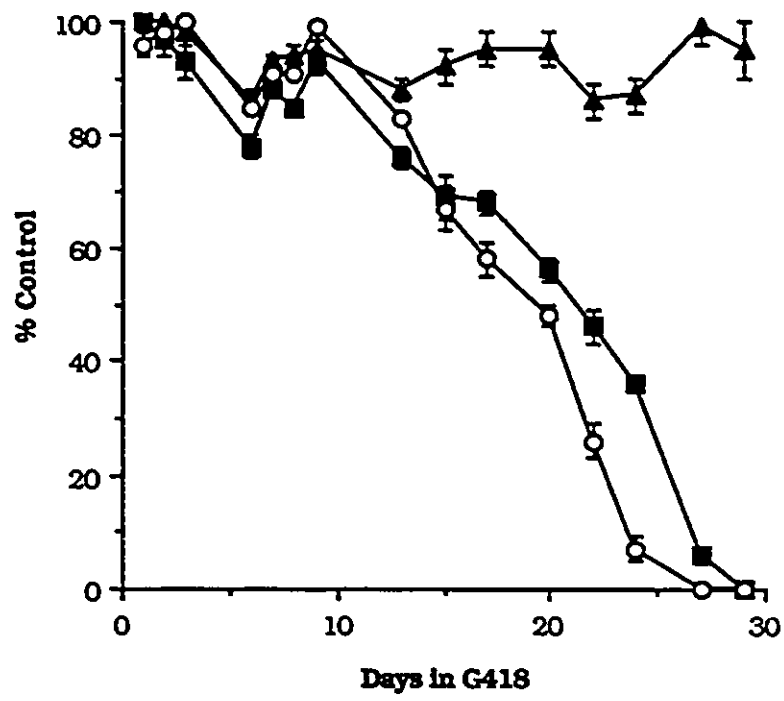


Figure 10B

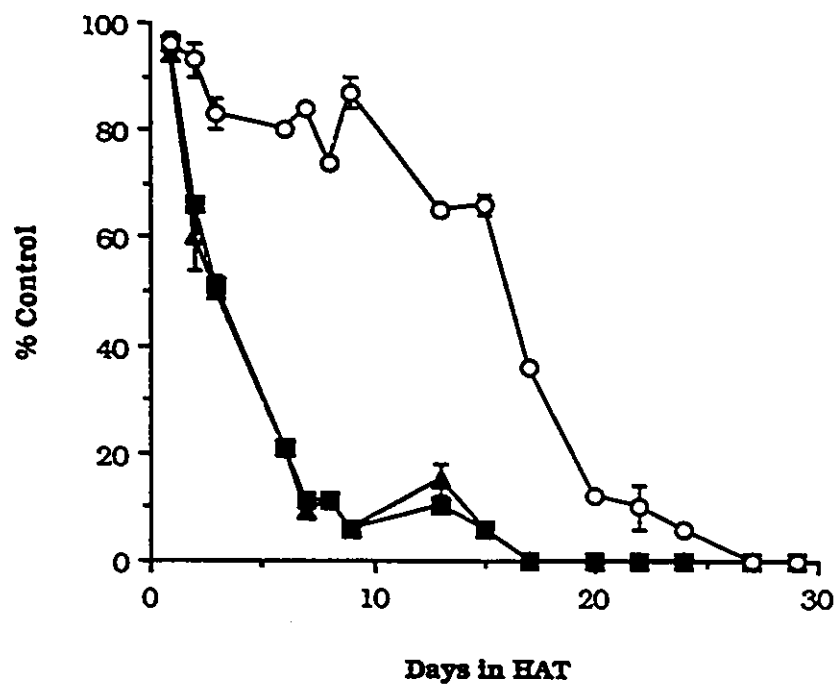
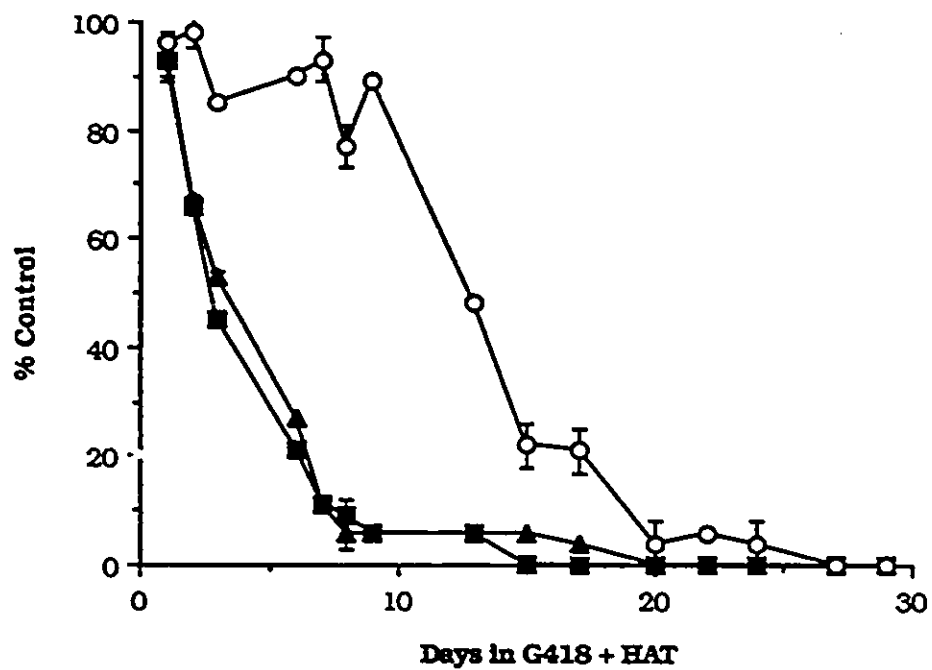


Figure 10C



Because approximately one third of the EBV-PBL retain HAT resistance in conditions of HAT exposure that kill off the MM-neo cells, it is presumed these HAT resistant EBV-PBL will confer HAT resistance to hybridomas resulting from fusion of these EBV-PBL to the MM-neo cells. The resulting hybridomas would thus be able to survive in HAT until all the unfused MM-neo cells would be killed.

In IMDM-15% FBS-HAT-0.75 mg/ml G418, one hundred per cent cell death was achieved by 27 days for EBV-PBL and by 20 days for MM-neo.

Fusion of EBV-PBL with MM-neo

A total of 10^8 EBV-PBL and 5×10^7 MM-neo were fused according to the basic fusion protocol essentially as described by (Foung et al., 1985) with some modifications. As controls, unfused MM-neo cells and EBV-PBL were each plated out at the same concentration in IMDM-15% FBS alone or containing: (1) HAT; (2) HT-0.75mg/ml G418; or (3) HAT-0.75 mg/ml G418. The fused cells were fed every 4-5 days with IMDM-15% FBS containing HAT and 0.75 mg/ml G418 while the control cells were fed with their respective medium. The cells were examined every 2-3 days for cell death.

After 22 days in culture, all the control unfused MM-neo cells were dead in HAT but were still alive in 0.75 mg/ml G418. At this point, the fused cells were fed with IMDM-15% FBS-HT-0.75 mg/ml G418. After 30 days in culture, all the control unfused EBV-PBL in HT-0.75 mg/ml G418 were dead while some still remained alive in HAT. It was at this point that the G418 was removed from the medium of the fused cells which were then maintained in IMDM-20% FBS-HT. At 40 days post fusion, the cells were dispersed and were maintained in IMDM-20% FBS containing 100 pg/ml interleukin-6 (IL-6) to enhance hybridoma growth (Tosato et al., 1988) until the hybridomas appeared.

The first well (12C1) containing hybridomas was observed 47 days post fusion. The

second well (12C4) containing hybridomas was observed 63 days post fusion. The fusion frequency of EBV-PBL with the MM-neo cells was found to be 2 clones in 1×10^8 EBV-PBL.

Screening of hybridomas for IAA production

The hybridomas 12C1 and 12C4 were grown to confluence in IMDM-20% FBS-100 pg/ml IL-6 and after seven days in the same medium, quadruplicate 100 μ l aliquots of the supernatant of the wells containing hybridomas were tested for insulin binding by ELISA. Included in this assay were quadruplicate humulin coated wells incubated with 100 μ l aliquots of: (1) PBS-1% BSA (blocking agent), (2) IMDM-20% FBS-100 pg/ml IL-6, (3) supernatant from confluent MM-neo cells which had also been grown in IMDM-20% FBS-100 pg/ml IL-6 and which was known to contain IgA λ and (4) serum from a patient with Type I DM at diagnosis which had been found to be IAA positive by a competitive radioimmunoassay and whose EBV-PBL were used in the fusion with MM-neo (Patient #18; chapter 1).

Table 3 shows the results obtained from the ELISA. The Abs readings of the supernatant of the two wells containing the hybridomas and the MM-neo confluent cells were compared with those containing IMDM-20% FBS-100 pg/ml IL-6 using the unpaired student's t-test. There was no significant difference between the Abs readings of the supernatant of the 12C1 hybridomas or the supernatant of the 12C4 hybridomas when compared with medium alone. There was no significant binding of the supernatant of the MM-neo cells which contained IgA to the insulin coated plates. There was also no significant difference of the Abs readings of the insulin coated wells incubated with PBS-1% BSA (no primary antibody) and the Abs readings of the supernatant as well as those of the medium alone. Thus, the hybridomas 12C1 and 12C4 do not secrete an insulin binding antibody. As expected, the diluted serum of Type I DM Patient #18 (chapter 1) demonstrated binding to the insulin coated plates.

Table 3

Measurement of Total Insulin Binding of Antibodies Secreted by Hybridomas

The 12C-1 and 12C-4 wells contained hybridomas which were grown in IMDM-20% FBS-100 pg/ml IL-6 and which resulted from the fusion of EBV-PBL and MM-neo cells. Quadruplicate 100 μ l aliquots of the supernatant of these confluent wells were transferred to wells of a 96-well ELISA microassay plate which had been previously coated with human recombinant insulin (2 μ g/well) and blocked with PBS-1% BSA. Other quadruplicate insulin coated wells contained 100 μ l aliquots of: 1) PBS, 1% BSA, 2) IMDM-20% FBS-100 pg/ml IL-6, 3) supernatant from confluent MM-neo cells which had been grown in IMDM-20% FBS-100 pg/ml IL-6 and 4) serum from a patient with Type I DM at diagnosis which had been previously found to be IAA positive (Patient #18, chapter 1).

The samples were incubated in the insulin coated wells for 1-2 h at room T and washed. The secondary antibody (a goat anti-human polyvalent antibody horseradish peroxidase conjugate diluted 1 in 3000 with PBS-1% BSA) was then added to each well (100 μ l/well) and incubated at room T for 1-2 h. The plates were washed and colour development was performed.

The specific absorbance was obtained by reading the Abs at 490 nm and correcting for non-specific absorbance with a reading at 450 nm. The results were analyzed using the unpaired student's t-test. The Abs readings of the supernatant were compared with those of medium alone.

Table 3

Test solution	Mean Abs $\pm$ SEM
PBS-1% BSA	0.022 $\pm$ 0.001
IMDM-20% FBS-100 pg/ml IL-6	0.019 $\pm$ 0.001
Supernatant 12C-1	0.018 $\pm$ 0.001
Supernatant 12C-4	0.019 $\pm$ 0.001
Supernatant MM-neo	0.020 $\pm$ 0.001
Serum patient #18 (1 in 30)	0.192 $\pm$ 0.034

Karyotyping of the hybridomas

The hybridomas 12C1 and 12C4 were subcloned by limiting dilution. One of the subclones of 12C1 (12C1-C12) as well as the MM-neo myeloma fusion partner were karyotyped by Dr. H. Wang at the cytogenetics laboratory at the Children's Hospital of Eastern Ontario. Numerous marker chromosomes in both the MM-neo and the 12C1-C12 hybridoma were detected which could be the result of complex rearrangement. The MM-neo cells were found to be near-triploidy while the 12C1-C12 cells were found to be near-tetraploidy (see table 4).

Table 4

Karyotyping of MM-neo and 12C1-C12 Cells

When MM-neo cells and 12C-C12 hybridomas reached confluency, colcemid was added to the culture (final concentration 0.04 µg/ml) for 30 min prior to harvest. The cells were then resuspended in 0.075 M KCl for 10 min followed by fixation in methanol:acetic acid (3:1). Chromosome preparation was performed by the air dry method, G-banded and analysed.

Table 4

Cell line	No. of Cells Analysed	Ploidy Level	Modal No. and Range
MM-neo	25	near-triploidy	73 (54-89)
12C1-C12	25	near-tetraploidy	86 (61-109)

Discussion

The goal of this study was to obtain immortalized B lymphocytes which secrete monoclonal IAA in order to study the autoimmune response to insulin in Type I DM. In EBV immortalization, it is not necessary to remove the T lymphocytes from the purified lymphocyte population before immortalization. T lymphocytes will not be transformed by EBV, however, they will survive in culture for a finite period and suppress the EBV-induced B lymphocyte proliferation (Moss et al., 1981). In order to overcome the inhibitory effect of T lymphocytes on B lymphocyte proliferation, CSA, an immunosuppressant which is not antigen specific and which preferentially inactivates T lymphocytes, is added to the culture medium for 10 days at a concentration of 1 µg/ml (Bejarano et al., 1985).

EBV immortalization ensures a continuous supply of PBL from the patients with Type I DM at the moment of diagnosis. This is important in studying the autoimmune response to insulin because PBL isolated from patients with Type I DM treated with exogenous insulin can also secrete antibodies directed against this injected insulin which would be indistinguishable from IAA. This potential response to injected insulin makes it impossible to collect PBL after insulin therapy and be assured of obtaining a monoclonal IAA.

Total populations of PBL from several IAA seropositive patients with Type I DM at diagnosis (not yet injected with insulin) have been transformed with EBV and many cell lines have been established in our laboratory. One approach to obtain monoclonal IAA was to clone the EBV-PBL population and test for specific insulin binding monoclonal antibody-producing cultures. This method has been useful in isolating lymphoblastoid cell lines producing monoclonal antibodies against diphtheria toxoid (Tsuchiya et al., 1980), acetylcholine receptor (Kamo et al., 1982), tetanus toxoid (Zurawski et al., 1978)

and other antigens. A major disadvantage of the EBV immortalization approach, however, is that bulk cultures of EBV-transformed lines frequently lose their ability to produce specific antibody after long-term culture (Edwards and O'Hare, 1986). In addition to the instability of specific antibody production, a second limitation of the EBV immortalization technique is that low quantities of antibody (<1 µg/ml) are generally produced.

An alternative approach to the production of human monoclonal IAA was to fuse the EBV-PBL from patients with Type I DM at diagnosis with a human fusion partner which could rescue high amounts of antibody production in EBV-PBL (Kozbor and Roder, 1982). A major problem with the production of human hybridomas had been the lack of an ideal human fusion partner. Human myeloma lines are expected to be more efficient producers of Ig compared with lymphoblastoid lines, and hence would be superior for production of human hybridomas. At the time of undertaking of this study, only two true myelomas were widely available for human hybridoma work, namely, U-266 and RPMI-8226 (Campling et al., 1987). However, the frequency of fusion of these myeloma cell lines was inferior when compared with either mouse or myeloma fusion partners or lymphoblastoid fusion partners (Côté et al., 1983). The most widely used human lymphoblastoid fusion partner was the IgG secreting human lymphoblastoid cell line GM4672 (Shoenfeld and Schwartz, 1985). The advantage of this cell line as well as other lymphoblastoid cell lines over myeloma lines was that the growth rates were more rapid than with the available myeloma lines, and the fusion frequencies were superior. The major problem with the GM4672 line as well as most of the other lymphoblastoid fusion partners was that antibody levels were variable and often unstable.

A good choice for fusion partner at the time was one which combined certain

advantages of lymphoblastoid and myeloma lines (Kozbor and Roder, 1982). By fusing the KR-4 lymphoblastoid cells with the true myeloma RPMI-S226, a hybrid myeloma designated KR-12, with the phenotypic features of a myeloma (prominent rough endoplasmic reticulum and Golgi apparatus) and the rapid growth rate of a lymphoblastoid cell line, was obtained. This line had been found to have a high frequency fusion with EBV-PBL (1 in 10^5). Hybridomas resulting from the fusion of KR-12 with EBV-PBL were found to secrete 10 $\mu\text{g}/\text{ml}$ of specific IgM and were stable for at least a year in culture (Campling et al., 1987). KR-12 was found to secrete γ and κ chains from the KR-4 parent and λ L chains from the S226 parent.

The disadvantage of using KR-12 as well as nearly all of the available human fusion partners was that these secrete their own Ig which would dilute the specific Ig (in this case, IAA) produced by the hybridomas. Mixed molecules would also be secreted, made up of more or less random combinations of the various chains synthesized, so that in a hybrid between two IgG-producing cells only one molecule in 16 of the secreted immunoglobulins would have all four chains specified by the PBL fusion partner, unless preferential association occurred (Milstein et al., 1983). Only one molecule in 16 would then have two active binding sites for insulin. The ideal fusion partner for the EBV-PBL would be a myeloma cell line which was a non producer and which would give high fusion frequencies.

A human myeloma cell line (MM-BA) which was derived from a patient with IgA secreting myeloma was established by Dr. Izaguirre at the Children's Hospital of Eastern Ontario (Ottawa, Canada). This cell line was a promising fusion partner because it grew very well in culture and because, in Dr. Izaguirre's laboratory, Ig H chain was not detected by immunofluorescence. Immunofluorescence studies did however show that there was the production of Ig L chain. When we obtained the MM-BA cell line, it had already been mutagenized and selected for 6-thioguanine resistance, and

HAT sensitivity. Thus, although MM-BA cells were as yet an undefined system in human hybridoma technology, it was expected to be a better fusion partner than other lymphoblastoid cell lines since MM-BA was defined as producing an intracellular Ig L chain only and because it was a true myeloma cell line.

Before fusing, the MM-BA cells had to be adapted to a selectable marker. In fusions with spleen cells or even PBL, the fused cells are selected by virtue of their acquired immortality upon fusing with the myeloma cell. For the purpose of this study, previously immortalized EBV-PBL were to be fused. Thus, a selectable resistance had to be conferred on our cell line. Attempts were made at adapting the MM-BA cells to growth in concentrations of ouabain, however the maximum concentration reached was only 3×10^{-8} M which did not kill the EBV-PBL. Various means of transfecting the MM-BA cells with plasmid DNA conferring resistance to the antibiotics G418 (pSV2neo) or hygromycin B (pSV2hyg) were employed. Calcium phosphate coprecipitation yielded no transformant cells. A synthetic cationic lipid (lipofectin, BRL) was found to be toxic to MM-BA cells and attempts at transfection using this system produced no resistant clones. Finally, MM-BA cells were rendered G418 resistant by transfecting the DNA plasmid pSV2neo into the MM-BA cells by electroporation.

Preliminary experiments had revealed that the EBV-PBL were completely killed in IMDM-10% FBS-0.75 mg/ml G418 by 20 days in culture. Thus, the MM-BA cells which were also sensitive to G418 had to be rendered resistant to IMDM-10% FBS-0.75 mg/ml G418 in order to become an adequate fusion partner for the EBV-PBL. The DNA plasmid pSV2neo was transfected into the MM-BA cells by electroporation and grown in the presence of IMDM-10% FBS-0.75 mg/ml G418. The fastest growing G418 resistant clones were subcloned twice by limiting dilution in order to ensure clonal stability. The subclone D3-1C2-A9 (MM-neo) was the fastest growing clone which had been subcloned

twice with a doubling time of 36 h in IMDM-10% FBS-0.75 mg/ml G-418.

The MM-neo cell line was tissue typed in the laboratory of Dr. D.P.S. Sengar at the Ottawa General Hospital. HLA typing revealed the following haplotypes: A3, A24, Cw7, Bw4, Bw6. Although HLA-A and -C specific antigens were picked up, Dr. Sengar was unable to detect any specific HLA-B alleles. The cell line was positive for the two supertypic specificities (Bw4 and Bw6) of the HLA-B locus. These data confirm that MM-neo is of human origin.

The parental cell line MM-BA had been shown to be Ig H chain negative and Ig L chain positive by immunofluorescence in Dr. Izaguirre's laboratory. Therefore, it was postulated that the G418 resistant MM-BA myeloma cell line derivative, namely MM-neo, may also be a H chain loss variant and thus increase its value as candidate for fusion partner with EBV-PBL. It was however necessary to characterize the MM-neo cells before fusion. Western blot analysis of Ig H and L chains was performed on the parental MM-BA cell line to confirm the findings of Dr. Izaguirre and to establish Ig production of MM-neo. Supernatant from confluent IM-9 cells, which produce an IgG κ antibody (van Boxel and Buell, 1974), as well as human serum were included as controls. Western blot analysis revealed the presence of a band corresponding to the Ig α H chain in the MM-BA and MM-neo cell lysates as well as in the human serum but not in the IM-9 cell lysates as expected. It was somewhat surprising to find that MM-BA and MM-neo cells produced Ig α H chain by Western blot analysis in view of the negative immunofluorescence results found by Dr. Izaguirre. The discordance in the findings can be explained by an increased sensitivity of Western blot analysis compared with immunofluorescence analysis.

Ig L chain Western blot analysis revealed a band corresponding to the Ig λ L chain in the MM-BA and MM-neo cell lysates as well as in the human serum but not in the IM-9 cell lysate. Ig κ L chain Western analysis revealed the presence of a band corresponding

to the κ L chain in the human serum and the IM-9 cell lysate as expected, but not present in the MM-BA or MM-neo cell lysates. Thus, both the MM-BA parental cell line, and its G418 resistant MM-neo derivative, are IgA λ producing myeloma cell lines.

As a potential fusion partner for EBV-PBL, it was necessary to determine whether the MM-neo cells secreted the Ig H and L chains that they were found to produce. An ELISA for Ig α H chain and Ig λ L chain was therefore performed on the supernatant of confluent cultures of MM-neo and MM-BA grown in IMDM-15% FBS. The supernatant of confluent IM-9 cells which do not secrete Ig α H chain or Ig λ L chain (van Boxel and Buell, 1974) along with human serum were included as controls. The Ig α H and λ L chain were not detected in the IM-9 supernatant, however, they were present in the human serum as expected. MM-BA and MM-neo were found to secrete both the Ig α H and λ L chain into the medium.

In light of the finding that MM-neo was a IgA λ antibody secreting myeloma cell line, the attribute of non producer for a fusion partner could not be granted to these cells. The MM-neo cells did however grow very well in G418 for several months. Because of the stability of MM-neo, it was believed worthwhile to attempt fusions with EBV-PBL. Before fusion with EBV-PBL, it was necessary to confirm that the MM-neo cells retained the HAT sensitivity of their parental cell line MM-BA.

When a cell mixture, such as EBV-PBL and MM-neo, is subjected to reagents which promote fusion, the fusion events are poorly controlled. In addition to EBV-PBL/MM-neo fusions, it is expected that many fusions will be EBV-PBL/EBV-PBL or MM-neo/MM-neo. Thus, if it is desired to produce a long-term hybrid cell line from EBV-PBL and MM-neo, a selection procedure is required.

The most common selection procedure is HAT selection (Littlefield, 1964; Fig.11). Littlefield's procedure depends on the fact that when the main biosynthetic pathway for guanosine is blocked by the folic acid antagonist aminopterin, there is an alternative "salvage" pathway in which the nucleotide metabolites hypoxanthine and guanine are converted to their respective ribonucleotides (i.e. IMP and GMP) via the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT).

Aminopterin (4-aminofolic acid) inhibits dihydrofolate reductase and thus prevents de novo purine synthesis from glutamine (Bertino et al., 1975) as well as conferring an additional requirement for exogenous pyrimidines by inhibiting the tetrahydrofolate-dependent thymidylate synthetase. Addition of both a purine (hypoxanthine) and pyrimidine (thymidine) is therefore necessary for hybrid survival. Cells lacking HGPRT die in medium containing HAT, because both the main and the salvage pathways are blocked. However, enzyme-deficient mutant (HGPRT⁻) cells can be made to grow in HAT medium if it is provided with the missing enzyme by fusion with an HGPRT⁺ cell.

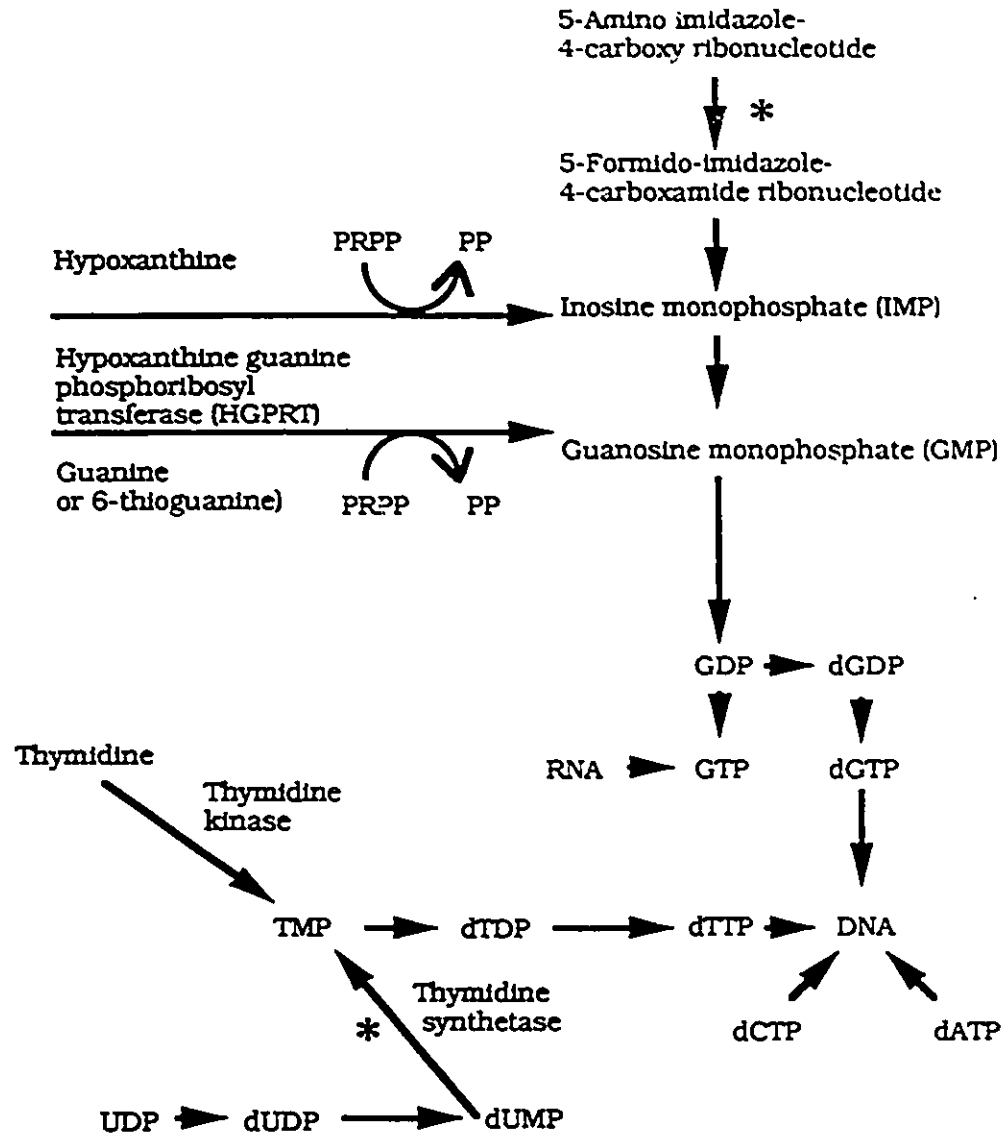
MM-BA cells had been rendered HAT sensitive by culturing the cells in medium containing the cytotoxic purine analogue 6-thioguanine. The original MM-BA cells which contained the enzyme HGPRT converted 6-thioguanine into the corresponding substituted nucleotides, which killed the cells, primarily by incorporation into DNA. Only HGPRT⁻ MM-BA variants survived in culture and hence the HAT sensitive MM-BA cell line was developed. HAT at the concentration of 100 μ M hypoxanthine, 1.6 μ M thymidine and 0.02 μ M aminopterin was found to be effective in the selection of human hybridomas using the human myeloma cell line SKO-007 (Olsson et al., 1983) and thus this concentration of HAT was used to determine the fusion parameters with MM-neo.

Figure 11

**Metabolic Pathways Relevant to Hybrid Selection in Medium Containing
Hypoxanthine, Aminopterin and Thymidine (HAT Medium)**

When the main synthetic pathways are blocked with aminopterin (*), the cell must depend on the "salvage" enzymes HGPRT and thymidine kinase. HGPRT⁻ cells can be selected by growth in medium containing 6-thioguanine or 8-azaguanine, which are incorporated into the cellular DNA via HGPRT. Only HGPRT⁻ cells survive. HGPRT⁻ cells cannot grow in HAT medium unless they are fused with HGPRT⁺ cells.

Figure 11



* Blockage with aminopterin

It was found that both the MM-neo and the MM-BA cells were completely killed in IMDM-15% FBS-HAT by 17 days in culture. The EBV-PBL were also sensitive to IMDM-15% FBS-HAT, although to a lesser degree. The differential HAT sensitivity of the MM-neo cells and the EBV-PBL could therefore serve as a means for selecting unfused MM-neo cells: when all control unfused MM-neo cells are dead in HAT medium, some of the hybridomas with sufficient levels of HGPRT conferred by the EBV-PBL upon fusion could survive. The aminopterin would then be removed from the medium which would prevent death of the hybridomas while ensuring unfused MM-neo cell death.

Unfortunately only approximately 36% of the EBV population survived the same exposure to HAT which completely killed the MM-neo cells. This lowered the chance of obtaining a hybridoma which could survive HAT selection and which also would secrete the antibody of interest, namely IAA.

It was necessary to determine the length of exposure to IMDM-15% FBS-0.75 mg/ml G418 which would kill the EBV-PBL but not the MM-neo cells. The MM-neo cells remained resistant to IMDM-15% FBS-0.75 mg/ml G418 for the 30 day period of study while the EBV-PBL had achieved one hundred percent cell death by 27 days in the same medium. Thus, exposure to IMDM-15% FBS-0.75 mg/ml G418 for at least 27 days would provide an adequate selectable marker for fusions in which the unfused EBV-PBL would be killed but not the hybridomas which would have acquired the G418 resistance from the MM-neo myeloma fusion partner.

The EBV-PBL used for fusion with MM-neo in order to obtain a monoclonal IAA were obtained from a patient with Type I DM at the time of diagnosis (Patient #18; chapter 1). The serum from this patient had been found to have a specific insulin binding level which was greater than 3 SD above control specific insulin binding levels.

The first well (12C1) containing hybridomas was observed 47 days post fusion, while the second well (12C4) was observed 63 days post fusion. In general, human hybridomas tend to have a delayed growth after fusion. The reasons for this delay may relate to thymidine toxicity, they may reflect the relative paucity of purine metabolizing enzymes in typical lymphocyte populations or they may be the consequence of an extended period of chromosomal rearrangement and/or instability (Edwards and O'Hare, 1986).

The hybridomas grew very well in IMDM-15% FBS. The confluent hybridoma supernatants were tested for the presence of insulin binding antibodies. However, both 12C1 and 12C4 supernatant were found to be negative. The hybridomas were easily subcloned in the absence of a feeder layer. The resulting subclones also grew well in IMDM-15% FBS-0.75 mg/ml G418 with a doubling time of 59 h. Because of this G418 resistance, it was assured that the hybridomas were not merely unfused EBV-PBL.

Karyotyping of MM-neo and the subcloned hybridoma 12C1-C12 was performed. Chromosomal analysis revealed that MM-neo was near-triploidy with the presence of marker chromosomes which could be the result of complex rearrangement. Chromosomal analysis also revealed that the 12C1-C12 cells were near tetraploidy with the presence of the same MM-neo myeloma marker chromosomes. The increased number of chromosomes in the 12C1-C12 cells compared with the MM-neo cells were contributed by the EBV-PBL while the marker chromosomes were contributed by the MM-neo cells upon fusion. The finding that 12C1-C12 is near-tetraploidy suggests that it is a hybridoma and not unfused MM-neo cells. This finding along with the demonstration that 12C1-C12 is a G418 resistant cell line, confirm that 12C1-C12 is a hybridoma resulting from the fusion of MM-neo with EBV-PBL.

The fusion frequency of EBV-PBL with the MM-neo cells was found to be 2 clones in 10^8 EBV-PBL. Although only one fusion was performed, it was necessary to assess whether it was worth continuing the fusions of EBV-PBL with MM-neo in order to obtain a IAA-secreting hybridoma. Table 5 compares the fusion frequency obtained with MM-neo with those of other human fusion partners. The fusion frequency of MM-neo with EBV-PBL was of the same order of magnitude as fusions performed with the SKO-OO7 human myeloma cell line with PBL (Côté et al., 1983) but was 15 fold less than the human myeloma GK-5 and PBL (Taub et al., 1985). Compared with the fusion frequencies obtained with the fusion of KR-4 or KR-12 with EBV-PBL (Kozbor and Roder, 1982, Campling et al., 1987), the fusion frequency of MM-neo was approximately 500 fold less.

The MM-neo fusion frequency was also considerably lower than those obtained for other lymphoblastoid cell lines. This low frequency of fusion, coupled with the fact that total populations of EBV-PBL were being fused, as well as with the finding that MM-neo is an IgA λ antibody secretor, makes the chance of obtaining a clone of IAA secreting-hybridomas using MM-neo as fusion partner minimal. Many fusions would have had to be performed with a very low chance of success. Although the fusion frequency of MM-neo could probably have been improved by varying the parameters of fusion (I.e myeloma: EBV-PBL ratio, the use of a feeder layer, the concentration and type of PEG used, etc.), to continue along this line of investigation or even to try fusions with KR-4 or KR-12 would have been very costly and would not have assured being able to achieve the primary goal of this project: to study the autoimmune response to insulin by nucleotide sequence analysis. It was therefore decided to attempt to directly clone the EBV-PBL populations from Type I DM patients in order to obtain a monoclonal IAA secreting lymphoblastoid cell line which would serve for nucleotide sequence analysis.

Table 5

Summary Information of Various Human Cell Lines Used as Fusion Partners

The fusion frequency is calculated from the number of wells containing hybridomas divided by the total number of PBL used for fusion ($\times 10^7$). NA = data not available.

References:

- 1)** Schoenfeld and Schwartz (1985)
- 2)** Côté et al. (1983)
- 3)** Kozbor et al. (1982)
- 4)** Taub et al. (1985)
- 5)** Campling et al. (1987)

Table 6

Cell line	Reference	Description	Ig secreted	Type of fused cells	No. of fusions	Fusion frequency
GM4672	1	Lymphoblastoid (GM1500 derived)	γ, κ	PBL	18	5.4
LJCR-2	2	Lymphoblastoid	γ, κ	PBL	4	3.2
KR-4	3	Lymphoblastoid (GM1500 derived)	γ, κ	PBL	3	2
KR-4	3	Lymphoblastoid (GM1500 derived)	γ, κ	EBV-PBL	3	112
KR-12	5	Hybrid myeloma (KR-4 and RPMI-8226 derived)	γ, κ, λ	EBV-PBL	NA	100
SKO-007	2	Myeloma (U266 derived)	ϵ, λ	PBL	4	0.76
GK-5	4	Myeloma	κ	PBL	53	3
MM-neo		Myeloma (MM-BA derived)	α, λ	EBV-PBL	1	0.2

Chapter 3: Generation of a Human Monoclonal IAA-Secreting Cell Line

Introduction

To date, no group had been successful in cloning and characterizing insulin autoantibodies (IAA) from a non-insulin treated Type I DM patient at the time of diagnosis. In Chapter 2, attempts at generating a human hybridoma IAA-secreting cell line were described. Although hybridomas were generated by the fusion of EBV-PBL from Type I DM patients with a novel human myeloma fusion partner (MM-neo), the hybridomas did not secrete antibodies with insulin binding activity.

An alternative approach had to be undertaken for the generation of monoclonal IAA. PBL isolated from Type I DM patients seropositive for IAA (Chapter 1) have been transformed with EBV to establish continuous cultures (Chapter 2). These lymphoblastoid cells could be cloned and screened directly for insulin binding activity. The supernatant of the resulting monoclonal IAA-secreting EBV-PBL could be used for antigen binding studies to determine the specificity of insulin binding and to possibly provide information on the epitope to which the antibody is directed.

One of the disadvantages of this experimental approach is that only a very small number of cells in the initial polyclonal population can be cloned and will be good stable secretors of antibody (Edwards and O'Hare, 1986): it would therefore be likely that many EBV-PBL would have to be screened before identifying an IAA-secreting cell line. One way of increasing the chances of isolating a IAA-secreting cell line would be to screen EBV-PBL from a Type I DM patient with high titres of serum IAA. However, another disadvantage of this experimental approach is that EBV-PBL often stop dividing after months or even only weeks in culture (Edwards and O'Hare, 1986). It would therefore be necessary to identify the insulin antibody secreting clones and characterize the antibodies prior to the cell lines expiring.

The main objective of this project is to characterize IAA by sequence analysis. RNA could be isolated from the monoclonal IAA-secreting EBV-PBL cells and used for cDNA synthesis. The polymerase chain reaction could be performed in order to specifically amplify the cDNA corresponding to the variable regions of the heavy chain (V_H) and the light chains (V_L) of the IAA for sequence analysis. It would first be necessary to determine the isotype of the H and the L chain of the monoclonal IAA prior to PCR amplification in order to employ the correct isotype-specific oligonucleotide primers.

Chapter 3 describes the cloning of lymphoblastoid cell lines that produce and secrete antibodies with insulin binding activity. The fine specificity of insulin binding as well as the antibody H and L chain isotype determinations are also outlined.

Materials and Methods

Materials

Tissue Culture

The materials used for the isolation, transformation and culture of EBV-PBL were the same as described in Chapter 2.

ELISA for Screening EBV-PBL for Total Insulin Binding Activity

The antibodies (goat anti-human polyvalent antibody horseradish peroxidase conjugate reactive with IgG, IgA, IgM, Bence Jones κ , and Bence Jones λ) were purchased from Sigma Chemical Co.

Determination of the H Chain Isotype of the Monoclonal Insulin Antibodies

The purified human immunoglobulin of the μ isotype (IgM) and the polyclonal goat anti-human IgM (μ -chain specific), conjugated to horseradish peroxidase, were purchased from Sigma Chemical Co.

Determination of the L Chain Isotype of the Monoclonal Insulin Antibodies

The polyclonal rabbit anti-human λ L chain antibody (IgG fraction of serum) and the polyclonal rabbit anti-human κ L chain antibody (IgG fraction of serum) were purchased from Sigma Chemical Co. The polyclonal goat anti-rabbit IgG horseradish peroxidase conjugated antibody was purchased from Bio-Rad Laboratories.

Antigen Binding Determination

The polyclonal goat anti-human IgM (μ chain specific) conjugated to horseradish peroxidase was purchased from Sigma Chemical Co. The porcine and the bovine insulins were purchased from Calbiochem Co.

Methods

Transformation of Peripheral Blood Lymphocytes (PBL) with Epstein-Barr Virus (EBV)

Blood was collected from patients with Type I DM at: (1) the time of diagnosis, (2) following injections with insulin, and (3) prior to the diagnosis of Type I DM (as described in chapter 1). The PBL were purified from the blood and transformed with EBV to establish lymphoblastoid cell lines (see chapter 2).

Screening of EBV-PBL for Insulin Binding Activity

EBV-PBL were plated out in 96-well tissue culture plates in IMDM-15% FBS at a concentration of 10 cells per well and incubated at 37°C in a 5% CO₂ atmosphere. Fresh medium was provided every 4-5 days. After 30-60 days in culture, the wells contained confluent cells. At this time, the medium was left for 7 days without replacement prior to assaying for insulin binding activity by ELISA.

Each well of 96-well ELISA microassay plates was coated with 2 µg humulin in 100 µl coating-buffer and incubated overnight at 4°C. The plates were washed with PBS and blocked for 2-3 h at 37°C with PBS-1% BSA. The plates were then washed with PBS-0.05% Tween 20 and PBS.

Aliquots of 100 µl of the supernatant of the EBV-PBL from the 96-well tissue culture plates were aspirated using a multichannel pipet and placed in corresponding wells of the 96-well ELISA microassay plate coated with humulin. Triplicate aliquots of 100 µl of IMDM-15% FBS were included as negative controls in this assay. The microassay plates were incubated for 1-2 h at room T after which time the plates were washed.

The goat anti-human polyvalent antibody horseradish peroxidase conjugate, diluted 1 in 3000 with PBS-1% BSA, was added to the wells (100 µl/well) and incubated for 1-2 h at room T after which time the plates were washed.

Colour development was performed as described for the ELISA in chapter 2. The specific Abs of the colorigenic product was obtained by reading the Abs at 490 nm and correcting for non-specific Abs with a reading at 450 nm.

When a particular well gave an Abs value greater than that of the triplicate mean of IMDM-15% FBS in the screening assay, the corresponding EBV-PBL were expanded to a 2 ml well in a 24-well tissue culture plate. The supernatant of the confluent cells was retested for insulin binding activity in microassay plates as described for the screening assay. Wells coated with coating-buffer alone were included to correct for nonspecific

binding to the plate. When the supernatant was positive for binding to humulin, the EBV-PBL were cloned by limiting dilution at theoretical concentrations of 5, 1 and 0.5 cells per well in IMDM-15% FBS. The EBV-PBL were grown to confluency and the resulting clones were retested for insulin binding activity by ELISA as previously described.

Over 8,000 wells containing confluent EBV-PBL from patients with Type I DM were screened using this assay. This extensive screening resulted in the generation of two cloned EBV-PBL cell lines which were found to secrete insulin antibody: (1) 3-0.5F10, derived from a non-insulin treated patient at diagnosis for Type I DM (Patient #3 from chapter 1), and which secretes a monoclonal IAA and, (2) 1-0.5G1, derived from a diabetic who had been injected daily with humulin since being diagnosed 9 months earlier with Type I DM (Patient #1 from chapter 1); it is not possible to distinguish between insulin antibody (IA) or IAA secretion in this cell line.

Determination of the H Chain Isotype of the Monoclonal Antibodies

The 3-0.5F10 and 1-0.5G1 cloned cell lines were plated out into 24-well plates in IMDM-10% FBS at a concentration of 10^4 cells per well. The cells were left for 42 days without a change of medium. At the end of this period, the supernatant was collected and frozen at -80°C .

The supernatant from a confluent population of EBV-PBL obtained from a patient at diagnosis for Type 1 DM (Patient #8 from chapter 1) was also tested for the H chain isotype of antibodies secreted in the medium. This lymphoblastoid cell line was chosen because the serum from the blood of Patient #8 from whom the cell line was derived was shown to be negative for IAA in the fluid phase competitive IAA assay (specific insulin binding of 30 nU [^{125}I] insulin bound/ ml serum; chapter 1). If these cells produced and

secreted antibodies which were of the same isotype as the insulin antibodies, but were not insulin specific, the supernatant from these cells could be used as a negative control for subsequent antigen binding determinations. The EBV-PBL from Patient #8 were plated out into wells of a 24-well plate in IMDM-10% FBS at concentration of 10^4 cells per well. The cells were then left for 15 days without a change of medium at the end of which time the supernatant was collected and stored frozen at -80°C . Aliquots of the same batch of IMDM-10% FBS used to plate out the cells was also stored at -80°C for control purposes.

Positive controls for this assay were diluted serum samples from Type I DM Patients #1, 3 and 8. A blood sample ranging from 8 to 15 ml was collected from each of the Type I DM patients at the time of diagnosis. This blood sample was made up to 35 ml with PBS for Ficoll purification of the lymphocytes (described in chapter 2). After centrifugation, the serum layer was collected from the Ficoll gradient and frozen at -80°C for subsequent determinations. For the determination of the H chain isotype, these serum samples were further diluted ten fold with PBS. Thus, the final dilution factors for the serum of Patients # 1, 3 and 8 were 180, 110 and 165 with PBS, respectively.

Preliminary experiments had revealed that the monoclonal antibodies secreted by the 1-0.5G1 and 3-0.5F10 cell lines were of the IgM isotype. In order to obtain a standard curve for the purpose of determining the concentration of IgM in the supernatants and in the serum samples, triplicate 100 μl aliquots of serial dilutions of purified human IgM in PBS were applied to wells and left to incubate overnight at 4°C . The supernatants from the two confluent cell lines and the serum samples were also plated in triplicate into wells (100 μl per well). Negative controls included triplicate wells containing IMDM-10% FBS, as well as triplicate uncoated wells.

The ELISA was performed as described above, using a polyclonal goat anti-human IgM conjugated to horseradish peroxidase, diluted 1 in 3000 with PBS-1% BSA, as secondary antibody. The Abs readings for the test solutions were blanked against the triplicate mean of the uncoated wells.

Determination of the L Chain of the Monoclonal Antibodies

The 3-0.5F10 and 1-0.5G1 cloned cells were each plated out in 24-well plates, in IMDM-10% FBS, at a concentration of 10^4 cells per well. At the end of 18 days without medium change, the supernatant was harvested and assayed for κ or λ L chain content.

Six wells of a 96-well ELISA plate were coated with 100 μ l aliquots of: (1) the supernatant of the 3-0.5F10 cells, (2) the supernatant of the 1-0.5G1 cells, (3) IMDM-10% FBS, and (4) serum samples from the Patients # 1 and 3 diluted by factors of 180 and 110 with PBS, respectively. Six wells were also left uncoated. The microassay plates were incubated for 4 h at room T and washed. The wells were blocked with PBS-5% BSA overnight at 37°C and washed.

Triplicate wells of each of the test solutions, as well as uncoated wells, were incubated for 2 h at room T with 100 μ l aliquots of either: 1) polyclonal rabbit anti-human κ L chain antibody, diluted 1 in 200 with PBS-1% BSA, or 2) polyclonal rabbit anti-human λ L chain antibody, diluted 1 in 200 with PBS-1% BSA. The wells were washed and polyclonal goat anti-rabbit IgG horseradish peroxidase conjugated antibody, diluted 1 in 3000 with PBS-5% BSA, was added to all the wells (100 μ l per well). The plates were incubated for 2 h at room T. The wells were washed and colour development was performed as previously described. The Abs readings of the test solutions were blanked against the triplicate mean of the uncoated wells.

Determination of the Insulin Binding Specificity of the Monoclonal Antibodies

The supernatants assayed for specific insulin binding activity were the same which had been used in the determination of IgM concentration. These included supernatants from: (1) 3-0.5F10 cells, (2) 1-0.5G1 cells, and (3) confluent EBV-PBL cells obtained from Patient #8. The serum samples from Patients # 1, 3 and 8 were diluted with PBS by a factor of 3.6, 2.2 and 3.3, respectively.

The specific insulin binding was determined by competitive ELISA essentially as described (Kuglin et al., 1988). The samples were preincubated with PBS (without excess humulin) to determine total binding to the humulin coated wells, or with excess humulin (16 μ M in PBS), to determine nonspecific binding to the humulin coated wells. Subtraction of the mean nonspecific binding from the mean total binding gave the specific insulin binding of the supernatant and serum samples expressed in Abs units.

ELISA microassay plates were coated overnight at 4°C with either: (1) 100 μ l humulin in coating-buffer, at a concentration of 0.005 μ g humulin per well, or (2) 100 μ l coating-buffer. The next day, the plates were washed and blocked for 2 h at 37°C with PBS-1% BSA.

In preliminary experiments, it was found that a dilution of 1 in 2 for the 1-0.5G1 supernatant and the serum samples in the preincubation period still enabled the detection of specific insulin binding. Thus, six times 50 μ l aliquots of : (1) supernatant from the 1-0.5G1 cells, (2) IMDM-10% FBS, and (3) serum samples (from Patients #1 and 3) were each preincubated for 90 min at room T with either: (a) 50 μ l of 32 μ M humulin in PBS, to give a final concentration of 16 μ M humulin; or (b) 50 μ l of PBS alone.

The results of preliminary experiments had demonstrated that the specific insulin binding of the 3-0.5F10 supernatant could not be detected when the final dilution of the supernatant in the preincubation mixture was 1 in 2. The preincubation in this

experiment was therefore performed with a final dilution of 1 in 1.11. Thus, 90 μ l aliquots of: (1) supernatant from 3-0.5F10 cells, (2) IMDM-10% FBS, and (3) supernatant from Patient #8 cells were each preincubated for 90 min at room T with either: (a) 10 μ l of 160 μ M humulin in PBS, to give a final concentration of 16 μ M humulin; or (b) with 10 μ l of PBS alone.

After 90 min, the preincubated samples were added in triplicate to the blocked humulin coated wells and in triplicate to the blocked coating-buffer coated wells. The plates were incubated for 2 h at room T after which time they were washed with PBS-0.05% Tween 20 and PBS. Polyclonal goat anti-human IgM conjugated to horseradish peroxidase, diluted 1 in 3000 in PBS-1%BSA, was added to the wells (100 μ l per well) and incubated at room T for 2 h. At the end of this incubation, the plates were washed. Colour development and Abs readings were performed as previously described.

The total binding component of the test solutions was obtained by subtracting the mean Abs readings of the triplicate coating-buffer coated wells from the mean Abs readings of the triplicate humulin coated wells. The nonspecific binding component for the test solutions was obtained by subtracting the mean Abs readings of the triplicate coating-buffer wells from the mean Abs readings of the triplicate humulin coated wells. Finally the specific insulin binding was obtained by subtracting the nonspecific binding component from the total binding component. The specific insulin binding was expressed in Abs units.

The same supernatants and the serum samples were also tested for their ability to bind to BSA in order to determine whether the IgM bound to proteins nonspecifically. The supernatants and the serum samples were added in triplicate to wells which had been coated overnight at 4°C with either: 1) PBS-5% BSA; or 2) PBS.

The ELISA was performed as previously described. The total binding component of the test solutions to the BSA was obtained by subtracting the mean Abs readings of the triplicate PBS-coated wells from the mean Abs readings of the triplicate BSA-coated wells.

Competition Binding Curves

A. Serum

Wells from a ELISA microassay plate were coated overnight at 4°C with: (1) 0.005 µg humulin per well, in 100 µl coating-buffer; or (2) 100 µl coating-buffer. The next day, the plates were blocked at 37°C for 2 h with PBS-1% BSA.

The serum from Patients #1 and #3 were diluted with PBS by a factor of 7.2 and 2.2, respectively. Six 50 µl aliquots of: (1) the Patient #1 serum, and (2) the Patient #3 serum were incubated with 50 µl of PBS containing humulin, porcine insulin or bovine insulin (final concentrations ranging from 0-32 µM insulin). The samples were preincubated for 90 min at room T.

The preincubated samples were then added in triplicate to the humulin coated wells and to the coating-buffer coated wells and were incubated at room T for 2 h. The plates were washed and polyclonal goat anti-human IgM, diluted 1 in 3000 with PBS-1% BSA, was added to the wells (100 µl/well). The plates were incubated at room T for 2 h after which time the plates were washed. Colour development and Abs readings were obtained as previously described for the screening ELISA.

The total insulin binding for each concentration of competitor was obtained by subtracting the mean Abs of the triplicate wells coated with coating-buffer alone from that of the mean Abs value of the triplicate humulin coated wells.

The % inhibition of humulin binding was obtained as follows:

$$\begin{array}{l} \text{\% inhibition of} \\ \text{humulin binding} \end{array} = \frac{\text{Mean Abs (PBS)} - \text{Mean Abs (PBS + competitor)}}{\text{Mean Abs (PBS)}} \times 100$$

B. Supernatant of confluent cells

The supernatant of 1-0.5G1 cells and the IMDM-10% FBS were of the same batches as those used in the determination of the H chain. The competition studies were performed on these samples as described for the serum samples.

The competition studies were also performed on the supernatant of confluent (1) 3-0.5F10 cells, and (2) EBV-PBL obtained from Patient #8 at the time of diagnosis with Type I DM, as well as on the IMDM-10% FBS, as described for the serum samples with the following modification: 90 µl aliquots were incubated with 10 µl of PBS containing various concentrations of humulin, porcine insulin or bovine insulin, ranging in final concentration from 0-32 µM competitor. The other steps of the ELISA were performed as previously described.

Results

Screening of EBV-PBL for Insulin Binding Activity

Two cloned EBV-PBL cell lines were obtained which secreted insulin binding antibodies. The first, **3-0.5F10**, was derived from a patient at diagnosis for Type I DM who had not been injected with exogenous insulin (Patient # 3, chapter 1). Therefore, 3-0.5F10 is a monoclonal IAA secreting cell line. This cell line was obtained by plating the EBV-PBL from Patient #3 in six 96-well tissue culture plates at a concentration of 10 cells per well in IMDM-15% FBS. When the cells had reached confluence, the wells were

screened for humulin binding activity by ELISA. The twelve most positive wells (Abs readings greater than the IMDM-15% FBS alone) were pooled and then cloned by limiting dilution. The resulting clones were rescreened for insulin binding activity. The supernatant of the F10 well of the plate containing cells which had been plated out at a theoretical concentration of 0.5 cells per well (1 cell per 2 wells) was found to have a higher Abs reading than the IMDM-15 % FBS alone. This well contained a single clone of cells which was observed under a light microscope. Because the EBV-PBL were derived from Patient # 3, this cell line was named 3-0.5F10. Further attempts were made to subclone this cloned cell line. However, the resulting subclones had lost their ability to secrete IAA.

The second cloned EBV-PBL cell line which was found to secrete antibodies with insulin binding activity was 1-0.5G1. This cell line was derived from a patient with Type I DM (Patient #1, chapter 1) who had been injected daily with humulin since being diagnosed 9 months earlier. Because this patient was injected with humulin, it was not possible, using the binding assay or any other available method, to distinguish between an antibody directed against exogenous humulin (insulin antibody, IA) or an antibody directed against endogenous human insulin (IAA). The EBV-PBL derived from this patient were cloned by limiting dilution. The ELISA screening assay revealed that the supernatant of the well G1, of the plate containing 0.5 cells per well, was positive for insulin binding when compared with the IMDM-15% FBS. This well contained a single clone of cells which was observed under the light microscope. Because the EBV-PBL were derived from Patient # 1, the clone was named 1-0.5G1. Attempts were made at subcloning the cloned cell line. However, the resulting subclones could not be maintained in culture. As noted already, it had been previously observed that EBV-PBL tend to be unstable in culture (Edwards and O'Hare, 1986).

Determination of the H Chain Isotype of the Monoclonal Antibodies

A standard curve was obtained for the Abs values measured by solid phase ELISA in relation to human IgM concentration (Fig. 12). The Abs values of the supernatants of the confluent cells and of the serum samples were converted to $\mu\text{g IgM/ml}$ (Table 6). The IgM concentration of the supernatants of the 1-0.5G1 and 3-0.5F10 cloned EBV-PBL were significantly greater than the IMDM-10% FBS ($p < 0.005$; Table 6). Thus, the monoclonal antibodies secreted by the clones were of the IgM isotype.

The supernatant from the EBV-PBL population from the Type I DM Patient #8 at the time of diagnosis also contained IgM antibodies ($p < 0.005$ when compared with IMDM-10% FBS, Table 6). The concentration of the IgM antibodies in the EBV-PBL Patient #8 supernatant was approximately the same as that of the 1-0.5G1 supernatant and was approximately 2.4 times greater than that of the 3-0.5F10 supernatant. The serum samples also contained IgM antibodies as expected.

Determination of the L Chain of the Monoclonal Antibodies

The L chain isotypes of the antibodies secreted by 1-0.5G1 and 3-0.5F10 cells were determined (Table 7). Both monoclonal antibodies had κ L chains since the Abs of the supernatants tested with the anti- κ antibody were significantly greater than the IMDM-10% FBS ($p < 0.005$, Table 7). There was no significant difference between the Abs of the supernatants and the IMDM-10% FBS when tested with the anti- λ antibody. As expected, the serum samples contained both κ and λ L chain.

Figure 12

Standard Curve: Absorbance as a Function of IgM Concentration

A standard curve was obtained for the purpose of determining the concentration of IgM in the supernatants and in the serum samples. Triplicate 100 µl aliquots of serial dilutions of purified human IgM in PBS were applied to wells of a 96-well ELISA microassay plate. Triplicate uncoated wells were included in this assay. The plate was incubated at 4°C overnight.

The next day, the plates were washed and blocked for 2 h at 37 °C with PBS-1% BSA. Polyclonal goat anti-human IgM conjugated to horseradish peroxidase, diluted 1 in 3000 with PBS-1% BSA, was used as secondary antibody. The plates were incubated at room T for 2 h and washed.

Colour development was performed and the Abs readings were obtained. The Abs readings of the IgM dilutions were blanked against the triplicate mean of the uncoated wells. The results are expressed as the mean log Abs readings in relation to the log concentration of human IgM (µg/ml).

Figure 12

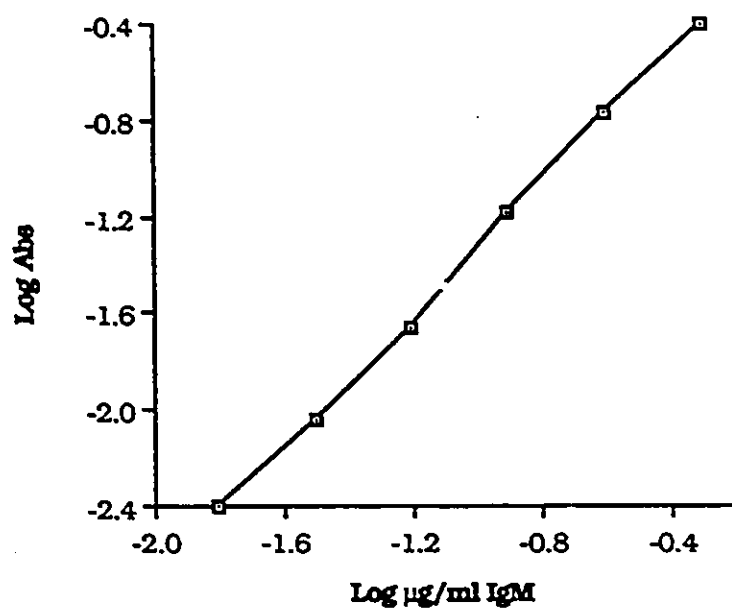


Table 6

Determination of the IgM Concentration

Six wells of a 96-well ELISA plate were coated with 100 µl aliquots of: 1) supernatant from confluent 3-0.5F10 cells, 2) supernatant from confluent 1-0.5G1 cells, 3) IMDM-10% FBS, and 4) serum Type I DM Patient # 1 (diluted by a factor of 180 with PBS), 5) serum from Type I DM Patient #3 (diluted by a factor of 110 with PBS), 6) serum from Type I DM Patient #8 (diluted by a factor of 165 with PBS), 7) IMDM-10% FBS, and 8) human IgM standards (see Fig. 12). Triplicate uncoated wells were also included. The microassay plate was incubated overnight at 4°C.

The next day, the plates were washed and blocked for 2 h at 37 °C with PBS-1% BSA. Polyclonal goat anti-human IgM conjugated to horseradish peroxidase, diluted 1 in 3000 with PBS-1% BSA, was added to the wells. The plates were incubated at room T for 2 h and washed.

Colour development was performed and Abs readings were obtained. The Abs readings for the test solutions were blanked against the triplicate mean of the uncoated wells. The results are expressed as the mean $\pm$ SEM; the Abs readings were converted into µg/ml IgM by intrapolation of the standard curve (Fig. 12).

* = $p < 0.005$ when compared with IMDM-10% FBS (unpaired student's t-test)

Table 6

Test Solution	Abs	µg/ml IgM	Dilution Factor	Corr. µg/ml IgM
EBV-PBL #8 supernatant	0.015 ± 0.001	0.041 ± 0.003	1	0.041 ± 0.003 *
1-0.5G1 supernatant	0.017 ± 0.000	0.046 ± 0.000	1	0.046 ± 0.000 *
3-0.5F10 supernatant	0.006 ± 0.000	0.017 ± 0.000	1	0.017 ± 0.000 *
IMDM-10% FBS	0.001 ± 0.000	0.003 ± 0.000	1	0.003 ± 0.000
Patient #8 Serum	0.197 ± 0.006	0.470 ± 0.017	165	77.6 ± 2.8
Patient #1 Serum	0.077 ± 0.001	0.193 ± 0.003	180	34.7 ± 0.5
Patient #3 Serum	0.376 ± 0.010	0.865 ± 0.028	110	95.2 ± 3.1

Table 7

Light Chain Isotype Determination

Six wells of a 96-well ELISA plate were coated with 100 µl aliquots of: 1) supernatant from confluent 3-0.5F10 cells, 2) supernatant from confluent 1-0.5G1 cells, 3) IMDM-10% FBS, and 4) serum samples from the Type I DM Patients # 1 (diluted 1 in 180 with PBS) and #3 (diluted 1 in 110 with PBS). The plate was incubated for 4 h at room T after and washed. The wells were blocked with PBS-5% BSA overnight at 37°C and washed.

Triplicate wells of each of the test solutions, as well as triplicate uncoated wells, were incubated for 2 h at room T with 100µl of either : 1) polyclonal rabbit anti-human κ L chain antibody, diluted 1 in 200 with PBS-1% BSA, or 2) polyclonal rabbit anti-human λ L chain antibody, diluted 1 in 200 with PBS-1% BSA. The wells were washed and polyclonal goat anti-rabbit IgG horseradish peroxidase conjugated antibody, diluted 1 in 3000 with PBS-5% BSA, was added to the wells. The plates were incubated for 2 h at room T with and washed.

Colour development was performed and Abs readings obtained. The Abs readings of the test solutions were blanked against the triplicate mean of the uncoated wells. The results are expressed as mean Abs readings ± SEM.

* = $p < 0.005$ when compared with IMDM-10%FBS (unpaired student's t-test).

Table 7

Test Solution	κ Abs	λ Abs
IMDM-10% FBS 1-0.5G1 Supernatant 3-0.5F10 supernatant patient #1 serum (1 in 110) patient #3 serum (1 in 180)	0.031 $\pm$ 0.006 0.077 $\pm$ 0.005 * 0.077 $\pm$ 0.009 * 1.006 $\pm$ 0.011 1.020 $\pm$ 0.011	0.075 $\pm$ 0.007 0.072 $\pm$ 0.009 0.065 $\pm$ 0.006 0.841 $\pm$ 0.007 0.878 $\pm$ 0.011

Determination of the Insulin Binding Specificity of the Monoclonal Antibodies

The humulin binding specificities of the monoclonal antibodies secreted by 1-0.5G1 and 3-0.5F10 were tested by a competitive solid phase ELISA (Table 8). The supernatant of the 1-0.5G1 cells contained IgM monoclonal antibodies which bound humulin specifically since the Abs was significantly greater than that of the IMDM-10 %FBS at the same dilution ($p < 0.005$, Table 8).

The supernatant of the 3-0.5F10 cells contained IgM antibodies which bound humulin specifically since the Abs was significantly greater than that of the IMDM-10% FBS at the same dilution ($p < 0.005$, Table 8). Patient #3 from whom the 3-0.5F10 cells were derived had never been treated with insulin. Therefore, 3-0.5F10 cells secrete IAA.

The supernatant of the EBV-PBL from the Type I DM Patient #8, at the time of diagnosis, had been shown to contain IgM at the same concentration as the supernatant of the 1-0.5G1 cells. These IgM antibodies did not bind to humulin specifically.

The serum sample from the Type I DM Patient #1, obtained after 9 months of daily humulin injections, as well as the serum samples from the Type I DM Patients #3 and #8 at the time of diagnosis, contained IgM antibodies which bound humulin specifically.

Binding of the supernatant to BSA was included in order to determine whether the supernatants and the serum samples bound protein nonspecifically (Table 8). The supernatant and the serum samples did not bind to wells coated with PBS-5% BSA.

Table 8

IgM Insulin and BSA Binding

Wells of 96-well ELISA microassay plates were coated overnight at 4°C with either: 1) 100 µl humulin in coating-buffer, at a concentration of 0.005 µg humulin per well, 2) 100 µl coating-buffer, 3) PBS, or 4) PBS-5% BSA. The next day, the plates were washed and blocked for 2 h at 37°C with PBS-1% BSA.

Six times 50 µl aliquots of : 1) supernatant from confluent 1-0.5G1 cells, 2) IMDM-10% FBS, and 3) serum samples from Type I DM Patients #1, #3 and #8, diluted with PBS by a factor of 3.6, 2.2 and 3.3, respectively, were each preincubated for 90 min at room T with either i) 50 µl of 32µM humulin in PBS, to give a final concentration 16µM humulin, or ii) with 50 µl of PBS alone. 90 µl aliquots of: 1) supernatant from confluent 3-0.5F10 cells, 2) IMDM-10% FBS, and 3) supernatant from confluent EBV-PBL from Patient #8 were each preincubated for 90 min at room T with either a) 10 µl of 160 µM humulin in PBS, to give a final concentration 16 µM humulin, or b) with 10 µl of PBS alone.

After 90 min at room T, the preincubated samples were added, in triplicate, to humulin coated wells and to coating-buffer coated wells. Triplicate 100 µl aliquots of each test solution were also added to the PBS-5% BSA and PBS coated wells. The plates were incubated for 2 h at room T and washed. Polyclonal goat anti-human IgM conjugated to horseradish peroxidase, diluted 1 in 3000 with PBS-1%BSA, was added to the wells. The plates were incubated at room T for 2 h and washed.

Colour development was performed and the Abs readings of the humulin coated wells were blanked against the mean of the coating-buffer coated wells. The Abs readings of the PBS-5% BSA coated wells were blanked against the mean of the PBS coated wells.

The specific insulin binding was obtained by subtracting the mean nonspecific binding component from the total binding component. The results are expressed as mean Abs ± SEM.

a $p < 0.005$ when compared with IMDM-10% FBS (1 in 2); unpaired student's t-test

b $p < 0.005$ when compared with IMDM-10% FBS (1 in 1.11); unpaired student's t-test.

Table 8

Test Solution	Abs Total Binding	Abs Nonspecific Binding	Abs Specific Insulin Binding	Abs PBS-5% BSA
1-0.5G1 (1 in 2) supernatant IMDM-10% FBS (1 in 2)	0.129 ± 0.001 a 0.010 ± 0.001	0.076 ± 0.003 a 0.010 ± 0.001	0.053 ± 0.003 a 0.000 ± 0.001	0.000 ± 0.001 0.001 ± 0.002
3-0.5F10 (1 in 1.11) supernatant EBV-PBL #8 (1 in 1.11) supernatant IMDM-10% FBS (1 in 1.11)	0.025 ± 0.002 b 0.004 ± 0.002 0.000 ± 0.000	0.000 ± 0.000 0.003 ± 0.002 0.000 ± 0.000	0.025 ± 0.002 b 0.001 ± 0.002 0.000 ± 0.000	0.000 ± 0.000 0.002 ± 0.002 0.001 ± 0.000
Patient #1 serum (1 in 7.2) Patient #3 serum (1 in 4.4) Patient #8 serum (1 in 6.6)	0.183 ± 0.006 0.508 ± 0.012 0.025 ± 0.008	0.146 ± 0.007 0.475 ± 0.020 0.000 ± 0.000	0.039 ± 0.005 0.033 ± 0.011 0.025 ± 0.008	0.002 ± 0.002 0.001 ± 0.002 0.001 ± 0.001

Competition Binding Curves

The serum from Type I DM Patient #1, who had been injected with humulin daily for 9 months, contained IgM antibodies whose binding to humulin could be completely inhibited by preincubation with 4 μ M humulin, but only partially inhibited (30-40%) by bovine or porcine insulin at the same concentration ($p < 0.005$; Fig. 13A). The final dilution of the serum in the preincubation mixture was 1 in 14.4.

The binding to humulin of the serum IAA from Type I DM Patient #3 could be entirely inhibited by preincubation with either 0.5 μ M humulin, bovine or porcine insulin (Fig. 13B). The final dilution of the serum in the preincubation mixture was 1 in 4.4. There was no significant difference in the % inhibition of humulin binding between the humulin, bovine or porcine insulin for the concentrations of competitor employed in the assay.

The supernatant from the 1-0.5G1 cloned cells (which were derived from Patient #1 after 9 months of injections with humulin) contained IgM monoclonal antibodies whose binding to humulin could be entirely inhibited by preincubation with either humulin, bovine or porcine insulin (Fig. 13C). There was no significant difference in the % inhibition of humulin binding between the humulin, bovine or porcine insulin for the concentrations of competitor employed in the assay. One μ M of competitor completely inhibited humulin binding of the monoclonal antibody secreted by 1-0.5G1.

The humulin binding of the IgM IAA secreted by 3-0.5F10 cells could be entirely inhibited by preincubation with either humulin, bovine or porcine insulin (Fig. 13D). There was no significant difference in the % of inhibition of humulin binding between the humulin, bovine or porcine insulin for the concentrations of competitor employed in this assay. At a 0.5 μ M concentration of competitor, all three variants of insulin completely inhibited humulin binding of the monoclonal IAA secreted by 3-0.5F10.

Figure 13

% Inhibition of Humulin Binding

Humulin binding inhibition studies by Type I DM Patient #1 serum (Fig. 13A), Type I DM Patient #3 serum (Fig. 13B), supernatant from 1-0.5G1 cells (Fig. 13C), and supernatant of 3-0.5F10 cells (Fig. 13D) were performed to establish antigen binding specificity.

Six 50 µl aliquots of: 1) Type I DM Patient #1 serum, diluted 1 in 7.2 with PBS, 2) Type I DM Patient #3 serum, diluted 1 in 2.2 with PBS, and 3) supernatant from 1-0.5G1 cells, were incubated with 50 µl of PBS containing increasing concentrations of either humulin, porcine insulin or bovine insulin. Six 90 µl aliquots of supernatant of 3-0.5F10 cells, were incubated with 10 µl of PBS containing increasing concentrations of either humulin, porcine insulin or bovine insulin.

Wells from a 96-well ELISA microassay plate were coated overnight at 4°C with: 1) 0.005 µg humulin per well, in 100 µl coating-buffer, or 2) 100 µl coating-buffer. The next day, the plates were blocked at 37°C for 2 h with PBS-1% BSA.

The preincubated samples were added in triplicate to the humulin coated wells and to the coating-buffer coated wells and were incubated at room T for 2 h. The plates were washed and polyclonal goat anti-human IgM, diluted 1 in 3000 with PBS-1% BSA, was added to the wells. The plates were incubated at room T for 2 h and washed. Colour development was performed and Abs readings were obtained.

The mean Abs readings of the triplicate wells coated with coating-buffer alone was subtracted from the mean Abs readings of the humulin coated wells for each type and concentration of competitor. The % inhibition of humulin binding was obtained as follows:

$$\% \text{ inhibition of humulin binding} = \frac{\text{Mean Abs (PBS)} - \text{Mean Abs (PBS + competitor)}}{\text{Mean Abs (PBS)}} \times 100$$

Results are expressed as the mean % inhibition ± SEM.

Squares, humulin

Circles, porcine insulin

Triangles, bovine insulin

Figure 13A

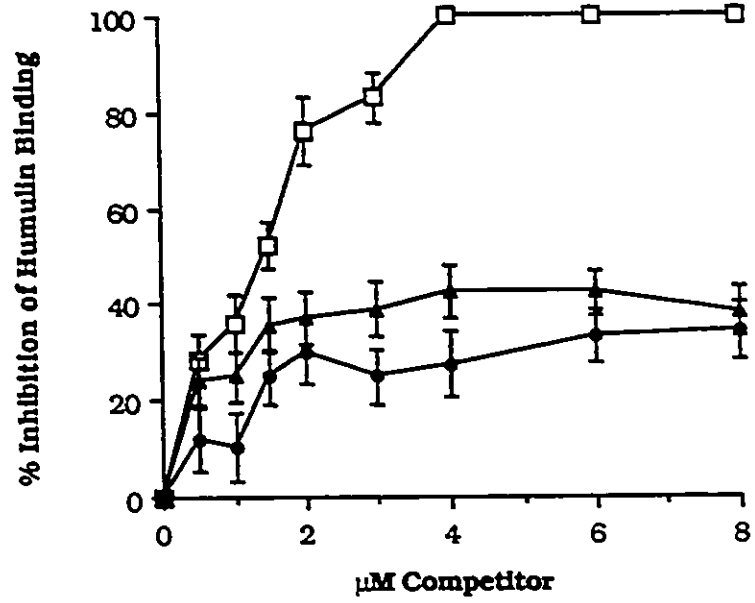


Figure 13B

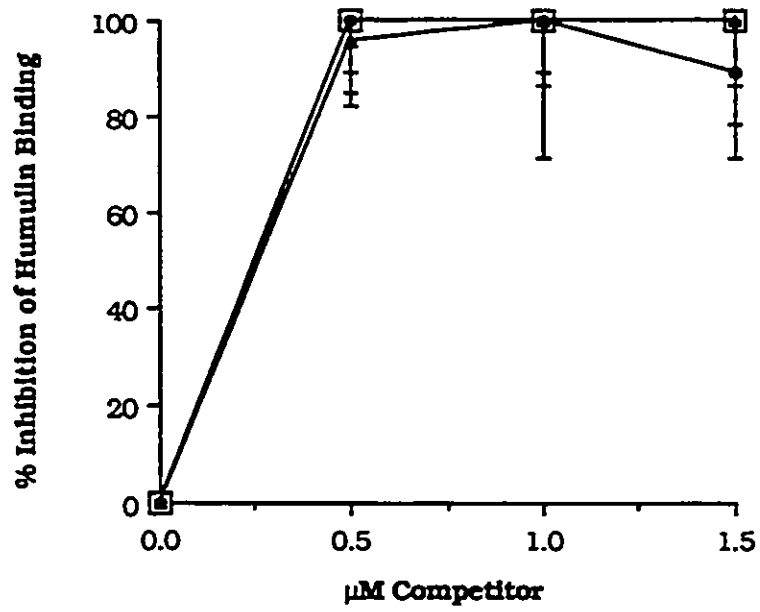


Figure 13C

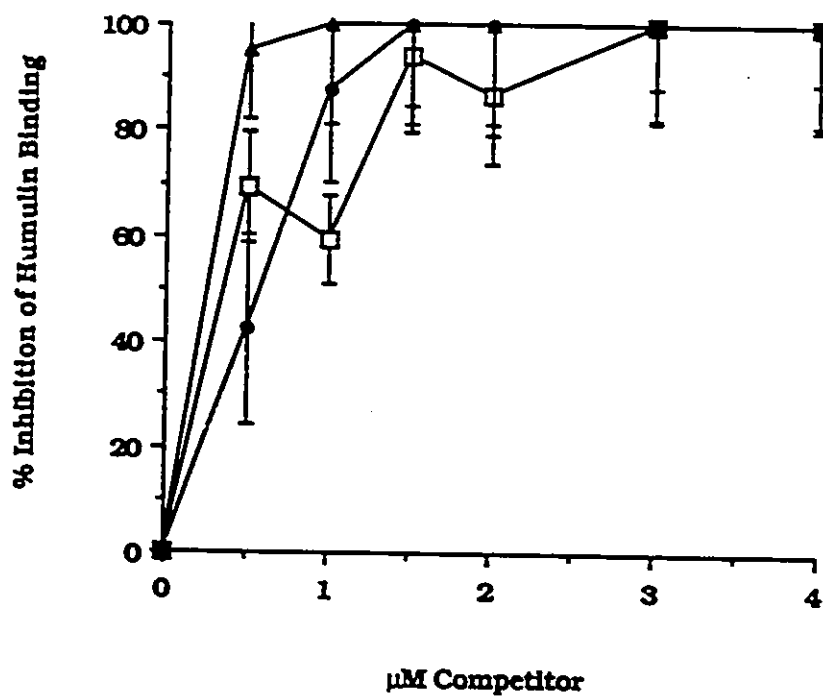
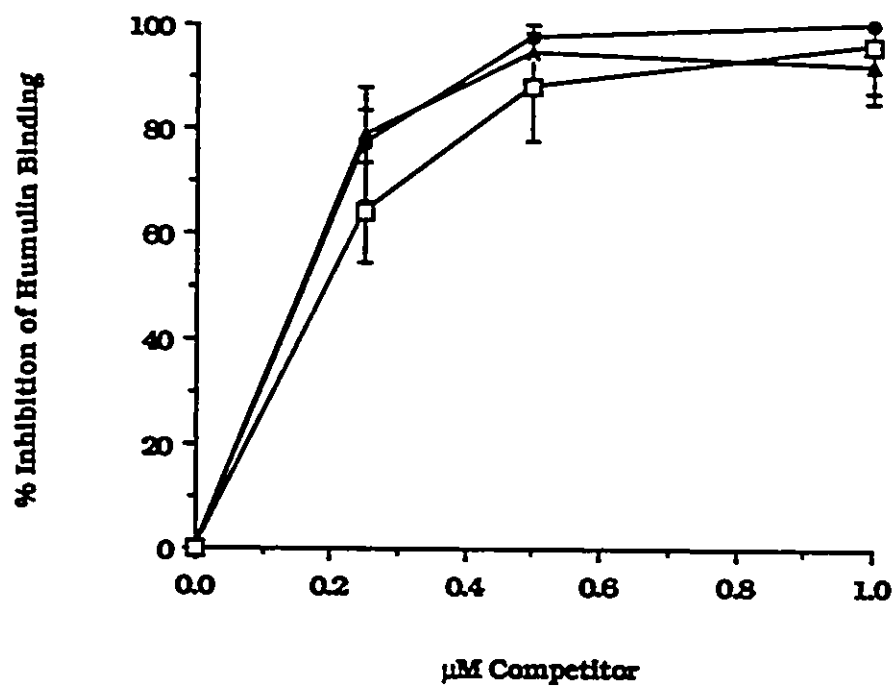


Figure 13D



The supernatant from confluent EBV-PBL from Type I DM Patient #8, at the time of diagnosis, contained the same concentration of IgM as the supernatant from 1-0.5G1 cells and 2.4 times that of 3-0.5F10 cells (Table 6). This supernatant was included in the competition as negative control. For all concentrations of competitor studied, binding of the Patient #8 EBV-PBL supernatant to the humulin coated plates was not detectable. IMDM-10% FBS was also included as negative control in this assay; binding to the humulin-coated plates was not detectable.

Discussion

EBV-PBL from Type I DM patients were cloned directly and tested for secretion of antibodies with insulin binding activity. One disadvantage to this experimental approach is that only a very small number of cells in the initial polyclonal population can be cloned and will be good stable secretors of antibody (Edwards and O'Hare, 1986). Another disadvantage of directly cloning EBV-PBL is that some of the lines die out after some weeks or months in culture (Edwards and O'Hare, 1986). With these considerations in mind, a screening assay had to be used which would allow: (1) many supernatants from wells of confluent EBV-PBL (up to 1000 wells at a time) to be tested for insulin binding activity, and (2) insulin binding activity to be detected immediately in order to expand and clone promising populations of EBV-PBL before these could die out.

Although the fluid phase competitive insulin autoantibody assay (CIAA) described in chapter 1 was the most useful method to identify Type I DM patients with high levels of IAA in the serum, this assay was not suitable for screening a great number of wells containing confluent EBV-PBL all at one time. For each sample tested by CIAA, six supernatants and six pellets were counted (triplicate total binding and triplicate nonspecific binding), a total of 12 tubes per sample. The sheer bulk of tubes would limit the number of supernatants from 96-well tissue culture plates which could be tested. Because the incubation in the CIAA was for one week at 4°C, immediate identification of positive wells would not be feasible. Another disadvantage to the fluid phase CIAA as screening assay was the relatively expensive cost of the [125 I] radiolabeled insulin which would be required in large amounts.

A solid phase ELISA was developed as screening assay. This solid phase ELISA did not discriminate between various isotypes of insulin binding Ig because an anti-human polyvalent antibody conjugated to horse radish peroxidase was employed. Insulin is a

component of the FBS in the IMDM-15% FBS culture medium in which EBV-PBL were grown. A previous study had demonstrated that removal of insulin from antibody by acid charcoal extraction prior to performing the ELISA made little difference to the measurement of IAA (Wilkin et al., 1985). It was therefore decided that the supernatants and serum samples tested by solid phase ELISA not be stripped of insulin prior to performing the assay.

Results arising from the Fourth International Workshop on the Standardization of IAA Measurement, as well as other studies, indicated that fluid phase assays may be superior to solid phase assays for identifying disease associated IAA signals (Kuglin et al., 1990, Greenbaum et al., 1992, Levy-Marchal et al., 1991). Solid phase ELISA is thermodynamically quite different from liquid phase radiobinding systems. In the fluid phase RIA, signal strength depends on the binding by relative excess antibody and a small quantity of ligand. When clones of different affinity are present, the high affinity antibodies will bind preferentially so that low affinity clones may not even be represented in the binding. The reverse is true for the ELISA. The ligand is present in large excess and is available to antibody in high concentration immobilized on solid phase. The amount of antibody which binds under these circumstances is not limited to a small amount of ligand, so that it may be expected that even the lowest affinity clones will be represented in the reaction. Given the differences in measurement of IAA by ELISA and RIA, it is not surprising that IgM IAA were detected by ELISA in the serum of Type I DM Patient #8 at the time of diagnosis, while this patient's serum was negative for IAA (Chapter 1).

Patient #3 had the highest serum levels of specific insulin binding detected by CIAA of all the Type I DM patients studied at the time of diagnosis (Chapter 1). A monoclonal IAA-secreting cell line (3-0.5F10) was obtained by screening the EBV-PBL for insulin

binding by solid phase ELISA. Whether the IgM IAA secreted by 3-0.5F10 cells detected by solid phase ELISA were the same as those detected in Patient #3's serum by the fluid phase RIA is not known. It has been documented that EBV-PBL may die after several weeks or months in culture. In fact, the 3-0.5F10 monoclonal IAA secreting cells did die after approximately one month in culture: enough supernatant from confluent cells was obtained to perform the H and L chain isotype determinations, as well as the competition studies with the solid phase ELISA, but unfortunately, there was not enough supernatant to determine binding of the monoclonal IgM IAA by fluid phase RIA.

The lymphoblastoid cell line 3-0.5F10 obtained from Type I DM Patient #3 (Chapter 1) at the time of diagnosis was shown to secrete monoclonal antibodies (IgM, κ) which bound specifically to humulin. Because Patient #3 had not been treated with exogenous insulin, these cells secreted monoclonal IAA. The monoclonal IAA humulin binding in solid phase ELISA could be inhibited to the same extent by humulin, porcine or bovine insulin. This monoclonal antibody did not bind to BSA. The serum from Patient #3 at the time of diagnosis also demonstrated specific humulin binding which could be inhibited to the same extent by humulin, porcine or bovine insulin. The monoclonal IAA secreted by 3-0.5F10 cells may represent a major population of IAA-secreting B cells in the serum because it demonstrated the same pattern of insulin cross-reactivity as that of Patient #3's serum at the time of diagnosis with Type I DM.

The lymphoblastoid cell line 1-0.5G1 was obtained from Type I DM Patient #1 after 9 months of daily injections with humulin. This cloned cell line was shown to secrete antibodies (IgM, κ) which bound humulin specifically but did not bind to BSA. Because this cell line was derived from a patient who had received exogenous humulin for 9 months, it is not possible to distinguish whether the monoclonal antibodies secreted by 1-0.5G1 were directed towards exogenous or endogenous human insulin.

The 1-0.5G1 cells which secreted antibodies with insulin binding activity as detected by solid phase ELISA died after a few weeks in culture: there was not enough supernatant to test insulin binding by fluid phase RIA. Therefore, it is not known whether the monoclonal insulin binding antibody secreted by 1-0.5G1 was represented in the binding observed in the RIA on the serum from Patient #1 after 9 months of treatment. The humulin binding of the monoclonal antibody secreted by 1-0.5G1 detected by solid phase ELISA could be inhibited to the same extent by humulin, porcine or bovine insulin. However, the serum from Patient #1 after 9 months of treatment with humulin showed humulin binding which could be entirely inhibited by preincubation with 4 μ M humulin but only partially inhibited by preincubation with porcine and bovine insulin at the same concentration. Because the monoclonal antibody from 1-0.5G1 cells showed a different pattern of reactivity as that of the serum, the IgM response to insulin in Patient #1 after insulin therapy is not monoclonal.

Both the monoclonal IAA from 3-0.5F10 cells and the monoclonal insulin binding antibody secreted by 1-0.5G1 cells showed the same pattern of cross-reactivity between humulin, porcine and bovine insulin. Based on the similarity of specificity of insulin binding exhibited by the monoclonal antibodies secreted by 1-0.5G1 and 3-0.5F10, it is possible that these monoclonal antibodies are directed towards the same epitope on the insulin molecule.

Interestingly, Wilkin and co-workers (1988) found that IAA detected by solid phase ELISA with cross-reactivity between bovine, porcine and human insulin are Type I DM related (Wilkin et al., 1988): cross-reactivity was seen in 98% of Type I DM-related persons (siblings of Type I DM patients, newly diagnosed but non-insulin treated Type I DM patients, and identical discordant twins for Type I DM) but only in 39% of persons who were not Type I DM related. Thus, it is possible that IAA in Type I DM are clonally

restricted and recognize an epitope that is common to human, porcine and bovine insulin.

The primary structures of insulin from more than 28 species have been determined (Dayhoff, 1972). Those of human, bovine and porcine insulin are shown in Fig. 14. High resolution X-ray analyses of the crystal structures of several species of insulins (beef, pork, human and hagfish) are all remarkably similar (Schroer et al., 1983). Circular dichroism studies of insulin in solution have indicated that the percentage of helix is close to that of the crystal structure, suggesting insulin's shape does not change dramatically in solution (Schroer et al., 1983). All species of insulins have been studied for their bioactivity and their potency always correlates with an undisturbed 3D structure (Schroer et al., 1983). This last observation coupled with structural studies of species variants of insulin suggest that the porcine, bovine and human insulins possess the same basic 3D structure with only minor changes in side chains.

In a theoretical model generated from X-ray coordinates, insulin emerged as a compact globular protein with very few amino acid residues buried in the hydrophobic core (Schroer et al., 1983); the A chain was found to have two helical portions separated by a loop region formed by intrachain disulfide bond between A chain cysteine residues 7 and 11, and the B chain was found to consist of pleated sheet segments at the amino and carboxy termini, joined by a central helical region intertwined with the carboxy-terminal A chain helical region.

Figure 14

Amino Acid Sequences of Insulin Variants

Comparison of the amino acid sequences (Dayhoff, 1972) of humulin, bovine and porcine insulin used in this study. Identities are shown by solid lines.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Human	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn
Pork																					
Beef								Ala				Val									

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Human	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu
Pork	_____														
Beef	_____														
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Human	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Thr
Pork	_____														Ala
Beef	_____														Ala

The insulin antibodies secreted by 1-0.5G1 and 3-0.5F10 are reactive towards the human (recombinant), porcine and bovine insulins. In a previous study, 4 out of 5 cross-reactive IAA sera studied bound exclusively A chain determinants or determinants dependent on an intact tertiary structure: there was absence of binding to human and porcine B chains (Diaz and Wilkin, 1987). Tainer and colleagues (Tainer et al., 1984) have suggested that the antigenic sites on proteins are closely related to local mobility of the peptide backbone. The A chain displays two particular sites at A4 and A8-10 that, along with the B3 and B28-30 sites, all occupy a band on the surface of the insulin molecule that shows high mobility: if mobility is an important factor in antigenicity, the A4 residue is a strong candidate for the reactions displayed by the cross-reactive monoclonal insulin binding antibodies obtained in this study (Diaz and Wilkin, 1987). Human, porcine and bovine insulins all contain glutamic acid at A4 (Fig. 14).

The polyclonal IgM humulin binding in the serum from Type I DM Patient #1 after 9 months of insulin injection was completely inhibited by preincubation with 4 μ M humulin but only partially inhibited by preincubation with bovine or porcine insulin at the same concentration. Porcine insulin differs from human insulin only at residue B30 (alanine instead of threonine, Fig. 14). Bovine insulin also has a alanine at position B30. Thus, the IgM polyclonal insulin response in the serum of Patient #1 after injection with human insulin may be largely directed towards the residue B30. The B30 residue has high mobility (Schroer et al., 1983), which may explain determinant restriction near the threonine B30 of the human insulin molecule.

The IgM in the serum of the insulin-treated Type I DM Patient #1 had higher affinity for human insulin than porcine or bovine insulin, while the IgM in the serum of the non-insulin treated Type I DM Patient #3 demonstrated cross-reactivity to the same degree between all three insulin variants. Human insulin specific IAA in the

serum of Type I DM individuals before insulin treatment was observed in only 1 out of 42 of Type I DM related patient sera (Wilkin et al., 1988). One possible explanation for the apparent absence of B30-directed antibodies in newly diagnosed Type I DM patients may involve the availability of antigen. Endogenous insulin is released into the portal circulation as a dilute monomeric solution (Steiner, 1970), whereas formulated insulins used in the treatment of diabetics are injected subcutaneously, where they polymerize and remain at a relatively high temperature for long periods before release into the systemic circulation (Kurtz and Nabarro, 1980). Polymerization greatly increases the antigenicity of insulin and may conceivably do so to the advantage of some determinants and the detriment of others (Diaz and Wilkin, 1987). Thus, polymerization may increase the antigenicity of a B30-dependent epitope.

The two cell lines 1-0.5G1 and 3-0.5F10 both secreted IgM antibodies with κ L chains. Interestingly, a human monoclonal antibody to insulin which was derived from an insulin treated Type I DM patient was also found to be an IgM antibody with a κ L chain (Livneh et al., 1986). In a few patients with the insulin autoimmune syndrome, the IAA have been characterized and have been found to be IgG with one L chain isotype (either κ or λ) dominating the autoimmune response (Nakagawa et al., 1973, Uchigata et al., 1989, Wasada et al., 1989). Nell et al. (1989) determined that, for IAA in patients with polyimmunity, one L chain isotype (either κ or λ) also usually dominated the autoimmune insulin response, whereas in sera of treated diabetics, a heterogeneous response was found. Whether the IAA that occur in the insulin autoimmune syndrome are similar to the IAA that occur as part of the autoimmune process in Type I DM has not been determined. Because only one monoclonal IAA antibody and one monoclonal insulin binding antibody (IA or IAA) have been characterized in this present study, it is not possible at this stage to draw any conclusions as to the degree of clonal restriction

of the H and L chains employed in the autoimmune response to insulin in Type I DM.

Both cell lines died out after several weeks in culture. However, enough 3-0.5F10 monoclonal IAA-secreting cells were obtained to allow the isolation of RNA. This RNA could be transcribed into cDNA. IgM μ H chain and κ L chain specific amplimers could be used for the PCR amplification of the cDNA corresponding to the variable regions of the H chain (V_H) and of the L chain (V_L) of the IAA secreted by 3-0.5F10. The V_L and the V_H cDNA of the IAA secreted by 3-0.5F10 could be sequenced and the degree of restriction of the usage of the V_L and the V_H genes in Type I DM could be determined.

Chapter 4: Cloning and Characterization IAA V_L and V_H cDNA

Introduction

The characterization of a monoclonal IgM- κ IAA has been described in Chapter 3. This IAA was secreted by the cell line 3-0.5F10, which was derived from a patient with Type I DM at the time of diagnosis. The next objective of this project is the cloning and sequence analysis of the variable region (V) of both the light chain (V_L) cDNA and the heavy chain (V_H) cDNA of this monoclonal IAA.

Immunoglobulins are encoded by large multigene families that potentially express an almost unlimited degree of diversity at the level of the serum antibody. A number of processes contribute to this phenomenon: (1) there are a large number of L and H chain germ line V-region genes (Berman et al., 1988, Lorenz et al., 1987); (2) the V-regions of both the L and H chains are encoded by multiple genetic elements: V_L and J_L (light chain joining region) in the L chain (Max et al., 1979) and V_H, D_H (heavy chain diversity region) and J_H (heavy chain joining region) in the heavy chain (Sakano et al., 1980); the number of protein structures that can be generated by various combinations of these segments constitutes a major contributor to the total diversity; (3) the joining of the various gene segments is imprecise, creating sequence variation at the points of recombination (Max et al., 1979); (4) gene conversion may occur between related members of immunoglobulin families (Clarke et al., 1982); and (5) somatic point mutation can generate structural alterations (Rudikoff et al., 1984).

Determination of the nucleic acid sequence of the V_L and V_H of the human IAA would provide information on the immune response to endogenous insulin. It would allow the assignment of genes to defined germ-line elements (V_H and V_L subfamilies), would allow analysis of D and J segment usage, and would permit the evaluation of the influence of somatic hypermutation in the clonal evolution of this response.

A technical advance which aids the cloning of Ig chain V-region cDNAs has been the development of the polymerase chain reaction (PCR) (Saiki et al., 1985). In the past, the PCR technique has been successfully applied to clone the V domains of mouse and human monoclonal antibodies (Orlandi et al., 1989, Trainor et al., 1990, Deane and Norton, 1990). Furthermore, a general method has been described to directly obtain the DNA sequence of the V_H and V_L of any immunoglobulin using PCR and a mixture of oligonucleotide primers (Larrick et al., 1989). In this method, mixed oligonucleotide primers corresponding to the 5' signal peptide and a conserved 3' constant region (C-region) primer are used for the enzymatic amplification of cDNAs corresponding to the V_H and V_L of monoclonal antibodies.

PCR is ideal for human monoclonal antibodies: it has the advantage that it allows amplification of very low abundance messages or messages of moderate abundance isolated from a small number of cells. Both these advantages are attractive when dealing with EBV immortalized lymphoblastoid cell lines which produce low levels of antibody and which may die out after a few weeks in culture. This chapter describes the PCR amplification, cloning and sequencing of cDNAs corresponding to the V_H and V_L of the monoclonal IAA secreted by the 3-0.5F10 cells. Furthermore, the degree of restriction of the IAA V_H and V_L gene usage within a Type I DM population at the time of diagnosis is also investigated.

Materials and Methods

Materials

Tissue Culture

The materials used for the isolation, transformation and culture of EBV-PBL were the same as described in Chapter 2.

Isolation of Total Cellular RNA

The chemicals used for making the buffers and the solutions were of reagent grade and were purchased from Fisher Scientific. Distilled and demineralized water was treated with diethylpyrocarbonate (DEPC, Fisher Scientific) to ensure that the water was RNase free. The reagents used for the electrophoresis of RNA through gels containing formaldehyde were purchased from Fisher Scientific.

PCR Amplification of V_L and V_H cDNA

The GeneAmp RNA PCR Kit was purchased from Perkin-Elmer Cetus. This kit included reagents to perform the reverse transcription of RNA to cDNA using cloned Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT), and oligo dT. Subsequent amplification is performed using the AmpliTaq DNA polymerase. A recombinant RNase inhibitor was included in the kit. The mineral oil used in the PCR was purchased from Sigma Chemical Co.

The mixed oligonucleotide primers used for PCR amplification were synthesized at the Biotechnology Institute of the University of Ottawa. The PHC-2 Dri-Block was used for the PCR and was manufactured by Techne. The columns used for the purification of the PCR amplified products were purchased from Qiagen.

Cloning of the PCR Amplified IAA V_L and V_H cDNAs into the pGEM-7Zf(+) Vector

The *Eco*RI and *Hind*III restriction enzymes were purchased from BRL. The reagents used for the agarose gel electrophoresis of DNA were purchased from Fisher Scientific. The klobase (kb) marker, λ DNA/*Hind*III, was purchased from BRL. The pGEM-7Zf(+), the *E. coli* NM522 bacterial cell line and the T4 DNA ligase were purchased from Promega. The calf intestinal phosphatase (CIP) was purchased from Boehringer Mannheim.

Sequencing of the Cloned IAA V_L and V_H cDNA

The T7 Sequencing Kit was purchased from Pharmacia. This kit provided all the solutions and reagents for the dideoxy sequencing reactions using T7 DNA polymerase. The pUC/M13 forward and reverse sequencing primers were purchased from Promega. The deoxyadenosine 5'-[α -thio] triphosphate ([α -³⁵S]dATP, 500 Ci/mmol) was purchased from New England Nuclear/DuPont. The restriction enzyme *Sca* I was purchased from BRL. The premixed, preweighed, acrylamide/bisacrylamide (29:1) was purchased from BioRad. The sodium persulfate, the ammonium peroxodisulfate, and the N,N,N',N'-tetramethylene (TEMED) were purchased from BDH. The urea was purchased from Fisher Scientific. The amberlite was purchased from ICN Biochemicals. The X-ray films were manufactured by Kodak.

PCR Amplification of cDNA Synthesized from EBV-PBL RNA, Using IAA-specific V_L and V_H Synthetic Oligonucleotide Primers

The oligonucleotide primers corresponding to the specific sequences within the IAA V_L and V_H cDNAs were synthesized at the Biotechnology Institute of the University of Ottawa.

Southern Analysis of the PCR Amplified Products Using IAA Sequence Specific Probes

The oligonucleotide primers used in Southern analysis were synthesized at the Biotechnology Institute of the University of Ottawa. The reagents used for agarose gel electrophoresis were purchased from Fisher Scientific. The kilobase (kb) marker, ϕ X174 DNA, cut with *Hinc* II, was purchased from Promega. The nylon membrane was purchased from Amersham. The adenosine 5-triphosphate tetra (triethylammonium) salt ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 3000 Ci/mmol) was purchased from New England Nuclear/DuPont. The T4 polynucleotide kinase was purchased from BioLabs.

Sequence Analysis

V_H and V_L sequences were analyzed using the PC/Gene Software and the EMBL Data Library kindly made available by Dr. M. Tenniswood, Department of Biochemistry, University of Ottawa.

Methods

Tissue Culture

The monoclonal IAA-secreting lymphoblastoid cell line, 3-0.5F10, was grown in IMDM-15% FBS in a 5% CO_2 atmosphere at 37°C . A total of 7×10^6 cells were pelleted at 1200 rpm for 10 min at 4°C with the brake off. The pellet was washed with PBS and frozen at -80°C for 2 months.

Isolation of Total Cellular RNA

The total cellular RNA was isolated from the 3-0.5F10 cells by the method of LiCl/urea (Auffray and Rougeon, 1980). The yield was 50 μg RNA per 10^6 cells as determined by an Abs reading at a wavelength of 260nm (an Abs of 1 corresponds to 40 μg RNA/ml)

(Maniatis et al., 1982). The integrity of the RNA was assessed by electrophoresis of the RNA through a gel containing formaldehyde and visualized by staining with ethidium bromide (Maniatis et al., 1982).

Synthesis of cDNA and PCR Amplification of the IAA V_L and V_H cDNA

First strand cDNA was directly synthesized from total RNA at 37°C for 1 h in a 20 µl reaction volume with oligo-dT priming using the Perkin-Elmer Cetus Kit as suggested by the manufacturer. Twenty µl of this reaction mixture contained: 1.0 µl MMLV RT (50 U/µl), 1.0 µl RNase inhibitor (20 U/µl), 2.0 µl 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 1.0 µl oligo d(T)₁₄ (50 µM), 2.0 µl of each dNTP (dATP, dCTP, dTTP, dGTP; 10 mM), 4.8 µl of a 25 mM MgCl₂ solution (final concentration of 6 mM MgCl₂), 2.2 µl RNA in DEPC-treated H₂O (ranging from 0.25-0.5 µg/µl). After the incubation period, the reaction mixture was heated to 95°C for 10 min.

For a given Ig chain, the sequence encoding the V-region was enzymatically amplified from the cDNA, using a downstream primer corresponding to a sequence of the conserved C-region and a mixture of oligonucleotide primers corresponding to the leader sequence (Larrick et al., 1989), as shown in Fig. 15. The PCR primers were designed with restriction sites at their 5' ends, which allowed subcloning of the amplification product after digestion with restriction enzymes.

Eighty µl of PCR mix was added to the 20 µl of first strand cDNA. The PCR mix contained: 68.3 µl H₂O, 1.2 µl of a 25 mM MgCl₂ solution (final concentration of 1.5 mM MgCl₂), 8.0 µl 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3) and 2.0 µl of primers to give a final primer concentration of 1 µM. Thus, for amplification of the V_H cDNA, a mixture of 0.25 µM each of the group HS-1, group HS-2, and group HS-3 primers, as well as 0.25 µM of the µ-H chain C-region primer, in a total volume of 2.0 µl H₂O were added. For amplification of the V_L cDNA, a mixture of 0.5 µM κ-leader and

0.5 μM $\kappa\text{-C}$ -region primers in a total volume of 2.0 μl H_2O were added. Finally, 0.5 μl of the AmpliTaq DNA polymerase (5 U/ μl) was added and the solutions were well mixed. RNase-free mineral oil was layered on top to prevent evaporation during PCR.

The mixture was subjected to PCR amplification using the Techne thermo cycler set for 35 cycles. The conditions used for the PCR were: (1) melt 94°C , 1 min, ramp 4 ($48^\circ\text{C}/\text{min}$); (2) primer anneal 50°C , 2 min, ramp 3 ($30^\circ\text{C}/\text{min}$); (3) primer extension, 72°C , 3 min, ramp 3; (4) 60°C , 7min, ramp 3; and (5) 22°C , up to overnight, ramp 1 ($1^\circ\text{C}/\text{min}$) at the end of the 35 cycles (Larrick et al., 1989).

The mineral oil was extracted from the PCR amplified products with chloroform. The PCR amplified products were run on a 2% agarose gel, with λ DNA/*Hind*III as marker and bands corresponding to the amplified V_L and V_H cDNAs were recovered by electroelution (Maniatis et al., 1982). The PCR amplified products were further purified by passing them through a Qiagen column as recommended by the manufacturer.

Cloning of the PCR Amplified V_L and V_H cDNA into the pGEM-7Zf(+) Vector

The purified PCR amplified cDNA products were cut for 4 h at 37°C with *Eco*RI and *Hind*III in a final volume of 12.0 μl containing: 9.0 μl of cDNA (100 ng) in H_2O , 1.0 μl 10X KGB buffer (1 M potassium glutamate, 250 mM tris-acetate, pH 7.6, 100 mM magnesium acetate, 500 $\mu\text{g}/\text{ml}$ BSA, 5 mM 2-mercaptoethanol) (McClelland et al., 1988), 1.0 μl of *Eco*RI (10 U/ μl), 1.0 μl of *Hind*III (10 U/ μl). After incubation, the volume was made up to 100 μl with H_2O , phenol: chloroform (1:1) extracted twice, chloroform: isoamyl (24:1) extracted once and ethanol precipitated. The pellet was washed with 70% ethanol, lyophilized, and resuspended in 3.0 μl H_2O to give a final concentration of 0.1 μg DNA/ μl . The *Eco*RI and *Hind*III cut PCR amplified V_L and V_H cDNAs were run on a 2% agarose gel, with λ DNA/*Hind*III as marker.

Figure 15

Design of Synthetic Oligonucleotide Primers for PCR Amplification of V-region cDNA

Synthetic oligonucleotide primers were synthesized which correspond to: **A)** immunoglobulin leader sequences and **B)** the N-terminal part of immunoglobulin C-regions (Larrick et al., 1989).

¹ Bases in parentheses represent substitutions at a given position, for example (AT) means both A and T were present in equimolar amounts during the synthesis of a particular position. *EcoRI* sites are underlined. Two Gs are added 5' of the restriction endonuclease sites to facilitate enzyme cleavage.

² Each of these sequences is unique and 100% complementary with germ-line C-region sequences. *HindIII* sites are underlined. An extra two Cs are added 5' of the restriction endonuclease sites to facilitate enzyme cleavage.

Figure 15

(A) immunoglobulin leader sequences ¹:

Human H chain:

Group HS1 (mixture of 8 primers):

5'—GGGAATTCATGGACTGGACCTGGAGG(AG)TC(CT)TCT(GT)C—3'

Group HS2 (mixture of 16 primers):

5'—GGGAATTCATGGAG(CT)TTGGGCTGA(CG)CTGG(CG)TTT(CT)T—3'

Group HS3 (mixture of 4096 primers):

5'—GGGAATTCATG(AG)A(AC)(AC)(AT)ACT(GT)TG(GT)(AT)(CGT)
C(AT)(CT)(CG)CT(CT)CTG—3'

Human κ—L chain (mixture of 3456 primers):

5'—GGGAATTCATGGACATG(AG)(AG)(AG)(AGT)(CT)CC(ACT)(ACG)
G(CT)(GT)CA(CG)CTT—3'

(B) the N-terminal part of immunoglobulin C-regions ²:

Human μ—H chain (amino acid positions 120-125):

5'—CCAAGCTTAGACGAGGGGAAAAGGGTT—3'

Human κ—L chain (amino acid positions 117-125):

5'—CCAAGCTTCATCAGATGGCGGAAGAT—3'

The pGEM-7Zf(+) vector DNA was used for the cloning of the V-region cDNA. This vector is 3000 bp with some of the following features: (1) T7 RNA polymerase transcription initiation site; (2) T7 RNA polymerase promoter; (3) multiple cloning sites including the *EcoRI* and the *HindIII* restriction sites; (4) the lac Z start codon, operon sequences and operator sequences; (5) the β -lactamase coding region; and (6) the binding sites for the pUC/M13 forward and reverse sequencing primers.

The pGEM-7Zf(+) vector DNA was cut for 4 h at 37°C with *EcoRI* and *HindIII* in volumes of 20.0 μ l containing: 3.0 μ l pGEM-7Zf(+) vector DNA (20 μ g), 2.0 μ l 10X KGB buffer, 2.0 μ l of *EcoRI* (10 U/ μ l), 2.0 μ l of *HindIII* (10 U/ μ l), and 11.0 μ l H₂O. After incubation, the cut pGEM-7Zf(+) vector DNA was extracted from a 1.0% low melting point agarose gel (Maniatis et al., 1982). The purified *EcoRI/HindIII* cut pGEM-7Zf(+) vector DNA was phosphatased using CIP (Maniatis et al., 1982).

Ligations were performed overnight, at room T, in a volume of 10.0 μ l containing: 1.0 μ l of phosphatased *EcoRI/HindIII* cut pGEM-7Zf(+) vector DNA (80 ng), 1.0 μ l of PCR amplified and *EcoRI/HindIII* cut V_L or V_H cDNA (100 ng), 1.0 μ l of 10X ligation buffer (300 mM Tris-HCl, pH 7.8, 100 mM MgCl₂, 100 mM DTT, 10 mM ATP), 6.6 μ l H₂O and 0.4 μ l T4 DNA ligase (1.2 Weiss units). Competent NM522 cells were transfected with the ligated DNA and plated with 50 μ l of 2% 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-GAL) in dimethyl formamide and 30 μ l isopropyl- β -D-thiogalactoside (IPTG; 100 mM in H₂O) onto LB agar plates containing 100 μ g/ml ampicillin (Maniatis et al., 1982).

Fifteen white colonies were picked from each of the plates containing the NM522 cells transfected with vector DNA harbouring either the IAA V_L or V_H cDNA insert. Minipreps of plasmid DNA were performed by the method of alkaline lysis (Maniatis et al., 1982). The plasmid DNA from each colony was cut with *EcoRI* and *HindIII* as

previously described and was run on a 2% agarose along with λ DNA/ *HindIII* to check for the presence of cDNA insert. Large-scale isolation of plasmids containing the V_L cDNA insert from two separate clones and the V_H cDNA insert from two separate clones was performed by the method of alkaline lysis followed by purification by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Maniatis et al., 1982). The amplified DNA was cut with: (1) *EcoRI* and *HindIII* to check for the presence of the cDNA insert, and (2) *Sca I* to verify that the plasmid with the cDNA insert could be linearized; the cut DNA was run on a 2% agarose along with λ DNA/ *HindIII*, as molecular weight marker.

Sequencing of the Cloned IAA V_L and V_H cDNA

The plasmids containing the V_L cDNA insert from two separate clones and the V_H cDNA insert from two separate clones were linearized with *Sca I*, phenol:chloroform (1:1) extracted twice and ethanol precipitated. The pellet was washed with 70% ethanol and then lyophilized. The DNA was resuspended in H_2O at a final concentration of 0.2 $\mu\text{g}/\mu\text{l}$ and sequenced by the dideoxy method (Sanger et al., 1977).

Sequencing was performed as recommended by the kit manufacturer, using T7 DNA polymerase, as follows: a 10.0 μl volume (2.0 μg) of the *Sca I* linearized plasmid containing the cDNA insert was boiled for 5 min and left on dry ice for 15 min. The pUC/M13 forward or reverse sequencing primer in a volume of 2.0 μl was added to the linearized plasmid to give a final molar primer:template ratio of 5. Two μl of annealing buffer (500 mM Tris-HCl, pH 9.0, 150 mM MgCl_2) was also added and the reaction mixture was incubated for 20 min at 37°C, followed by a 10 min incubation at room T.

The labeling and termination reactions were performed as recommended by the manufacturer which allowed sequences to be read up to approximately 500 nucleotides from the primer, using [α - ^{35}S]dATP.

The samples were heated to 80°C for 2 min and loaded onto a pre-electrophoresed 0.2 mm thick, 60 cm long, 6.0 % acrylamide gel using a shark's-tooth comb. The gel was run at 56 W until the xylene cyanol reached 4 cm from the bottom of the gel. Another set of samples was loaded onto the gel which was then run at 56 W until the xylene cyanol again reached 4 cm from the bottom of the gel. A final set of samples was loaded onto the gel which was run until the bromophenol blue reached 4 cm from the bottom of the gel.

The gel was soaked for 20 min in a tray containing 10% acetic acid-10% methanol in distilled water. The gel was removed from the tray, the top third of the gel cut horizontally and the two pieces of gel transferred to two sheets of Whatman Number 1 paper. The papers were covered with plastic wrap and dried using a vacuum gel dryer.

After drying the gel, the two filter-paper supports containing the gel were placed in a cassette along with an X-ray film next to the gel. The film was exposed overnight at room T and developed. The sequence of the DNA was read from the pattern of bands on the autoradiogram.

PCR Amplification of cDNA Synthesized from EBV-PBL RNA, Using IAA V_L and V_H Specific Synthetic Oligonucleotide Primers

The IAA V_L and V_H cDNA sequences were analyzed with the PC/Gene software with the EMBL data library. The complementarity determining regions (CDR) and the framework regions (FR) were identified by sequence homology with other antibodies and immunoglobulin family associations (Kabat et al., 1991). From these defined regions, IAA-specific V_L and V_H oligonucleotides were designed (Fig. 16). The oligonucleotides corresponding to sequences of the CDR1 and CDR3 of IAA V_L and V_H were used for PCR.

Figure 16

Design of IAA-specific Oligonucleotide Primers

The oligonucleotide primers are 100% complementary to complementarity determining region (CDR) cDNA sequences corresponding to the V_L and V_H of the monoclonal IAA secreted by the cell line 3-0.5F10 established from the blood of a Type I DM patient at the time of diagnosis.

Figure 16

(A) IAA V_H oligonucleotide primers:

i. V_H CDR1:

5'—ACCTATGCCATGAGC—3'

ii. V_H CDR2:

5'—GCGTAGTATGTCCTACCACCACTATC—3'

iii. V_H CDR3.1:

5'—GTAGTCAAAAGAATTAGG—3'

iv. V_H CDR3.2:

5'—GGATAGTAGGCACTCCA—3'

(B) IAA V_L oligonucleotide primers:

i. V_L CDR1:

5'—AGGTCTAGTCAAAGCCTCGTACAC—3'

ii. V_L CDR2:

5'—GAGAACCGGTTAGAAATCTT—3'

iii. V_L CDR3:

5'—GAGAGGGAATTGTGTAGCTTGC—3'

Total cellular RNA was isolated from EBV-PBL cell lines derived from Type I DM patients at the time of diagnosis (Patients #1, 4, 11, 13, 15, 16, 19, 20, 21 from chapter 1) and cDNA was synthesized as previously described. PCR amplification was performed under the same conditions as previously described, using IAA-specific V_L or V_H oligonucleotide primers. Two strategies were used to test the specificity of the PCR amplification: 1) two primer:template molar ratios (one ten times less than the other) and 2) the use of two separate pairs of primers. For the amplification of the V_L cDNA, PCR amplification was performed at a final concentration of 1.0 μ M primer and repeated with a final concentration of 0.1 μ M without changing the concentration of template. For the amplification of the V_H cDNA, two separate pairs of primers were used: 1) CDR1 and CDR 3.1 and 2) CDR1 and CDR 3.2 (Fig. 16). The IAA V_L and V_H cDNA which were obtained by PCR amplification of cDNA synthesized from RNA isolated from the IAA secreting 3-0.5F10 cell line were used as positive controls in the PCR reaction.

Southern Analysis of the PCR Amplified Products Using IAA Sequence Specific Probes

The PCR amplified products were run on a 1.3% agarose gel along with the ϕ X174 DNA/*Hinc* II marker. The gel was stained with ethidium bromide for visualization of the DNA fragments which were subsequently transferred from the gel to a nylon membrane (Ausubel et al., 1989). The oligonucleotides corresponding to the CDR2 regions of the IAA V_L and V_H (Fig. 16) were end-labeled with [γ - 32 P]ATP using T4 polynucleotide kinase (Ausubel et al., 1989). Hybridization of the probes to the prehybridized Southern blots was performed at respective hybridization temperatures (T_H) (Davis et al., 1986). The T_H of the Southern blots probed with synthetic 32 P end-labeled oligonucleotides was 57°C for the IAA V_H CDR2 probe and 45°C for the IAA V_L CDR2 probe (Davis et al., 1986). After an overnight hybridization, the blots were

washed three times with 2 X SSC (300 mM sodium chloride, 30 mM sodium citrate), containing 0.1% SDS, at room T. The blots were placed in a cassette along with an X-ray film. The film was exposed for 1-24 h and developed.

Results

Synthesis of cDNA and PCR Amplification of V_L and V_H cDNA

PCR was carried out on the cDNA synthesized from total cellular RNA isolated from the IAA-secreting 3-0.5F10 cells. The amplified products corresponding to V_H and V_L cDNA, along with λ DNA/*HindIII* as marker were run on a 2% agarose gel which was stained with ethidium bromide for visualization of the fragments (Fig. 17). The major band resulting from the PCR amplification of the V_H cDNA was approximately 480 bp (lane 1). The major band resulting from the PCR amplification of the V_L cDNA was approximately 420 bp (lane 2).

Cloning of the PCR Amplified V_L and V_H cDNAs into the pGEM-7Zf(+) Vector

The major bands corresponding to the PCR amplified IAA V_L and V_H cDNAs were gel purified, digested with specific endonucleases and cloned into the pGEM7Zf(+) vector. The miniprep plasmid DNA was isolated from separate clones and cut with *EcoRI* and *HindIII* (Fig. 18). The arrow in panel A indicates the position of the IAA V_L cDNA insert at approximately 420 bp. The plasmids run in lanes 1, 2, 3, 6, 7 and 8 contain the cDNA insert. The plasmids shown in lanes 3 and 8 which contained the IAA V_L cDNA insert were used for subsequent sequencing.

The arrow in panel B indicates the position of the IAA V_H cDNA insert at approximately 480 bp. The plasmids in lanes 3 and 6 contain the cDNA insert and were used for subsequent sequencing.

Figure 17

PCR Amplification of the V_H and V_L cDNA from 3-0.5F10 Cells

First strand cDNA was synthesized from total RNA isolated from the IAA-secreting 3-0.5F10 cells for use in PCR. The V_H and V_L cDNAs were enzymatically amplified using a downstream primer corresponding to a sequence of the conserved C-region and a mixture of oligonucleotide primers corresponding to the leader sequence. The PCR primers contained restriction sites at their 5' ends.

After PCR, one tenth of the reaction mixture (2.0 μ l) from the amplification of the V_H cDNA (lane 1) and the V_L cDNA (lane 3), was run on a 2% agarose gel along with λ DNA/*Hind*III as kb marker (lane 2). The DNA fragments were stained with ethidium bromide. The arrow indicates the position of the bands corresponding to the major PCR amplified products: (1) for V_H cDNA (lane 1), the major band is at approximately 480 bp and, (2) for V_L cDNA (lane 3), the major band is at approximately 420 bp.

Figure 17

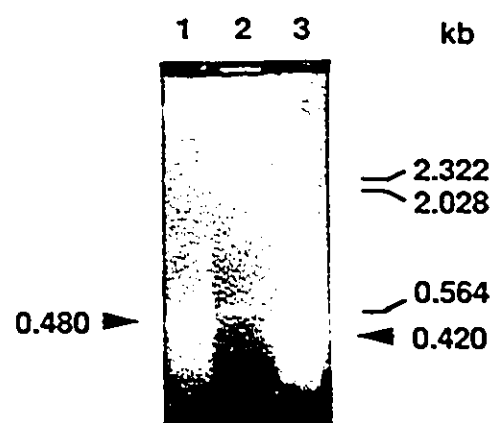


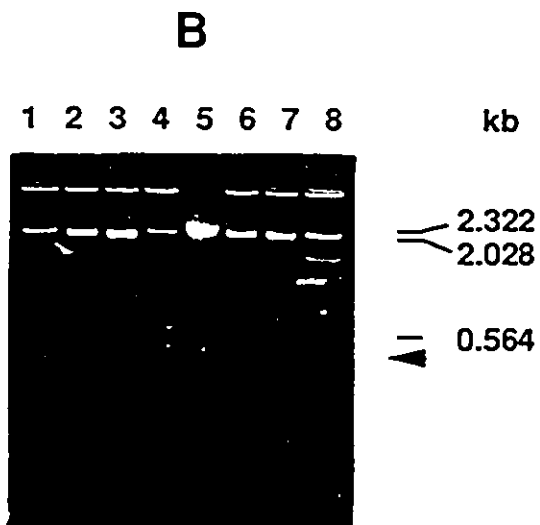
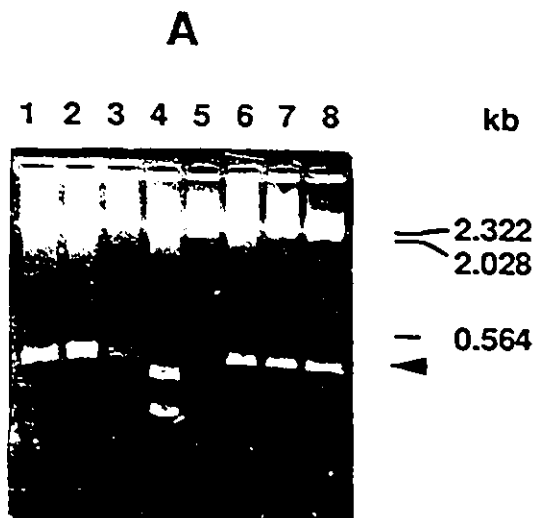
Figure 18

Cloning of the PCR Amplified IAA V_H and V_L cDNA

The PCR amplified IAA V_H and V_L cDNAs were gel purified. The cDNAs were cut with *Eco*RI and *Hind*III and ligated to the pGEM7Zf(+) vector which had also been cut with *Eco*RI and *Hind*III. Competent *E. coli* NM522 cells were transfected with the ligated DNA and plated onto LB agar plates containing ampicillin, X-GAL, and IPTG. White colonies were picked and the plasmids were isolated. The miniprep plasmid DNA was cut with *Eco*RI and *Hind*III and run on a 2% agarose gel along with λ DNA/*Hind*III as kb marker. The DNA fragments were stained with ethidium bromide.

The figures shows miniprep plasmid DNA of colonies transfected with vector harbouring: **1)** the PCR amplified IAA V_L cDNA insert (**panel A**), and **2)** the PCR amplified IAA V_H cDNA insert (**panel B**). The plasmid DNA was isolated from seven separate colonies (lanes 1,2,3,4,6,7,8) and cut with *Eco*RI and *Hind*III. The λ DNA/*Hind*III is in lane 5. The arrow indicates the position of the bands corresponding to the cDNA inserts: **1)** approximately 420 bp for the V_L cDNA insert (**panel A**); and **2)** approximately 480 bp for the V_H cDNA insert (**panel B**).

Figure 18



Sequencing of the Cloned IAA V_L cDNA

The plasmids containing: (1) the V_L cDNA insert from two separate clones and (2) the V_H cDNA insert from two separate clones were sequenced by the dideoxy method (Sanger et al., 1977). The sequence of the DNA was read from the pattern of bands on the autoradiogram. The restriction sites *EcoRI* and *HindIII*, indicating the beginning and the end of the cDNA sequences, were identified along with the PCR oligonucleotide primer sequences. Since multiple samples were loaded onto one gel, overlaps of at least 20 bands between the patterns from successive sets of samples were identified. In this way, it was possible to read the complete sense and antisense sequences of: (1) IAA V_L cDNA from two separate clones, and (2) the IAA V_H cDNA from two separate clones. The duplicate sequences of both the PCR amplified IAA V_L and IAA V_H cDNA from two separate clones were identical.

Sequencing of the Cloned IAA V_L Chain cDNA

The sequence obtained for the nucleic acid sequence and the deduced amino acid sequence of the cloned IAA V_L cDNA from 3-0.5F10 cells is shown in Fig. 19. The cloned IAA V_L cDNA is 414 bp in length. The subregions of the immunoglobulin cDNA were identified by comparison with other antibody sequences (Kabat et al., 1991). The complementarity determining regions (CDR), also known as the hypervariable regions, as well as the framework regions (FR) were identified. The IAA V_L is part of the V_KII subfamily (Kabat et al., 1991).

The sequences corresponding to IAA V_L CDR1 and CDR3 were used in the design of IAA specific cDNA synthetic oligonucleotides for use in PCR (Fig. 19). The sequences corresponding to the CDR2 were used in the design of synthetic oligonucleotides for use in Southern analysis (Fig. 19).

Figure 19

The Nucleic Acid Sequence and the Corresponding Amino Acid Sequence of the Cloned IAA V_L cDNA from 3-C.5F10 Cells

Both the sense (5' → 3') and the antisense (3' → 5') strands were sequenced. The restriction cloning sites *EcoRI* and *HindIII* are indicated. Some of the pGEM7Zf(+) vector DNA sequences are shown. The one letter abbreviations of deduced amino acids are indicated above the corresponding codon sequences. The arrows above the sequence delineate the subregions of the immunoglobulin V-region cDNA (Kabat et al., 1991). The sequences which are underlined were used in the design of IAA V_L cDNA synthetic oligonucleotides for use in PCR and Southern analysis.

FR = framework regions

CDR = complementarity determining regions

Figure 19

```

<-----Leader Region-----
<-----Vector DNA-----> [EcoRI] M D M G F Q V Q
5' GCATGCTCCTCTAGACTCGAGGAATTCATGGACATGGGGTTCCAAGTGCAGC
3' CGTACGAGGAGATCTCACATCCTTAAGTACCTGTACCCCAAGGTTACGTCG
><-----
L L G L L M L W V P G S S G D I V M
TTCTGGGGCTGCTAATGCTCTGGGTCCCTGGATCCAGTGGGGATATTGTGATG
AAGACCCCGACGATTACGAGACCCAGGGACCTAGGTCACCCCTATAACACTAC
-----FR 1-----
T Q T P L S S P V T L G Q P A S I
ACCCAGACTCCACTCTCCTCACCTGTACCCTTGGACAGCCGGCCTCCATCT
TGGGTCTGAGGTGAGAGGAGTGGACAGTGGGAACCTGTGGCCGGACCTAGA
><-----CDR 1-----
S C R S S Q S L V H S D G N T Y L S
CCTGCAGGTCTAGTCAAAGCCTCGTACACAGTGATGGAAACACCTACTTGAG
GGACGTCCAGATCAGTTTCGGAGCATGTGTCACTACCTTTGTGGATGAACTC
><-----FR 2-----><-----
W L Q Q R P G Q P P R L L I Y K I
TTGGCTTCAGCAGAGGCCAGGCCAGCCTCCAAGACTCCTAATTTATAAGATT
AACCGAAGTCGTCTCCGGTCCGGTCCGAGGTTCTGAGGATTAAATATTCTAA
-----CDR 2-----><-----
S N R F S G V P D R F S G S G A G
TCTAACCGGTTCTCTGGGGTCCCAGACAGATTTCAGTGGCAGTGGGGCAGGGA
AGATTGGCCAAGAGACCCAGGGTCTGTCTAAGTCACCGTCACCCCGTCCCT
-----FR 3-----
T D F T L K I S R V E A E D V G V Y
CAGATTTCACTGAAAATCAGCAGGGTGGAGCTGAGGATGTCGGGGTTTA
GTCTAAAGTGTGACTTTTAGTCGTCCACCTTCGACTCCTACAGCCCCAAAT
><-----CDR 3-----><-----FR 4-----
Y C M Q A T Q F P L T F G G G T K
TTACTGCATGCAAGCTACACAATTCCCTCTCACTTTCGGCGGAGGGACCAAG
AATGACGTACGTTTCGATGTGTTAAGGGAGAGTGAAAGCCGCCTCCCTGGTTC
><-----Constant Region-----
V E I K R T M A A P S A F I F P P
GTGGAGATCAAACGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCAT
CACCTCTAGTTTGCTTGACACCGACGTGGTAGACAGAAGTAGAAGGGCGGTA
----->
S D [HindIII] <-----Vector DNA----->
CTGATGAAGCTTGGATCCGGAGAGCTCC 3'
GACTACTTCGAACCTAGGCCTCTCGAGG 5'

```

Fig. 20 shows the V_L nucleotide and deduced amino acid sequence of IAA and of a monoclonal rheumatoid factor derived from a patient with monoclonal gammopathies (RFTS3) (Pascual et al., 1990), compared to the spliced version of the highly homologous A23 germ-line gene (Straubinger et al., 1988). The IAA V_L nucleotide sequence was found to be 99.7 % homologous with that of the A23 germ-line gene. There was only one base substitution which resulted in a silent mutation at amino acid position 101. The deduced amino acid composition of IAA V_L is 100% homologous with that of the spliced A23 germ-line gene. The IAA V_L was found to be 100% homologous in nucleotide and deduced amino acid sequence with V_L of a monoclonal rheumatoid factor derived from a patient with monoclonal gammopathies (RFTS3) (Pascual et al., 1990).

The nucleotide and deduced amino acid sequence of the IAA J_L was compared to that of the highly homologous J_L human germ-line gene ($J_{\kappa 4}$) (Hieter et al., 1982) in Fig. 21, panel A. The nucleotide sequence of the IAA J_L chain is 97.4% homologous with the $J_{\kappa 4}$ germ-line gene. There is only one base substitution which results in a silent mutation. Therefore, the deduced amino acid composition of the IAA J_L is 100% homologous with that of the $J_{\kappa 4}$ germ-line gene.

As expected, the nucleotide and deduced amino acid sequence of the IAA C_L are both 100% homologous to the human κ (INV3 allele) germ-line gene (Hieter et al., 1980) as shown in Fig. 21, panel B.

Sequencing of the Cloned IAA V_H Chain cDNA

The nucleic acid and the deduced amino acid sequences of the cloned IAA V_H cDNA from 3-0.5F10 cells are shown in Fig. 22. The cloned V_H cDNA is 469 bp in length. The CDR and FR of the immunoglobulin cDNA were identified by comparison with other antibody sequences (Kabat et al., 1991). The IAA V_H was found to belong to the V_{HIII} subfamily (Kabat et al., 1991).

Figure 20

**Homologous V_L Nucleotide and Deduced Amino Acid Sequences
of Various Monoclonal Antibodies**

Sequences homologous to the spliced version of the A23 germ-line gene (Straubinger et al., 1988) are shown. RFTS3 is a monoclonal rheumatoid factor derived from a patient with monoclonal gammopathies (Pascual et al., 1990). The FR and CDR are labeled (Kabat et al., 1991). Dashes denote nucleotide identity compared to the germ-line sequence. Differences in sequences are indicated where appropriate. Numbering is according to Kabat et al. (1991).

	FR1															
	<1----->															
A23	gat	att	gtg	atg	acc	cag	act	cca	ctc	tcc	tca	cct	gtc	acc	ctt	gga
IAA	D	I	V	M	T	Q	T	P	L	S	S	P	V	T	L	G
RFTS3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	20-----><-----CDR1-----															
A23	cag	ccg	gcc	tcc	atc	tcc	tgc	agg	tct	agt	caa	agc	ctc	gta	cac	agt
IAA	Q	P	A	S	I	S	C	R	S	S	Q	S	L	V	H	S
RFTS3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	40><-----FR2-----															
A23	gat	gga	aac	acc	tac	ttg	agt	tgg	ctt	cag	cag	agg	cca	ggc	cag	cct
IAA	D	G	N	T	Y	L	S	W	L	Q	Q	R	P	G	Q	P
RFTS3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	><-----CDR2-----60-----><-----															
A23	cca	aga	ctc	cta	att	tat	aag	att	tct	aac	cgg	ttc	tct	ggg	gtc	cca
IAA	P	R	L	L	I	Y	K	I	S	N	R	F	S	G	V	P
RFTS3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	FR3-----80-----															
A23	cca	gac	aga	ttc	agt	ggc	agt	ggg	gca	ggg	aca	gat	ttc	aca	ctg	aaa
IAA	D	R	F	S	G	S	G	A	G	T	D	F	T	L	K	I
RFTS3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	><-----															
A23	atc	agc	agg	gtg	gaa	gct	gag	gat	gtc	ggg	ggt	tat	tac	tgc	atg	caa
IAA	S	R	V	E	A	E	D	V	G	V	Y	Y	C	M	Q	A
RFTS3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	CDR3-----100----->															
A23	gct	aca	caa	ttt	cct											
IAA	A	T	Q	F	P											
RFTS3	---	---	---	---	---											
	*	*	*	*	*											
	---	---	---	---	---											
	*	*	*	*	*											

Figure 21

A. Comparison of the Nucleotide and Deduced Amino Acid Sequence of the IAA J_L with the Germ-line Gene Counterpart

The nucleotide and deduced amino acid sequence of the J-region of the IAA V_L is compared to that of the highly homologous κ -L chain J-region (J κ 4) human germ-line gene (Hieter et al., 1982).

B. Comparison of the Nucleotide and Deduced Amino Acid Sequence of the IAA C_L with the Germ-line Gene Counterpart

The nucleotide and deduced amino acid sequence of the IAA C_L is compared to that of the homologous human C κ (INV3 allele) germ-line gene (Kabat et al., 1991).

Dashes for nucleotides and asterisks for deduced amino acids denote identity of the regions of the IAA to the germ-line sequences. Differences in sequences are indicated where appropriate.

Figure 21

A

J_K4	ctc	act	ttc	gcc	gga	ggg	acc	aag	gtg	gag	atc	aaa	cgt
	L	T	F	G	G	G	T	K	V	E	I	K	R
IAA	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*

B

C_K	act	gtg	gct	gca	cca	tct	gtc	ttc	atc	ttc	ccg	cca	tct	gat
	T	M	A	A	P	S	A	F	I	F	P	P	S	D
IAA	---	---	---	---	---	---	---	---	---	---	---	---	---	---
	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Figure 22

The Nucleic Acid Sequence and the Corresponding Amino Acid Sequence of the Cloned IAA V_H cDNA from 3-O.5F10 Cells

Both the sense (5' → 3') and the antisense (3' → 5') strands were sequenced. The cloning sites *EcoRI* and *HindIII* are indicated. Some of the pGEM7Zf(+) vector DNA sequences are shown. The one letter abbreviations of deduced amino acids are indicated above the corresponding codon sequences. The arrows above the sequence delineate the subregions of the immunoglobulin V_H-region cDNA (Kabat et al., 1991). The FR and CDR are labeled (Kabat et al., 1991). The sequences which are underlined were used in the design of IAA specific V_H cDNA synthetic oligonucleotides for use in PCR and Southern analysis.

Figure 22

```

<-----Vector DNA ----->[EcoRI] M E F G L T W V
5'TCGCATGCTCCTCTAGACTCGAGGAATTCATGGAGTTTGGGCTGAGCTGGGTTT
3'AGCGTACGAGGAGATCTGAGCTCCTTAAGTACCTCAAACCCGACTGGACCCAAA
-----><-----Leader Region-----
S L V A I L K G V Q C E V Q L L E S
CTCTTGTGGCTATTTTAAAAGGTGTCCAGTGTGAGGTGCAGCTGTTGGAGTCTG
GAGAACACCGATAAAATTTTCCACAGGTCACACTCCACGTCGACAACCTCAGAC
-----FR 1-----
G G G L V Q P G G S L R L S C A A S
GGGGAGGCTTGGTCCAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTG
CCCCICCGAACCAGGTCGGACCCCCCAGGGACTCTGAGAGGACACGTCGGAGAC
-----><-----CDR 1-----><-----FR 2-----
G F T F S T Y A M S W V R Q A P G K
GATTCACCTTTAGCACCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAG
CTAAGTGGAAATCGTGGATACGGTACTCGACCCAGGCGGTCCGAGGTCCCTTC
-----><-----CDR 2-----
G L E W V S T I S D S G G R T Y Y A
GGGCTGGAGTGGGTCTCAACTATTAGTGATAGTGGTGGTAGGACATACTACGC
CCCGACCTCACCCAGAGTTGATAATCACTATCACCACCATCCTGTATGATGCG
-----><-----
D S V K G R F T I S R D N S K N T
AGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGC
TCTGAGGCACTTCCCGGCCAAGTGGTAGAGGTCTCTGTTAAGGTTCTTGTGCG
-----FR 3-----
L Y V Q M N S L R A E D T A V Y Y C
TGTATGTACAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGT
ACATACATGTTTACTTGTTCGGACTCTCGGCTCCTGTGCCGGCATATAATGACA
-----><-----CDR 3-----><-----
A K D Q F W S A Y Y P N S F D Y W G
GCGAAAGATCAATTTTGGAGTGCCTACTATCCTAATTCTTTTGACTIONGGG
CGCTTTCTAGTTAAACCTCACGGATGATAGGATTAAGAAAACCTGATGACCCC
-----FR 4-----><-----Constant Region-----
Q G T L V T V S S G S A S A P T L F
CCAGGGGACCCTGGTCACCGTCTCCTCAGGGAGTGCATCCGCCCCAACCCTTTT
GGTCCCCTGGGACCAGTGGCAGAGGAGTCCCTCACGTAGGCGGGGTGGGAAA
----->
P L V [HindIII] <-----Vector DNA----->
CCCCCTCGTCTAAGCTTGGATCCGGAGAGCTCC 3'
GGGGGAGCAGATTCGAACCTAGGCCTCTCGAGG 5'

```

The sequences corresponding to CDR1, CDR3.1 and CDR3.2 were used for IAA specific V_H cDNA synthetic oligonucleotides for use in PCR (Fig. 19). The sequence corresponding to the IAA V_H CDR2 was used to synthesize the oligonucleotide for use in Southern analysis (Fig. 19).

The IAA V_H was found to have high sequence homology with the spliced version of the V_H26 germ-line gene (Mathysens and Rabblitts, 1980). The nucleotide sequence (Fig. 23) and the deduced amino acid sequence (Fig. 24) of the IAA V_H and the spliced version of V_H26 germ-line gene were compared. The nucleotide sequence of IAA V_H is 96.6 % homologous with that of the V_H26 germ-line gene.

There are eight base substitutions, two of which are silent mutations and which occur outside of the CDRs (Fig. 24). A base substitution at amino acid position 31 in the IAA CDR1 nucleotide sequence leads to a change in amino acid from a serine to a threonine. Three nucleotide substitutions lead to three changes in amino acids in the CDR2: from alanine to threonine at position 50, from glycine to aspartic acid at position 53, and from serine to arginine at position 57. Two nucleotide substitutions in FR3 lead to one change in amino acid composition from leucine to valine at position 83. The deduced amino acid homology between IAA V_H and the spliced version of the highly homologous V_H26 germ-line gene is 94.7%.

The nucleotide sequence (Fig. 23) and the deduced amino acid sequence (Fig. 24) of the IAA V_H were also compared to the homologous V_H sequences of other antibodies: (1) 4G12, a human monoclonal antibody secreted by 4G12 hybridoma cells, with broad reactivity to malignant tumor cells (Kishimoto et al., 1989); (2) 30pl, a 130-day human fetal-derived H chain cDNA transcript (Schroeder Jr. and Wang, 1990); (3) 18/2, an anti-DNA autoantibody (Dersimonian et al., 1987); (4) Ab18, a polyreactive monoclonal antibody isolated from EBV-PBL derived from a normal individual (Sanz et al., 1989).

Figure 23

**V_H Nucleotide Sequences of Various Monoclonal Antibodies Which
are Homologous to the Spliced Version of the V_H26 Germ-line Gene**

Sequences homologous to the spliced version of the V_H26 germ-line gene (Mathysens and Rabbitts, 1980). The FRs and CDRs are labeled (Kabat et al., 1991). Dashes denote nucleotide identity of the various antibodies to the germ-line sequence. Differences in sequences are indicated where appropriate.

IAA is a monoclonal autoantibody derived from a Type I DM patient at the time of diagnosis which binds porcine, bovine and human insulin with equal affinity. 4G12 is a human monoclonal antibody secreted by hybridoma cells (Kishimoto et al., 1989). 30pl is a 130-day human fetal-derived H chain cDNA transcript (Schroeder Jr. and Wang, 1990). 18/2 is an anti-DNA autoantibody (Dersimonian et al., 1987). Ab18 is a polyreactive monoclonal antibody isolated from EBV-PBL derived from a normal individual (Sanz et al., 1989). ED8.4 is an antibody directed towards the capsular polysaccharide of *Haemophilus influenzae* type b (Adderson et al., 1991).

Figure 23

	<+1	FR1															
VH26	gag	gtg	cag	ctg	ttg	gag	tct	ggg	gga	ggc	ttg	gta	cag	cct	ggg	ggg	tcc
IAA	---	---	---	---	---	---	---	---	---	---	---	---c	---	---	---	---	---
4G12	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
30p1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
18/2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ab18	---	---	---	---	g	---	---	---	---	---	---	---	---	---	---	---	---
ED8.4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

	+20	>< CDR1															
VH26	ctg	aga	ctc	tcc	tgt	gca	gcc	tct	gga	ttc	act	ttt	agc	agc	tat	gcc	atg
IAA	---	---	---	---	---	---	---	---	---	---	---c	---	---	---c	---	---	---
4G12	---	---	---	---	---	---	---	---	---	---	---c	---	---	---c	---	---	---
30p1	---	---	---	---	---	---	---	---	---	---	---c	---	---	---	---	---	---
18/2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ab18	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---c	---	---
ED8.4	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

	><	+40	FR2														><
VH26	agc	tgg	gtc	cgc	cag	gct	cca	ggg	aag	ggg	ctg	gag	tgg	gtc	tca	gct	att
IAA	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	a	---
4G12	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
30p1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
18/2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ab18	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
ED8.4	---	---	---	---t	---	c	---	---	---	---	---	---	---	---	---	---	---

	CDR2 +60																><
VH26	agt	ggt	agt	ggt	ggt	agc	aca	tac	tac	gca	gac	tcc	gtg	aag	ggc	cgg	ttc
IAA	---	---a	---	---	---	---g	---	---	---	---	---	---	---	---	---	---	---
4G12	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
30p1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
18/2	---	---	---	---	---	---	---	---	---	---c	---	---	---	---	---	---	---
Ab18	---	---	---	---	---	---a	---	---	---	---	---	---	---	---	---	---	---
ED8.4	---	---	---	---	---	---a	---	---	---	---	---	---	---	---	---	---	---

	FR3 +80																
VH26	acc	atc	tcc	aga	gac	aat	tcc	aag	aac	acg	ctg	tat	ctg	caa	atg	aac	agc
IAA	---	---	---	---	---	---	---	---	---	---	---	---	g-a	---	---	---	---
4G12	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
30p1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
18/2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Ab18	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
ED8.4	---	---	---	---	---	---g	---	---	---	---	---	---	g	---	---	---	---

	>																
VH26	ctg	aga	gcc	gag	gac	acg	gcc	gta	tat	tac	tgt	gcg	aaa				
IAA	---	---	---	---	---	---	---	---	---	---	---	---	---				
4G12	---	---	---	---	---	---	---	---	---	---	---	---	---				
30p1	---	---	---	---	---	---	---	---	---	---	---	---	---				
18/2	---	---	---	---	---	---	---	---	---	---	---	---	---				
Ab18	---	---	---	---	---	---	---	---	---	---	---	---	---				
ED8.4	---	---	---	---	---	---	---	---	---	---	---	---	---				

Figure 24

**V_H Deduced Amino Acid Sequences of Various Monoclonal Antibodies
Which are Homologous to the Spliced Version of the V_H26 Germ-line Gene**

Homologous sequences to the spliced version of the V_H26 germ-line gene (Mathysens and Rabbits, 1980) are shown. The FRs and CDRs are labeled. Dashes for amino acids, denote identity of the region of the of the various antibodies to the germ-line sequence. Differences in sequences are indicated were appropriate.

IAA is a monoclonal autoantibody derived from a Type I DM patient at the time of diagnosis which binds porcine, bovine and human insulin with equal affinity. 4G12 is a human monoclonal antibody secreted by hybridoma cells (Kishimoto et al., 1989). 30pl is a 130-day human fetal-derived H chain cDNA transcript (Schroeder Jr. and Wang, 1990). 18/2 is a systemic lupus erythematosus-derived anti-DNA autoantibody (Derstimonian et al., 1987). Ab18 is a polyreactive monoclonal antibody isolated from EBV-PBL derived from a normal individual (Sanz et al., 1989). ED8.4 is an antibody directed towards the capsular polysaccharide of *Haemophilus influenzae* type b (Adderson et al., 1991).

Table 9

Comparison of Various Antibody V_H Sequences with IAA V_H

The V_H of: 1) 4G12, a human monoclonal antibody secreted by hybridoma cells (Kishimoto et al., 1989); 2) 30pl, a 130-day human fetal-derived H chain cDNA transcript (Schroeder Jr. and Wang, 1990); 3) 18/2, a systemic lupus erythematosus-derived anti-DNA autoantibody (Dersimonian et al., 1987); 4) Ab18, a polyreactive monoclonal antibody isolated from EBV-PBL derived from a normal individual (Sanz et al., 1989); and 5) ED8.4, an antibody directed towards the capsular polysaccharide of *Haemophilus influenzae* type b (Adderson et al. 1991) were compared with IAA V_H . IAA is a monoclonal autoantibody derived from a Type I DM patient at the time of diagnosis which binds porcine, bovine and human insulin with equal affinity. The % nucleotide and deduced amino acid homologies are shown.

Table 9

Antibody	% homology with IAA VH	
	nucleotide	amino acid
VH26	97.6	94.9
4G12	98.3	95.9
30pl	97.6	94.9
18/2	96.9	94.9
Ab 18	96.6	92.9
ED8.4	96.3	93.9

and (5) ED8.4, an antibody directed towards the capsular polysaccharide of *Haemophilus influenzae* type b (Adderson et al., 1991). The % nucleotide and deduced amino acid sequence homologies of the V_H of these antibodies compared with IAA V_H are shown in Table 9.

The third hypervariable region (CDR3) of the H chain is generated by $V_H-D_H-J_H$ joining. The IAA D_H is 36 bp in length (12 amino acids) and makes up the first portion of the CDR3 (Fig. 25). Random nucleotide additions (N insertions) and junctional flexibility makes assignment of the D-region origin difficult. However, a core of IAA D_H shares 15 bases of identity with the DXP4 germ-line gene (Ichihara et al., 1988) (Fig. 25, panel A). This suggests that the IAA D_H is encoded by the DXP4 germ-line gene.

The nucleotide and deduced amino acid sequence of the IAA J_H was compared to that of the highly homologous human J_H4 germ-line gene (Ravetch et al., 1981) (Fig. 25, panel B). There is only one silent base substitution in the IAA J_H compared with the J_H4 germ-line gene (Fig. 25, panel B). Therefore, the deduced amino acid composition of the IAA J_H is 100% homologous with that of the J_H4 germ-line gene.

As expected, the nucleotide and deduced amino acid sequence of the IAA C_H were both 100% homologous to the human IgM C_H1 germ-line gene (Dolby et al., 1980) (Fig. 25, panel C).

Ab2022 is a IgM- κ monoreactive anti-insulin antibody from a Type I DM patient treated with heterologous insulin that binds human and animal insulins with equal affinity (Sanz et al., 1989). The V_H nucleotide and deduced amino acid sequence of Ab2022 and IAA were compared to the spliced version of the V_H251 germ-line gene which belongs to the V_HV subfamily (Humphries et al., 1988) in Fig. 26.

Figure 25

**A. Comparison of the Nucleotide and Deduced Amino Acid Sequence
of the IAA D_H with the Germ-line Gene Counterpart**

The nucleotide and deduced amino acid sequence of the IAA D_H is compared to that of the homologous D-region DXP4 human germ-line gene (Ichihara et al., 1988).

**B. Comparison of the Nucleotide and Deduced Amino Acid Sequence
of the IAA J_H with the Germ-line Gene Counterpart**

The nucleotide and deduced amino acid sequence of the IAA J_H is compared to that of the highly homologous HUMJ_H4 human germ-line gene (Ravetch et al., 1981).

**C. Comparison of the Nucleotide and Deduced Amino Acid Sequence
of the IAA C_H with the Germ-line Gene Counterpart**

The nucleotide and deduced amino acid sequence of the IAA C_H is compared to that of the homologous human IgM C_H1 germ-line gene (Dolby et al., 1980).

Dashes for nucleotides and asterisks for deduced amino acids denote identity of the regions of the IAA to the germ-line sequences. Differences in sequences are indicated where appropriate.

Figure 26

**Comparison of the IAA and Ab2022 V_H Nucleotide and
Deduced Amino Acid Sequences with the V_H251 Germ-line Gene**

The V_H nucleotide and deduced amino acid sequence of IAA and of an insulin antibody from a Type I DM patient injected with heterologous insulin, Ab2022, (Sanz et al., 1989) is compared to the spliced version of the V_H251 germ-line gene (Humphries et al., 1988). The FR and CDR are labeled. Dashes for nucleotides and asterisks for deduced amino acids, denote identity of the sequences to the V_H251 germ-line sequence. Differences in sequences are indicated where appropriate. Numbering is according to Kabat et al. (1991).

	<1	FR1												
VH251		gag	gtg	cag	ctg	gtg	cag	tct	gga	gca	gag	gtg	aaa	aag
		E	V	Q	L	V	Q	S	G	A	E	V	K	k
Ab2022		---	---	---	---	---	---	---	---	---	---	---	---	---
		*	*	*	*	*	*	*	*	*	*	*	*	*
IAA		---	---	---	---	t--	g--	---	--g	-g-	-gc	t--	gtc	c--
		*	*	*	*	L	E	*	*	G	G	L	V	Q
		20												
VH251		ccc	ggg	gag	tct	ctg	aag	atc	tcc	tgt	aag	ggt	tct	gga
		p	G	E	S	L	K	I	S	C	K	G	S	G
Ab2022		---	---	---	---	---	---	---	---	---	---	---	---	---
		*	*	*	*	*	*	*	*	*	*	*	*	*
IAA		--t	---	-g-	--c	---	-ga	c--	---	---	gca	-cc	---	---
		P	*	G	*	*	R	L	*	*	A	A	*	*
		>< CDR1 ><												
VH251		tac	agc	ttt	acc	agc	tac	tgg	acc	ggc	tgg	gtg	cgc	cag
		Y	S	F	T	S	Y	W	T	G	W	V	R	Q
Ab2022		---	--t	---	-g-	--t	---	---	-t-	---	---	---	---	---
		*	R	*	S	*	*	*	I	*	*	*	*	*
IAA		-t-	-c-	---	-g-	-c-	--t	gcc	-tg	a--	---	--c	---	---
		F	T	*	S	T	*	A	M	S	*	*	*	*
		40 ><												
VH251		atg	ccc	ggg	aaa	ggc	ttg	gag	tgg	atg	ggg	atc	atc	tat
		M	P	G	K	G	L	E	W	M	G	I	I	Y
Ab2022		---	---	---	---	---	c--	---	---	---	---	---	---	---
		*	*	*	*	*	*	*	*	*	*	*	*	*
IAA		gct	--a	---	--g	--g	c--	---	---	g-c	tca	-ct	--t	ag-
		A	*	*	*	*	*	*	*	V	S	T	*	S
		CDR2 60												
VH251		cct	ggt	gac	tct	gat	acc	aga	tac	agc	ccg	tcc	ttc	caa
		P	G	D	S	D	T	R	Y	S	P	S	F	Q
Ab2022		---	---	---	---	---	---	---	---	---	---	---	c--	---
		*	*	*	*	*	*	*	*	*	*	*	L	*
IAA		ga-	a--	-gt	gg-	agc	--a	tac	---	gca	gac	---	g-g	a-g
		D	S	G	G	R	*	Y	*	A	D	*	V	K
		>< FR3												
VH251		ggc	cag	gtc	acc	atc	tca	gcc	gac	aag	tcc	atc	agc	acc
		G	Q	V	T	I	S	A	D	K	S	I	S	T
Ab2022		---	---	---	---	gg	---	---	---	---	---	---	---	---
		*	*	*	*	R	*	*	*	*	*	*	*	*
IAA		---	-g-	t--	---	---	--c	aga	---	--t	---	-ag	-a-	--g
		*	R	F	*	*	*	R	*	N	*	K	N	*
		80												
VH251		gcc	tac	ctg	cag	tgg	agc	agc	ctg	aag	gcc	tcg	gac	acc
		A	Y	L	Q	W	S	S	L	K	A	S	D	T
Ab2022		---	---	---	---	---	---	---	---	---	---	---	---	---
		*	*	*	*	*	*	*	*	*	*	*	*	*
IAA		ctg	--t	g-a	--a	at-	-a-	---	---	-ga	---	ga-	---	--g
		L	*	*	*	M	N	*	*	R	*	E	*	*
		>												
VH251		gcc	atg	tat	tac	tgt	gcg	aga						
		A	M	Y	Y	C	A	R						
Ab2022		---	---	---	---	---	---	---						
		*	*	*										
IAA		---	g--	---	---	---	---	-a-						
		*	V	*	*	*	*	*						

Figure 26

Figure 26

The V_H of Ab2022 shares 97.1% nucleotide sequence homology and 94.5% deduced amino acid homology with the V_H251 germ-line gene. The Ab2022 V_H is likely encoded by the V_H251 germ-line gene. The V_H of Ab2022 and IAA share only 62.4% nucleotide sequence homology and 48.4% amino acid homology. Thus, IAA and Ab2022 are not encoded by the same V_H germ-line gene.

The nucleotide and deduced amino acid sequences of the IAA D_H and Ab2022 D_H were compared with the DLR1 germ-line gene in Fig. 27, panel A. The Ab2022 D_H is 42 nucleotides and 14 amino acids in length. It has a nucleotide core sequence that shares 22 bases of identity with the DLR1 germ-line gene (Ichihara et al., 1988). The IAA D_H is 36 nucleotides and 12 amino acids in length. The IAA D_H shares little homology with either the DLR1 germ-line gene or the Ab2022 D_H .

The nucleotide and deduced amino acid sequence of the IAA J_H and Ab2022 J_H were compared to the homologous J_H3 human germ-line gene sequences (Ravetch et al., 1981) in Fig. 27, panel B. The Ab2022 J_H shares 94.9% nucleotide sequence homology and 84.6% amino acid homology with the J_H3 germ-line gene. The IAA J_H shares 82.5% nucleotide homology and 92.3% homology with the J_H3 germ-line gene. The IAA J_H and Ab2022 J_H share 79.5% nucleotide sequence homology and 84.6% amino acid homology.

Figure 27

**A. Comparison of the IAA D_H and Ab2022 D_H Nucleotide and
Deduced Amino Acid Sequences with the DLR1 Germ-line Gene**

The D_H nucleotide and deduced amino acid sequences of IAA and Ab2022, a monoclonal insulin antibody derived from a Type I DM patient injected with heterologous insulin (Sanz et al., 1989), are compared to the D-region DLR1 human germ-line gene (Ichihara et al., 1988).

**B. Comparison of the IAA J_H and the Ab2022 J_H Nucleotide and
Deduced Amino Acid Sequences with the J_H3 Germ-line Gene**

The nucleotide and deduced amino acid sequences of the J_H of IAA and Ab2022 are compared to the homologous J_H3 human germ-line gene (Ravetch et al., 1981).

Dashes for nucleotides and asterisks for deduced amino acids, denote identity of the region of the sequences to the DLR1 germ-line sequence. Differences in sequences are indicated where appropriate.

Southern Analysis of PCR Amplified Products Using IAA V_L Sequence Specific Probes

The next objective was to determine the degree of restriction of the IAA V_L and V_H gene usage in Type I DM patients at the time of diagnosis. Fig. 28 shows the results of the Southern analysis of cDNA obtained from the PCR amplification of cDNA using IAA-specific V_L oligonucleotide primers and a IAA-specific V_L CDR2 radiolabeled oligonucleotide probe. Panel A shows the results obtained when PCR was performed with a final concentration of 1.0 μ M primer. A band of 234 bp, which corresponds to the length of the IAA V_L cDNA incorporating the two IAA V_L oligonucleotide primer sequences, is detected in Southern analysis for the positive control (lane 1, Fig. 28) as well as for the cDNA synthesized from RNA isolated from EBV-PBL cells derived from Type I DM patients at the time of diagnosis (lanes 2-10, Fig. 28). The 234 bp band remained apparent in the Southern analysis even though the concentration of primer was reduced ten fold. Therefore, the ten Type I DM patients at the time of diagnosis had B lymphocytes which were producing antibodies with IAA-homologous V_L.

Southern Analysis of PCR Amplified Products Using IAA V_H Sequence Specific Probes

Southern analysis of PCR amplified V_H cDNA is shown in Fig. 29. Panel A shows the results of a Southern blot of the PCR amplified products using the IAA-specific V_H CDR1 and CDR3.2 oligonucleotide primers and the IAA-specific V_H CDR2 radiolabeled oligonucleotide probe. The DNA fragments had been stained and visualized with ethidium bromide prior to Southern blotting. A major band of 229 bp was detected for the positive control after 1 h of exposure; the size of this band corresponds to the amplified IAA V_H cDNA incorporating the IAA V_H CDR1 and CDR3.2 primer sequences (lane 1, Fig. 29, panel A).

PCR amplification of IAA V_L and V_H cDNA from lymphocytes derived from Type I DM individuals at diagnosis was performed. When using the IAA V_L amplimers CDR1 and CDR3, the expected size of the amplified product is 234 bp. The use of IAA V_H amplimers CDR1 and CDR3.1 is expected to result in a PCR product of 245 bp. Finally, the expected size of the amplified product when using IAA V_H amplimers CDR1 and CDR 3.2 is 229 bp.

Probing of Southern blots containing the PCR products with oligonucleotides specific for these sequences reveals the presence of hybridising bands. Ideally, negative controls would have been included in this experiment. These controls are essential when using PCR based methodologies given the marked sensitivity of PCR to small concentrations of contaminating material. The use of a cDNA free system to rule out cross contamination of one or more of the PCR reagents would be important. Another negative control for the Southern transfer which could have been included is cDNA amplified from a cell line producing antibody of the same light and heavy chain isotypes as IAA, but with a different binding specificity.

Some hybridising bands of PCR amplified Type I DM patient cDNA are less intense than others in the Southern blots. To determine whether these bands represent a less abundant cDNA copy number, quantitation of DNA by PCR could be performed. The cDNA of Type I DM patients is prepared and its concentration is determined. A known amount of this DNA is then mixed with two sets of oligonucleotide primers, one set specific for the DNA of interest (IAA V_L or IAA V_H) and the other set specific for an internal control (a single-copy gene). The sequences between the primers are amplified, electrophoresed on a gel, transferred to a filter, and probed with oligonucleotides specific for each amplified product. The amounts of the amplified products from the DNA of interest can then be quantitated by comparison to the internal control.

Negative values of specific insulin binding in the CIAA are computational products arising from the fact that the counts following displacement with excess humulin were greater than those with tracer alone. These negative values were used as such for the determination of the mean and standard deviations of the normal population (n=74) and hence the cut-off point for "IAA positivity". This cut-off point was determined to be 99 nU [125 I-] insulin bound per ml serum. Using this cut-off point, fifty percent of the Type I DM patients at the time of diagnosis were "IAA positive". If the negative binding values were assigned as 0 nU [125 I-] insulin bound per ml serum, the cut-off point for "IAA positivity" would be 58 nU [125 I-] insulin bound per ml serum. With this new cut-off point, the incidence of "IAA positivity" of Type I DM patients at the time of diagnosis remains the same at fifty percent.

Figure 28

Southern Analysis of PCR Amplified IAA V_L cDNA

Total cellular RNA was isolated from EBV-PBL cell lines derived from Type I DM patients at the time of diagnosis and cDNA was synthesized. Oligonucleotides corresponding to sequences of the CDR1 and CDR3 of the cloned IAA V_L cDNA were used for PCR. The IAA V_L cDNA from 3-0.5F10 cells was also amplified using the CDR-specific oligonucleotides and served as positive control.

Ten μ l of the PCR reaction volume of amplified cDNA synthesized from RNA and 1.0 μ l of the PCR reaction volume of the amplified positive control were run on a 1.3% agarose gel along with the ϕ X174 DNA/*Hinc* II marker. The DNA fragments were stained with ethidium bromide and transferred to a nylon membrane. The IAA V_L CDR2 oligonucleotide was end-labeled with [γ -³²P]ATP. After hybridization, the blots were washed and exposed to X-ray film for 4 h at -80°C.

Panel A shows an autoradiogram of a Southern blot of the PCR amplified products where PCR was performed with a final concentration of 1.0 μ M primer. **Panel B** shows an autoradiogram of a Southern blot of the PCR amplified products where PCR was performed with the same amount of template but ten times less primer (final concentration of 0.1 μ M primer).

For both panels A and B, the PCR amplified IAA V_L cDNA obtained from 3-0.5F10 cells is shown in **lane 1**. The other lanes are PCR amplified cDNA from the following Type I DM patients: **lane 2**, Patient #1; **lane 3**, Patient #4; **lane 4**, Patient #11; **lane 5**, Patient #20; **lane 6**, Patient #16; **lane 7**, Patient #13; **lane 8**, Patient #19; **lane 9**, Patient #21; **lane 10**, Patient #15. The position of the bands of the ϕ X174 DNA/*Hinc* II kilobase marker (kb) are indicated. The arrows indicate the position of the 234 bp amplified product.

Figure 28

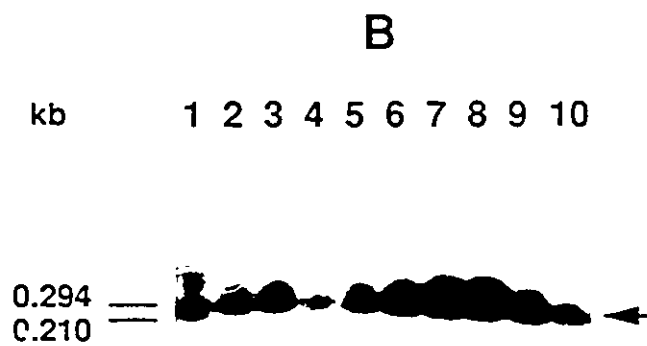
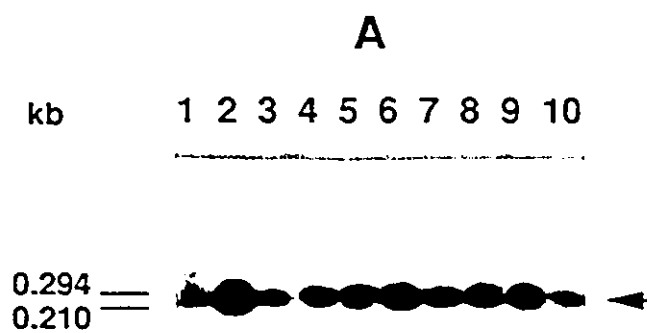


Figure 29

Southern Analysis of PCR Amplified IAA V_H cDNA

Total cellular RNA was isolated from EBV-PBL cell lines derived from Type I DM patients at diagnosis and cDNA was synthesized. Oligonucleotides corresponding to CDR sequences of the cloned IAA V_H cDNA were used for PCR. The IAA V_H cDNA from 3-0.5F10 cells was also amplified using the CDR-specific oligonucleotides and served as positive control.

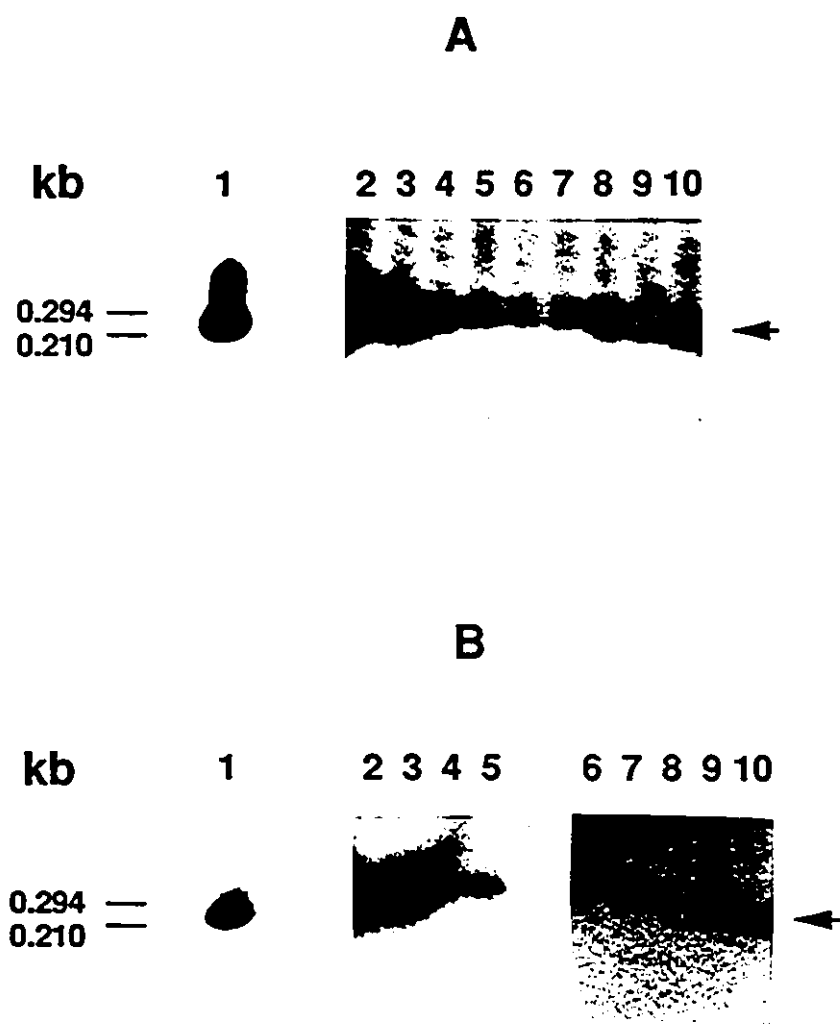
Ten μ l of the PCR reaction volume of amplified cDNA synthesized from EBV-PBL RNA and 2.0 μ l of the amplified positive control were run on a 1.3% agarose gel along with the ϕ X174 DNA/*Hinc* II marker. The DNA fragments were stained with ethidium bromide and transferred to a nylon membrane. The IAA V_H CDR2 oligonucleotide was end-labeled with [γ -³²P]ATP. After hybridization, the blots were washed and exposed for 1–24 h at -80°C.

For **panels A and B**, the PCR amplified IAA V_H cDNA from 3-0.5F10 cells is shown in **lane 1**. The other lanes are PCR cDNA from the following Type I DM patients: **lane 2**, Patient #1; **lane 3**, Patient #4; **lane 4**, Patient #11; **lane 5**, Patient #20; **lane 6**, Patient #16; **lane 7**, Patient #13; **lane 8**, Patient #19; **lane 9**, Patient #21; **lane 10**, Patient #15. The position of the bands of the ϕ X174 DNA/*Hinc* II kilobase marker (kb) are indicated.

Panel A shows the results of a Southern blot of the PCR amplified products using the IAA V_H CDR1 and CDR3.2 oligonucleotide primers. Lane 1: 1 h exposure. Lanes 2–10: 12 h exposure. The arrow indicates the position of a band of 229 bp.

Panel B shows the results of a Southern blot of the PCR amplified products using the IAA V_H CDR1 and CDR3.1 oligonucleotide primers. Lane 1: 1 h exposure. Lanes 2–5: 12 h exposure. Lanes 6–10: 24 h exposure. The arrow indicates the position of a band of 245 bp.

Figure 29



A larger quantity of the positive control cDNA was loaded compared to the other samples. A band of 229 bp was also detected after 12 h of exposure in lanes 2-10 (Fig. 29, panel A) which contained PCR amplified cDNA from Type I DM patients at the time of diagnosis.

Fig. 29, panel B, shows a Southern blot of the PCR amplified products using the IAA-specific V_H CDR1 and CDR3.1 oligonucleotide primers, and the IAA V_H CDR2 radiolabeled oligonucleotide as probe. The DNA fragments had been stained and visualized with ethidium bromide prior to Southern blotting. A major band of 245 bp was detected for the positive control: the size of this band corresponds to the length of the IAA V_H cDNA incorporating the IAA V_H CDR1 and CDR3.1 primer sequences (lane 1, Fig. 29, panel B). A larger quantity of the positive control cDNA was loaded compared to the other samples and, thus, the band of the positive control is best resolved after 1 h of exposure to the film. A band of 245 bp was detected in lanes 2—5 after 12 h exposure and in lanes 6—10 after 24 h of exposure (Fig. 29, panel B). The lanes 2—10 contained PCR amplified cDNA from Type I DM patients at the time of diagnosis. Thus, ten Type I DM patients at the time of diagnosis had B lymphocytes which were producing antibodies with IAA-homologous V_H .

Discussion

Sequencing of IAA V_L cDNA

The V_L of IAA belongs to the subfamily of V_KII (Kabat et al., 1991). There are approximately ten V_KII genes, many of them pseudogenes, in the human genome dispersed throughout more than 2 megabases on chromosome 2 (Lorenz et al., 1987). All of the functional V_K genes are found in six chromosomal regions, Aa, Ab, La, Lb, Oa, and Ob that have been formed by duplication of three regions (Straubinger et al., 1988). In this study, it was found that IAA V_L was 99.7% homologous in nucleotide sequence and 100% homologous in deduced amino acid sequence with the spliced version of the A23 germ-line gene in the chromosomal region Ab (Straubinger et al., 1988). Therefore, the A23 gene is a likely candidate for the gene coding for the IAA V_L protein sequence. The minor difference between the IAA V_L and the spliced version of the A23 germ-line gene may be attributed to allelic polymorphism (Karttinen et al., 1989, Van Dijk et al., 1989) or to somatic mutation (French et al., 1989).

It had originally been hypothesized that A23 was a potentially functional V_K gene on the basis that several deduced invariant amino acids of other V_L germ-line genes were replaced in A23, but most of them by similar ones, and the three dimensional structure of the protein would probable not be distorted by these changes (Straubinger et al., 1988). The IAA obtained in this study is an antigen-defined antibody with a V_K protein sequence encoded by the A23 germ-line gene. This finding confirms the hypothesis that A23 is a functional gene.

The IAA V_L nucleotide sequence was compared with the V_L of other human antibodies. The IAA V_L was found to be 100% homologous in nucleotide sequence with the V_L of a monoclonal rheumatoid factor derived from a patient with monoclonal gammopathies (RFTS3) (Pascual et al., 1990). However, the V_H of the RFTS3 antibody

shows little homology with IAA V_H . The RFTS3 antibody is monoreactive towards human IgG while IAA is a monoclonal antibody directed towards insulin even though these antibodies share the same V_L ($V_{\kappa II}$). This has been observed for other V_L subfamilies: V_L chain genes used among IgM autoantibodies relatively conserved to the $V_{\kappa III}$ subfamily, and showing little or no change from the germ-line sequence, have been found to have various specificities (Goñi et al., 1989, Chen et al., 1986, Atkinson et al., 1985, Spatz et al., 1990). Taken together, these findings suggest that, although critical in determining the gross conformation of some autoantibodies, L chains appear to be less important in defining their fine specificity.

An expressed gene is influenced by selective pressures, so its conservation in the germ-line implies an important physiologic role for its product, but the evolutionary forces that have conserved these autoantibody genes remain a mystery. A genetically determined and sequence-dependent CDR1, PSL2, has been observed to be extremely conserved among various autoantibodies with $V_{\kappa IIIb}$ L chains, and it has been suggested that this sequence may be relevant for the idiotypic network regulation of a family of autoantibodies (Goñi et al., 1989). The rare occurrence of somatic mutations in the $V_{\kappa II}$ L chain germ-line genes of the IAA and RFTS3 autoantibodies suggests that a similar genetically determined sequence of the $V_{\kappa II}$ L chains may also be relevant in the idiotypic network regulation of a different family of autoantibodies.

The persistence in the structure of an IgM directed against IgG (IgM-GII) obtained from a patient with Felty's syndrome was followed over a 4.5 y period (Abraham et al., 1978). The amino terminal amino acid sequences remained unchanged over this period and belonged to the $V_{\kappa II}$ subfamily. The persistence of the IgM autoantibody must have reflected the continued presence of the clone of cells which synthesized the IgM-GII during the 4.5 y interval. This persistent synthesis may have been dependent upon the continued presence of the original autoantigen or an antigen which was closely related

or structurally cross-reactive with the original immunogen. Whether the IAA obtained in this present study had also persisted in the Type I DM Patient #3 for a time prior to diagnosis of Type I DM or continued to exist after initiation of insulin therapy is not known.

The IAA J_L diverges from the J_K4 germ-line gene by a single silent point mutation that does not change the germ-line encoded amino acid sequence (Ravetch et al., 1981). Whether human autoantibodies with V_K regions encoded by the A23 germ-line gene preferentially recombine with J_K4 region genes remains unknown at this point in time since the J_K region sequence of RFTS3 was not sequenced. The J_K4 region is used by V_KIII human autoantibodies with anti-DNA, micelles of phosphatidic acid and myelin cross-reactivity (Spatz et al., 1990) and with anti-light density lipoproteins (anti-LDL) reactivity (Goñi and Frangione, 1987). Human autoantibodies have also been found to use the J_K1, J_K2 and J_K5 regions (Goñi et al., 1989). The J_K gene expression does not seem to reflect a restricted selectivity in human autoantibodies, although some of them occur more frequently in IgM-RF (Goñi et al., 1989).

As expected, the portion of the IAA C_K region sequenced (42 bp) did not diverge from the germ-line C_K gene (Kabat et al., 1991). This region is well conserved among antibodies in general.

Models for the Generation of Autoantibodies

One model for the generation of autoantibodies suggests that self-specific antibodies are encoded by the germ-line V gene repertoire and are thus present in both normal and diseased individuals. In normal individuals, self-reactive cells are suppressed, but in disease, an abnormal polyclonal B lymphocyte activation occurs resulting in the pathological secretion of autoantibodies (Teodorescu, 1981). This model predicts that autoantibodies resemble those derived after polyclonal activation

of normal B lymphocytes. The finding that the V_L of IAA is A23 germ-line gene encoded supports this model.

An alternative to the polyclonal activation model for the generation of autoantibodies invokes self-antigens as the driving force for the production of autoantibodies (Nelson et al., 1987). This 'antigen driven' model predicts that autoantibodies would resemble antibodies produced during the immune response to foreign antigens; these are usually derived from clonally related precursors, somatically mutated (often reflecting selection of mutations by antigen), and may be isotype switched.

In order to distinguish between polyactivation or antigen-driven activation with respect to IAA generation, the V_H sequence of IAA can be compared to highly similar germ-line or consensus sequences to identify mutations. If the IAA V_H is germ-line gene encoded, as is the IAA V_L , the polyclonal activation model for the generation of IAA would be supported. If the V_H of IAA is somatically mutated, particularly in the CDRs which determine the antigen specificity of the antibody (Amit et al., 1985, Chothia et al., 1989, Rini et al., 1992), the antigen-driven model would be supported.

Sequencing of IAA V_H cDNA

The V_{HIII} subfamily is one of the larger V_H subfamilies, with at least 30 members (Berman et al., 1988) encoded by genes located on chromosome 14 (Kabat et al., 1991). The finding that IAA V_H belongs to the V_{HIII} subfamily is not entirely surprising because many previously sequenced autoantibodies such as rheumatoid factors, anti-DNA antibodies, and polyreactive natural antibodies had also been found to belong to this subfamily (Hoch and Schwaber, 1987, Pascual et al., 1990, Sanz et al., 1989).

The IAA V_H nucleotide sequence was found to be 96.6% homologous to the V_H26 germ-line gene (Mathyssens and Rabbitts, 1980). The deduced amino acid V_H sequence of IAA was found to be 94.7% homologous to V_H26. This high homology suggests that the V_H26 gene is the germ-line gene counterpart of IAA V_H. The differences between IAA V_H and V_H26 could be due to either allelic polymorphism or to somatic mutation.

In the absence of selective pressures (under circumstances where there is neither selection "for" nor "against" replacement) the random ratio of replacement (R) mutation to silent (S) mutation (R:S ratio) would be 2.9:1 (Jukes and King, 1979). This ratio would be expected in regions of a protein which need not be conserved to maintain function. In regions such as the FR of antibodies which need to be conserved to maintain function, this ratio is expected to be lower and is about 1.5, based on the extent of conservation seen in comparison of diverse V-region sequences (Clarke et al., 1985). On the other hand, the ratio in CDRs of antibodies might approach 2.9 as there are relatively fewer conserved residues in CDRs (Schlomchik et al., 1987). But ratios significantly higher than 2.9 can only be achieved through positive selection of R mutations (Jukes and King, 1979). If an R mutation which increases the affinity of an antibody for antigen confers a selective advantage to a mutated B cell (that is a higher probability to divide compared to siblings), then this R mutation will be fixed and propagated in the antigen-specific population. Most of such mutations are expected to be in the CDRs, since these are thought to include most of the antigen contact residues (Amrit et al., 1985; Chothia et al., 1989; Rini et al., 1992).

The mutations of IAA V_H compared to the V_H26 germ-line gene counterpart show evidence of selection for R mutations in CDRs and perhaps against R mutations in FRs. For the CDRs, there were 4 R mutations and no S mutations. For the FRs, the R:S ratio is 1:1. This pattern of somatic mutations in the V_H of IAA can only be explained by

positive selection of certain B cells on the basis of their antigen receptors, presumably because these mutated receptors (in the CDR of the V_H) provide superior ability to bind antigen. This receptor mediated selection is consistent with the idea that IAA are the result of stimulation of B cells with antigen (most probably insulin but perhaps a cross-reacting antigen), but it is not predicted by the polyclonal activation model. The rise in IAA levels in certain individuals could correspond to the success of one or a few clones which had encountered antigen and grew with selective advantage.

It is known that H chain class switching is not required for somatic mutation to occur since IgM antibodies can undergo somatic mutation (Rudikoff et al., 1984, Rajewsky et al., 1987, Siekevitz et al., 1987). The IAA-secreting 3-0.5F10 B lymphocytes may have terminally differentiated into an IgM secreting plasma cell or they may not have yet have switched to a different isotype at the time of EBV infection.

Milstein and his co-workers showed that, in mice, antibodies of the IgM and IgG classes made during the first 7 days after immunization (early primary response) did not have somatic mutations, whereas antibodies made 14 days or more after a single antigenic stimulation (late primary response) had acquired significant numbers of base changes (Karttinen et al., 1983, Berek et al., 1985, French et al., 1989). The somatic mutations of IAA V_H compared with its germ-line gene counterpart V_H26 suggest a sustained exposure to insulin or some cross-reacting antigen.

The nucleotide sequence and the deduced amino acid sequence of the IAA V_H were compared to the V_H sequences of other antibodies and to the V_H26 germ-line gene. The V_H of 4G12, a human monoclonal antibody secreted by 4G12 hybridoma cells (Kishimoto et al., 1989) with broad reactivity to malignant tumor cells, was found to be homologous to IAA V_H both in nucleotide and amino acid sequence. The differences in nucleotide sequence between the V_H of IAA and that of 4G12 were found primarily in the

CDR2 which results in a three amino acid change in this region. Diamond and co-workers have shown that a single amino acid change in the CDR of the heavy chain resulted in a different antigen specificity (Diamond and Scharff, 1984). Thus, IAA will not necessarily cross-react with the tumor-associated antigen, of an Mr of 65,000, towards which the monoclonal antibody secreted by 4G12 is directed (Salto et al., 1988). Whether IAA cross-reacts with antigens other than bovine, porcine or human insulin is not known. The extent of IAA antigen binding analysis was limited by the amount of antibody produced by the 3-0.5F10 lymphoblastoid cell line before it stopped dividing and producing antibody in culture.

The V_H of 30pl is a 130-day human fetal-derived H chain cDNA transcript (Schroeder Jr. and Wang, 1990). The V_H of 30pl is 100% homologous to the deduced amino acid sequence of the V_H26 germ-line gene. It has been observed that fetal V_H utilization is consistently restricted with striking preference for specific V_{HIII} elements, one of which is the V_H of 30pl (Schroeder Jr. and Wang, 1990). This restriction implies that V_{HIII} -like elements play an important biologic role. One possibility is that V_{HIII} -like elements may have been selected in response to antigens shared by common pathogens.

With respect to this hypothesis, it is interesting to note that the V_H of ED8.4, an antibody directed towards the capsular polysaccharide of *Haemophilus influenzae* type b (Adderson et al., 1991), was found to be highly homologous to the following: (1) the V_H26 germ-line gene, (2) the 30pl transcript, (3) the V_H gene of Ab18, a polyreactive antibody isolated from EBV-PBL derived from a normal individual (Sanz et al., 1989); this antibody binds human insulin, IgG Fc fragment, DNA, thyroglobulin, tetanus toxoid, and various bacterial LPS and (4) the V_H of 18/2, a anti-DNA autoantibody (Dersimonian et al., 1987). This shared homology suggests that the production of polyreactive antibodies with V_{HIII} germ-line encoded elements early in development

may permit clonal B cell expansion in the absence of external environmental antigen. Such a preassembled immune repertoire may be important in the host's early defense against pathogens such as bacteria by enhancing phagocytosis or complement-mediated lysis or by amplifying an ongoing specific antibody response.

Cells bearing the B lymphocyte-specific CD5 surface marker (CD5⁺ B lymphocytes) are responsible for the production of Ab18, the V_H26 germ-line encoded polyreactive monoclonal antibody (Sanz et al., 1989). Moreover, it has been postulated that the CD5⁺ B lymphocyte subset is responsible for the production of polyreactive "natural antibodies" present in the circulation of healthy individuals (Casali et al., 1989a, 1989b). It has been observed that production of such antibodies is dramatically enhanced during the response to exogenous antigens, such as tetanus toxoid, or to bacterial lipopolysaccharides, or in the course of viral, bacterial or parasitic infections (Welch et al., 1983, Carson et al., 1978, Dresser, 1978, Izui et al., 1979, Coulic and Van Snick, 1983). Moreover, polyreactive IgM have been shown to potentiate antibody-dependent viral neutralization (Ashe et al., 1971) and to protect young rats from infection with *Trypanosoma levysii* (Clarkson Jr. and Mellow, 1981). The importance of these polyreactive antibodies in the defense against infections is further suggested by their representation in primordial phylogenetic stages: polyreactive IgM are the only antibodies in sharks and torpedo fish (Gonzales et al., 1988).

The autoreactivity of these germ-line encoded polyreactive antibodies towards insulin as well as other antigens such as DNA and thyroglobulin and their potency in antimicrobial defense may be interrelated: germ-line gene encoded antibodies could be targeted to epitopes on the microbes which have been acquired by molecular mimicry of insulin, DNA and thyroglobulin (Kocks and Rajewsky, 1989). Germ-line gene encoded polyreactive antibodies produced by CD5⁺ B lymphocytes may also be involved in avoiding autoimmune disease by blinding the immune system to environmental

epitopes cross-reactive with self. These autoantibodies could act as a filter (perhaps by passive blocking of the antigen) to ensure that only non-self epitopes impinge on the immune system and arouse a violent effector response (Cohen and Cooke, 1986).

By using an ultrasensitive assay, Fineberg and co-workers have discovered that nondiabetic individuals regularly have low levels of human insulin specific low-affinity IgG autoantibodies (Fineberg et al., 1991). This data support the idea that low-level insulin autoimmunity is a normal event, possibly essential as a first line defense in infection or, more interestingly, reflecting an active suppression of self-reactive B-lymphocyte clonal expansion and expression.

The low-affinity IAA measured were human insulin specific (Fineberg et al., 1991) while the monoclonal IAA derived from Type I DM Patient #3 was found to bind human, bovine and porcine insulins with equal affinity (chapter 3). The IAA detected by Fineberg and co-workers could possibly be encoded by germ-line genes and be "natural autoantibodies" such as the V_H26 germ-line gene encoded polyreactive Ab18 which binds human insulin with low affinity. Somatic mutation of the germ-line encoded genes, as observed for the IAA V_H , may lead to the generation of IAA with a higher affinity for human insulin and for IAA directed towards a different epitope on the insulin molecule. This difference in epitope specificity could lead to cross-reactivity with bovine, porcine and human insulin.

The V_H of ED8.4, the anti-Hib PS antibody, was found to be 96.3% homologous in nucleotide sequence and 94% homologous in deduced amino acid sequence to the IAA V_H . There is one amino acid difference in the CDR1 and two in the CDR2 between the two antibodies. It is not known whether IAA cross-reacts with the Hib PS but the mismatches in the CDRs between the ED8.4 V_H and the IAA V_H likely reflect separate antigen specificities.

The observation that the V_H of ED8.4 and the IAA from 3-0.5F10 cells are likely derived from the same germ-line gene leads to the following speculations concerning the generation of IAA. The V_H26 germ-line gene encoded antibodies may normally be expressed in $CD5^+$ B lymphocytes as a first line defense against pathogens such as *H. influenzae* type b. These unmutated germ-line gene encoded antibodies could bind various antigens including Hib PS and endogenous insulin with low affinity. In some B lymphocytes, the V_H26 encoded genes could undergo somatic mutation which results in a greater affinity for Hib PS. If the individual is infected with pathogen, these B lymphocytes will preferentially divide in order to better combat the infecting microorganism in accordance with the "antigen-driven" selection model. On the other hand, V_H26 genes could somatically mutate, resulting in the acquisition of specificity for a new epitope on the endogenous insulin molecule which could also be present in porcine and bovine insulins. These mutated V_H26 gene encoded IAA may bind circulating endogenous insulin with increased affinity and the clone of B lymphocytes would potentially expand according to the "antigen-selection" model. In support of the hypothesis that an antigen-driven process of positive selection and somatic point mutation shapes the anti-self response in autoimmunity is the finding that V segments encoding a number of different rheumatoid factors and anti-DNA antibodies (Schlomchik et al., 1987) harbour many somatic point mutations, suggesting that a non-random, antigen-driven positive selection is operative in the generation of these autoantibodies.

It has been shown that: (1) $CD5^+$ B lymphocytes represent the vast majority of B lymphocytes in the human fetal spleen (Antin et al., 1986) and cord blood (Gadol and Ault, 1986); (2) the V_H26 -homologous 30pl transcript is expressed preferentially early in life (Schroeder Jr. and Wang, 1990); (3) $CD5^+$ B lymphocytes produce V_H26 encoded polyreactive antibodies which can bind insulin with low affinity, such as Ab18 (Sanz et

al., 1989); and (4) a monoclonal IAA derived from a Type I DM patient at the time of diagnosis is an antigen-selected somatically mutated V_H26-encoded antibody (described in this thesis). These findings suggest that there may be more CD5⁺ B lymphocytes expressing V_H26 encoded antibodies in early life. With more V_H26 transcripts being expressed, there is a greater chance of somatic mutation of V_H26 encoded genes which could result in an increase in affinity for endogenous insulin. Therefore, at a young age, there is a greater likelihood of antigen-driven selection and clonal expansion of IAA. This could account for the higher prevalence and binding of IAA in younger aged individuals observed by many groups (McEvoy et al., 1986, Arslanian et al., 1985, Srikantha et al., 1986, Ziegler et al., 1989, Vardi et al., 1988, Sochett and Daneman, 1989).

The somatic mutations of the V_H of IAA could change the idiotype of the autoantibodies which in turn may allow them to escape idiotypic control. Antibody mutants may arise whose V-regions happen to be recognized as such by helper cells in the environment. In such situations mutant, autoreactive B-lymphocyte clones could be triggered into uncontrolled expansion. Interestingly, anti-insulin receptor antibodies, which are found in the serum of some recent onset Type I diabetic patients (Maron et al., 1983, Ludwig et al., 1987), have also been shown to be anti-idiotypic to IAA (Schechter et al., 1982, Uchigata et al., 1991), that is they bound to and could be blocked by IAA. The insulin receptor antibodies might be part of the body's natural attempt at down-regulating self-reactivity by binding and thereby inactivating the mutant, autoreactive IAA.

In Type I DM patients, increasing IAA levels may also be due to a failure of immune suppression. In support of this hypothesis, a temporal link has been observed between humoral and cellular immune alterations in a Type I DM related population. Faustman and co-workers have detected a linear relationship between the degree of

elevation of naive suppressor-inducer T lymphocytes to the reciprocal decrease of helper-inducer primed T lymphocytes, and the levels of IAA measured by competitive radioimmunoassay (Faustman et al., 1991). It was suggested that the unprimed T lymphocytes in prediabetic individuals may improperly fail to differentiate into memory T lymphocytes or that the helper-inducer primed T lymphocytes have been sequestered to the site of autoimmune attack in the islets. This speculation requires further investigation and understanding of the precise network of T lymphocyte immunity in the pathogenesis of Type I DM.

High levels of IAA in Type I DM patients could be a secondary result of the pancreatic β cell destruction. IAA may be directed towards insulin, or even proinsulin, which is being slowly leached out of the pancreatic β cell while it is being destroyed in an autoimmune attack. This leached (pro)insulin may be available in a conformation which renders it antigenic or which exposes previously masked epitopes. Somatic mutations in the V_H26 -encoded IAA V_H elements could generate antibodies with a greater affinity for these epitopes. Upon contact with this antigen, the B lymphocytes would expand clonally.

Sequence analysis of the CDR3 of IAAV_H

The third hypervariable region (CDR3) of the H chain is generated by $V_H-D_H-J_H$ joining. The diversity (D) segment of the IAA H chain was found to be 36 bp in length (12 amino acids) and to make up the first portion of the CDR3. A core of the IAA H chain D segment of the IAA H chain was found to share 15 bases of identity with the DXP4 germ-line gene (Ichihara et al., 1988). In as much as this core resembled the DXP4 germ-line gene, there were some substitutions. If indeed the IAA H chain D fragment was derived from DXP4, either the level of polymorphism or somatic mutation in this segment is

from DXP4, either the level of polymorphism or somatic mutation in this segment is high. Insertion of nucleotides at the 5' and 3' ends of the D-region by terminal deoxytransferase (N insertion) (Alt and Baltimore, 1982), D-D fusion (Pascual et al., 1990) and imprecise joining at these ends may also account for the divergence from the germ-line sequence at the 5' and 3' ends.

Much heterogeneity has been reported in the length and sequence of D segments found among autoantibodies (Hoch and Schwaber, 1987, Sanz et al., 1989). However Dersimonian and co-workers have identified a recurrent D segment among several anti-DNA antibodies that use members of different V_H families. This D segment is different in length (25-30 nucleotides) to that of the IAA (36 nucleotides). Conservation of CDR3 length has been observed among antibodies obtained from EBV transformed B lymphocytes that bind DNA and express the V_H6 gene (Logtenberg et al., 1989) but variation of CDR3 length has been observed in anti-DNA antibodies that use members of different V_H families (Dersimonian et al., 1989). Additional autoantibodies must be sequenced and their CDR3 compared to one another to sort out this confusion and to determine the significance of CDR3 length on antigen binding.

The IAA J_H chain is likely derived from the J_H4 human germ-line gene (Ravetch et al., 1981). There is only one silent base substitution which may be due to polymorphism of the J_H4 gene. The nucleotide sequence is six nucleotides shorter than the germ-line J_H4 at the 3' recombination site. The J_H4 region is frequently used by human autoantibodies with anti-DNA (Spatz et al., 1990, Dersimonian et al., 1987), and anti-Sm (Sanz et al., 1989) reactivities and is also frequently expressed in the early B cell repertoires (Schroeder Jr. et al. 1987).

One possible mechanism responsible for the generation of the CDR3 region of IAA is D-D fusion. The IAA D region cDNA shares homology with the DXP4 germ-line but not at the 5' or 3' ends. This lack of homology may be explained by the fusing of the DXP4 germ-line gene with another as yet unidentified D-region germ-line gene to form the IAA D-region. Indeed, other autoantibodies have been identified in which D-D fusions may have contributed to the generation of the CDR3 regions (Pascual et al., 1990; Sanz et al., 1989).

The possibility of D-D fusions raises the question of how such recombination could be brought about, either in the germ-line or within somatic cells during immunodifferentiation. The rule that recombination can occur only between properly spaced recombination signals would have to be violated if the D-D fusion were to occur somatically by the V-D-J recombination mechanism. Each germ-line D is flanked by two recombination signals, each of which contains a 12 base spacer that would theoretically mitigate against D-D joining. Furthermore, at least in the case of the human germ-line D segments, convincing recombination signals are not present elsewhere in the D segments (Ravetch et al., 1981). Such considerations point to the involvement of other recombinational mechanisms in the formation of putative D mosaics. For example, short, overlapping segments of homology such as those seen within the recombination signals or the D segments themselves may draw segments together either to be joined covalently or via a gene conversion mechanism. Such mechanisms might operate within the germ-line or within somatic cells (Ravetch et al., 1981).

Ab2022

Ab2022 is a IgM κ monoreactive anti-insulin antibody from a Type I DM patient treated with heterologous insulin that binds human, porcine and bovine insulins with equal affinity (Sanz et al., 1989). The V_H of Ab2022 is homologous to the V_H251 germ-line gene which belongs to the V_HV subfamily (Humphries et al., 1988), while the IAA V_H belongs to the V_HIII subfamily. Although the clone producing Ab2022 was derived from a Type I DM patient chronically treated with insulin, it likely constitutes the progeny of a B cell clone not involved in any process of antigen-driven clonal selection and somatic hypermutation because the ratio of R:S mutations in the CDRs of this antibody is only 2:1. In comparison however, the IAA V_H described in this thesis is somatically mutated and is likely antigen-selected. The A2022 D_H has a nucleotide core sequence that shares 22 bases of identity with the DRLI germ-line gene (Ichihara et al., 1988) and shares little homology with the IAA D_H. The Ab2022 J_H is likely derived from the J_H3 germ-line gene (Ravetch et al., 1981) and shares 79.5% nucleotide and 84.6% amino acid sequence homology with that of IAA. Both the IAA characterized in this present study and the Ab2022 antibody cross-react with bovine, porcine and human insulin antibodies. However, because of the varying V_H, D_H and J_H segments, these antibodies may recognize different epitopes of the insulin molecule.

Formulated insulins used in the treatment of diabetics are injected subcutaneously, where they polymerize and remain at a relatively high temperature for long periods before they are released into the systemic circulation (Kurtz et al., 1980). Polymerization greatly increases the antigenicity of insulin and may conceivably do so to the advantage of some determinants and the detriment of others (Díaz and Wilkin, 1987). It is conceivable that the Ab2022 antibody with a V_HV element is directed towards an epitope present in porcine, bovine and human insulins which has become unmasked upon polymerization of injected insulin. Upon contact with the injected

insulin, the B lymphocytes producing Ab2022 may have expanded clonally.

In this same context, a monoclonal insulin antibody secreting lymphoblastoid cell line, 1-0.5G1, was derived from a Type I DM patient who had been treated with recombinant human insulin (described in this thesis). This monoclonal antibody, like Ab2022, was found to bind human, porcine, and bovine insulins with equal affinity. One might speculate that these two antibodies are directed towards the same epitope on the insulin molecule and that the V_H of the insulin antibody secreted by 1-0.5G1 is homologous to that of Ab2022. The antigenicity of the epitope involved may have been unmasked upon polymerization of the injected insulin. On the other hand, the insulin antibody secreted by 1-0.5G1 cells may be directed towards the same epitope present on the endogenous insulin as that recognized by the IAA secreted by 3-0.5F10 cells. If this were the case, the V_H of the insulin antibody might be homologous to that of the IAA secreted by 3-0.5F10 cells. Alternatively, the insulin antibodies secreted by 1-0.5G1 cells and by 3-0.5F10 cells as well as Ab2022 may all recognize different epitopes on the insulin molecule. Further binding studies are required to address these possibilities.

The serum from Patient #1 (from whom 1-0.5G1 cells were isolated) was found to bind human insulin with greater affinity than porcine or bovine serum. Human insulin specific antibodies may employ V_H elements of a different subfamily (or a mutated version) than those used by Ab2022 or the antibodies secreted by 3-0.5F10 cells or 1-0.5G1 cells.

Only one monoclonal IAA was derived from the Type I DM Patient #3 in this study. This monoclonal IAA was found to bind porcine, bovine and human insulin with equal affinity. The serum from Patient #3 (from whom 3-0.5F10 cells were isolated) demonstrated the same profile of cross-reactivity of binding to porcine, bovine and human insulin. Therefore, it may be that the monoclonal IAA secreted by 3-0.5F10 cells represented the major population of IAA-secreting B lymphocytes in the blood of

Patient #3 at the time of diagnosis. Alternatively, insulin autoantibodies encoded by other V_H elements, such as that employed by Ab2022, may also have been present in Patient #3 's blood at the time of diagnosis with Type I DM.

Southern Analysis of the PCR Amplified Products Using IAA Sequence Specific Probes

It was not known whether the IAA secreted by 3-0.5F10 cells was also secreted by B lymphocytes of other Type I DM patients at the time of diagnosis. The experimental approach employed to address this question was to isolate RNA from EBV immortalized B lymphocytes which had been established from Type I DM patients at the time of diagnosis. cDNA was synthesized from the RNA and PCR amplification was performed using IAA-specific V_H and V_L primers. To determine the specificity of the amplification, Southern analysis of the PCR amplified products was performed using IAA CDR2-specific radiolabeled probes.

The A23 germ-line gene encodes the V_L of the IAA and the RFTS3 rheumatoid factor (Pascual et al., 1990). The V_L CDR1 and CDR3 oligonucleotide primers, as well as the CDR2 probe, were homologous to the regions of the A23 germ-line gene. Therefore detection of a band corresponding to an A23 V_L element would not necessarily ensure the detection of a light chain transcript specifically belonging to an IAA.

Southern analysis of cDNA obtained from the PCR amplification using IAA- V_L oligonucleotide primers and a IAA- V_L CDR2 radiolabeled oligonucleotide probe was performed. A band of 234 bp, which corresponds to the cDNA segment incorporating the IAA-specific V_L oligonucleotide primers, was detected for the positive control as well as for the cDNA synthesized from RNA isolated from EBV-PBL cells derived from nine Type I DM patients at the time of diagnosis. The 234 bp band did not disappear when the concentration of primer was reduced ten fold. Thus, ten Type I DM patients at

when the concentration of primer was reduced ten fold. Thus, ten Type 1 DM patients at the time of diagnosis had B lymphocytes which were producing antibodies with IAA-homologous V_L .

The V_H of the IAA secreted by the 3-0.5F10 cells is a somatically mutated version of the V_H26 germ-line gene. No other antibody V_H elements studied thus far have the same V_H sequence as the IAA V_H . The primers used for the PCR amplification of the IAA V_H cDNA were therefore IAA-sequence specific: **(1)** the CDR1 primer encompassed the entire CDR1 of the IAA V_H and contained 1 nucleotide substitution compared with the V_H26 germ-line gene counterpart (Fig. 23); **(2)** the CDR3.2 primer encompassed a portion of the D-region which was likely derived from the DXP4 germ-line gene but which contained 7 nucleotide substitutions; and **(3)** the CDR3.1 primer encompassed another portion of the CDR3 which contained a portion of the D_H -region (12 nucleotides, 9 of which were possibly derived by N insertion or D-D fusion) and a portion of the J_H -region (6 nucleotides which were likely derived from the J_H4 germ-line gene). Thus, amplification of cDNA using **a)** CDR1 and CDR3.2 and **b)** CDR1 and CDR3.1, would hopefully ensure amplification of a IAA-specific V_H element.

To further ensure that the amplified cDNA was a IAA-specific V_H element, Southern analysis was performed. The oligonucleotide probe which was used for Southern analysis corresponded to a portion of the CDR2 which contained 2 nucleotide substitutions compared with the V_H26 germ-line gene counterpart (Fig. 23). These nucleotide substitutions were not found in the V_H of other autoantibodies with V_H elements homologous to the V_H26 germ-line gene (Fig. 23). Thus, the CDR2 probe was specific for IAA V_H .

Southern analysis of the PCR amplified products using the IAA-specific V_H CDR1 and CDR3.2 oligonucleotide primers and the IAA-specific V_H CDR2 radiolabeled oligonucleotide probe was also performed. A band of 229 bp which corresponds to the

primers was detected for the positive control and for the cDNA from nine Type I DM patients at the time of diagnosis.

Southern analysis of the PCR amplified products using the IAA-specific V_H CDR1 and CDR3.1 oligonucleotide primers, as well as the IAA-specific V_H CDR2 radiolabeled oligonucleotide as probe, was performed to verify the specificity of PCR amplification of the V_H cDNA. A band of 245 bp which corresponds to the segment of IAA V_H cDNA incorporating the two IAA-specific V_H oligonucleotide primers was detected for the positive control as well as for the cDNA from the Type I DM patients at the time of diagnosis. Thus, the ten Type I DM patients at the time of diagnosis had B lymphocytes which were producing antibodies with IAA-specific V_H . It is not known whether the heavy chains with IAA-specific V_H elements were combined with the light chains with A23 germ-line gene encoded V_L elements in the nine Type I DM patients studied.

The Type I DM patients at diagnosis used in this study had been analyzed for IAA serum levels by a competitive fluid phase radioimmunoassay insulin autoantibody assay (CIAA; chapter 1). Type I DM Patients #1, 4, 11, 13, 16 and 20 were found to have IAA levels which were greater than 3 SD above the normal population ("IAA positive"). Type I DM Patients #15, 19 and 21 had IAA levels which fell within the range of the normal population. All of these Type I DM patients at the time of diagnosis had B lymphocytes which were producing IAA-specific V_H elements and A23 germ-line gene encoded V_L elements. Therefore, the presence of IAA-specific V_H and A23 germ-line gene encoded V_L elements at the time of diagnosis was not correlated with "positive IAA levels" measured by CIAA but was highly correlated with Type I DM onset.

The apparent lack of correlation between the presence of the IAA V_H and A23 germ-line gene encoded V_L elements and "IAA positivity" measured by CIAA at the time of diagnosis of Type I DM could be explained as follows: (1) either the fluid phase CIAA allows the measurement of a population of insulin binding antibodies other than that

secreted by 3-0.5F10 cells, or; (2) PCR amplification followed by Southern analysis is such a sensitive technique that it allows the detection of transcripts even though the titers in the patient serum measured by CIAA were not elevated. High IAA levels in some of the Type I DM patients at diagnosis may reflect a sustained immune response to insulin being produced by a residual β cell mass. The Type I DM patients with low levels at the time of diagnosis may not have had sufficient residual insulin production to ensure a sustained antigen-driven activation.

Wilkin and co-workers have shown that human insulin specific IAA were virtually never found in association with Type I DM while IAA which cross-reacts with bovine, porcine and human insulin was present in 41 out of 42 Type I DM-related patients (Wilkin et al., 1988). This observation considerably improved the specificity of IAA as a marker for Type I DM relatedness without compromising its sensitivity. The IAA derived from a Type I DM patient at the time of diagnosis, which was characterized in this present study, also bound bovine, porcine and human insulins with equal affinity. Moreover, B lymphocytes producing IAA-homologous V_H and V_L elements were present in all of the Type I DM patients studied at the time of diagnosis. Taken together, these observations suggest that the IAA characterized in this study may be a marker for Type I DM. Further studies are required to determine whether these IAA V elements are strictly restricted to Type I DM patients or whether they are also present in normal individuals as well.

Should these particular IAA V elements be present in normal individuals and in patients with Type I DM, this would suggest that these antigen-selected IAA are part of a normal low-level autoimmunity to endogenous insulin. Thus, a simple "yes or no" detection of IAA V_H and V_L transcripts alone would not be sufficient for the identification of individuals at risk for Type I DM.

However, if the antigen-selected IAA characterized in this study are specifically

restricted to Type I DM patients at the time of diagnosis, we could speculate that the detection of this particular IAA is reflective of an autoimmunity which can lead to the destruction of pancreatic β -cells. Should this be the case, the IAA-specific V_H element in combination with the A23-encoded V_L elements could be useful as a marker for Type I DM.

Prospective studies could be performed to determine the potential of IAA V_H and A23 germ-line gene encoded V_L elements to predict the onset of Type I DM. It would also be interesting to determine whether the IAA described in this study have different V elements than IAA which have been found to occur in other conditions such as the autoimmune insulin syndrome, or other autoimmune syndromes (Di Mario et al., 1990, Wilkin et al., 1988). However, the V-region sequences of these IAA are not yet available.

Ziegler and co-workers have observed a strong association of IAA with HLA-DR4, which indicates that the autoantibody responsiveness to autologous insulin may be HLA restricted (Ziegler et al., 1991). HLA-DR4 is a susceptibility haplotype for Type I DM: 95% of Type I DM patients possess either HLA-DR3 or -DR4, compared to 45-54% of the normal population (Todd et al., 1987). Future studies could be performed to address a possible HLA association with the monoclonal IAA described in this thesis.

Obviously, treatment for Type I DM will be most effective when the primary defect(s) and/or event(s) have been understood. Reports of benefit from immunosuppressive therapy in patients with recently diagnosed Type I DM have generated considerable enthusiasm for possible prevention (Jenner et al., 1992, Castaño et al., 1988), despite the great potential for toxicity of the immunosuppressive agents currently available. One particular immunosuppressive agent, cyclosporin A (CsA), was found to significantly suppress IAA levels in pre-diabetic children before insulin therapy was started, while it had only minor effects on the production of other islet cell

autoantibodies (Castaño et al., 1988). If IAA are markers of the β -cell destructive process, the blockade of their production might be a secondary result of the CsA-induced diminution of the β -cell lysis. If IAA are somehow directly involved in the autoimmune β -cell destruction of Type I DM, this observation supports a search for a relation between the favorable effect of CsA in Type I DM and its ability to specifically alter the production of IAA.

If successful and safe preventive therapy does become possible in the future, it would be ideal to institute this at a time when there is as much remaining pancreatic β cell function as possible. It is therefore imperative that individuals be identified in whom the autoimmune process is underway. Herein lies the value of a good marker for pancreatic β cell destruction. Also of importance is that the marker be fairly easy to identify. If the IAA characterized in this study are specifically restricted to Type I DM patients, the PCR amplification of IAA V elements followed by Southern analysis which was developed in this study could be easily applied to screen a population for individuals at risk of developing Type I DM.

If the IAA V elements described in this study are specifically restricted to Type I DM patients, we could speculate that these autoantibodies are somehow involved in the autoimmunity towards the insulin-secreting pancreatic β cells. In order to determine whether these IAA play a role in the pathogenesis of the disease, introduction of functionally rearranged and somatically mutated IAA V_H and rearranged IAA V_L into transgenic mice may generate a population of B lymphocytes that express IAA V_H and V_L encoded autoantibodies. Such a mouse model may be useful in the study of "autoaggressive" insulin antibodies and autoimmune disease.

Conclusion

Chapter 1

A competitive insulin autoantibody assay (CIAA) for the measurement of serum IAA levels was established and characterized. It was found that stripping the serum prior to performing the CIAA increased significantly the sensitivity and the specificity of the assay. Type I DM patient serum samples (before diagnosis, at diagnosis, and following months of insulin therapy) were assayed for specific insulin binding. None of the control subjects were IAA positive, while 50% of the Type I DM patients were positive for IAA at the time of diagnosis. One of the three pre-diabetic patients studied was positive for IAA prior to diagnosis.

Chapter 2

Many EBV-PBL lines were derived from patients with Type 1 DM at diagnosis. A novel human myeloma fusion partner, MM-neo, was developed for fusion with these EBV-PBL in order to stabilize the cells and boost the Ig production. The parameters for fusion of MM-neo with EBV-PBL were established and hybridomas were generated from this fusion. These hybridomas grew very well and were easily cloned by limiting dilution. Unfortunately, the hybridomas which resulted did not secrete IAA. Because of the low frequency of fusion obtained with MM-neo, the chances of obtaining an IAA-secreting human monoclonal hybridoma cell line using this fusion partner were minimal. It was therefore decided that the EBV-PBL established from Type 1 DM patients at diagnosis would be cloned directly and IAA secreting cultures would be selected.

Chapter 3

EBV-PBL derived from Type I DM patients were cloned and screened for insulin binding activity by solid phase ELISA. Two monoclonal insulin antibody-secreting cell lines were obtained: (1) 1-0.5G1 was obtained from a Type I DM patient treated with exogenous human insulin, and (2) 3-0.5F10 was obtained from a non-insulin treated Type I DM patient at the time of diagnosis; the monoclonal antibody secreted by 3-0.5F10 is therefore an IAA.

The insulin antibody secreted by the 1-0.5G1 cell line was found to be of the IgM κ isotype and bound human, porcine and bovine insulins with equal affinity. The serum IgM of Patient #1, from whom 1-0.5G1 was also derived, exhibited a higher affinity for humulin than for porcine or bovine insulin. Because the monoclonal antibodies secreted by the 1-0.5G1 cells demonstrated a different pattern of insulin binding specificity than that detected in the serum of Patient #1, it is concluded that the immunological response to insulin in this patient is not monoclonal.

The monoclonal IAA secreted by the 3-0.5F10 cell line was of the IgM κ isotype and bound human, porcine and bovine insulins with equal affinity. The serum IgM of Patient #3 at the time of diagnosis also bound humulin, porcine and bovine insulins with equal affinity. Therefore, the monoclonal IAA secreted by 3-0.5F10 cells may represent a major population of IAA-secreting B cells in Patient #3's serum at the time of diagnosis with Type I DM.

Chapter 4

The V_L and V_H cDNAs of the monoclonal IAA secreted by the 3-0.5F10 cell line were cloned and sequenced. The V_L of this IAA is encoded by the A23 germ-line gene (Straubinger et al., 1988) and is part of the $V_{\kappa}II$ subfamily (Kabat et al., 1991). This finding confirms the hypothesis that A23 is a functional gene. The IAA V_L was found to

be 100% homologous in nucleotide and deduced amino acid sequence with the V_L of RFTS3, a monoclonal rheumatoid factor monoreactive towards human IgG (Pascual et al., 1990). The IAA J_L chain was found to diverge from the $J_{\kappa}4$ germ-line gene by a single silent point mutation (Ravetch et al., 1981). The portion of the IAA C_L sequenced did not diverge from the germ-line C_{κ} gene (Kabat et al., 1991). Thus, IAA V_L is germ-line gene encoded.

The IAA V_H belongs to the V_{HIII} subfamily (Kabat et al., 1991). The IAA V_H was found to be homologous to the spliced version of the V_{H26} germ-line gene (Mathyssens and Rabbitts, 1980). The somatic mutations of IAA V_H when compared to the V_{H26} germ-line gene counterpart indicate a selection for R mutations in CDRs and against R mutations in FRs. This pattern of somatic mutations in the IAA V_H can be explained by positive selection of B cells on the basis of their antigen receptors, presumably because these mutated receptors provide superior ability to bind antigen. This receptor mediated selection is consistent with the idea that IAA are the result of stimulation of B cells with antigen (most probably insulin but perhaps a cross-reacting antigen), but it is not predicted by the polyclonal activation model.

IAA V_H was found to be homologous to the V_H of: (1) a human monoclonal antibody secreted by 4G12 hybridoma cells (Kishimoto, et al., 1989) with broad reactivity to malignant tumor cells; (2) a 130-day human fetal-derived H chain cDNA transcript (Schroeder Jr. and Wang, 1990); (3) an antibody directed towards the capsular polysaccharide of *Haemophilus influenzae* type b (Adderson et al., 1991); (4) Ab18, a polyreactive monoclonal antibody isolated from EBV-PBL derived from a normal individual which also binds insulin with low affinity (Sanz et al., 1989), and (5) an anti-DNA autoantibody (Dersimonian et al., 1987). All of these antibodies have V_H which are likely encoded by the V_{H26} germ-line gene.

The IAA D_H chain was found to be 36 bp in length (12 amino acids). A core of IAA D_H was found to share identity with the DXP4 germ-line gene (Ichihara et al., 1988). The IAA J_H is likely derived from the J_H4 region human germ-line gene (Ravetch et al., 1981).

Ab2022 is a IgM κ monoreactive anti-insulin antibody from a Type I DM patient treated with heterologous insulin that binds human, porcine and bovine insulins with equal affinity (Sanz et al., 1989). The V_H of Ab2022 is homologous to the spliced version of the V_H251 germ-line gene which belongs to the V_HV subfamily (Humphries et al., 1988). The D region of A2022 has a nucleotide core sequence that shares identity with the DRL1 germ-line gene (Ichihara et al., 1988). The Ab2022 J_H is likely derived from the J_H3 germ-line gene (Ravetch et al., 1981). Although Ab2022 and IAA both bind human, porcine and bovine insulins with equal affinity, these antibodies likely recognize different epitopes because of their different V_H, D_H and J_H segments.

PCR amplification using IAA V_H and V_L specific primers followed by Southern analysis using IAA specific oligonucleotide probes was performed. Ten Type I DM patients at the time of diagnosis had B lymphocytes which were producing antibodies with IAA-homologous V_L and V_H. Thus, the usage of IAA-homologous V_L and V_H elements in antibodies is restricted in Type I DM patients at the time of diagnosis.

If the antigen-selected IAA characterized in this study are specifically restricted to Type I DM patients, the IAA-specific V_H element in combination with the A23-encoded V_L elements could be useful as a marker for Type I DM.

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Appendix A

FORM 1. INFORMED CONSENT

(for blood sampling at CHEO)

1. I, _____ (type or print patient's name),
hereby consent to give 20-25 ml of blood (children 5 years or older) or 10 ml of blood
(children under 5 years of age) for research in Type I (insulin-dependent) diabetes
mellitus.
2. I understand that the blood will be taken at the time of diagnosis and again at 3-
monthly intervals for up to one year if required (4 times in total).
3. The purpose of the study is to learn more about antibodies in the blood that are
directed against insulin.
4. As far as possible the blood will be taken at the same time that other blood tests are
needed.
5. I understand that there will be no direct benefit to me from participating in this
study.
6. I also understand that I may withdraw from the study at any time, even after signing
this form, and that this will in no way affect the regular care that I will receive.
7. Any information that is collected about me during this study will be kept
confidential, and if the results are published, I will not be identified in any way.
8. _____
Patient's name (print) Signature Date
9. _____
Parent or guardian's Signature Date
name (print)
10. _____
Witness's name (print) Signature Date
11. I have explained the nature of the study to the patient and believe he/she has
understood it.
Dr. Etienne Sochett
or
Fleur-Ange Lefebvre PhD Signature Date

Appendix B

Abbreviations:

α : alpha; as in α -cell or α -heavy chain

Abs: Absorbance

dATP: deoxyadenosine 5'-[α -thio] triphosphate

ATP: adenosine 5'-triphosphate tetra (triethylammonium) salt

β : beta; as in β -cell

BB: BioBreeding; as in BioBreeding rat

bp: base pair(s)

BSA: bovine serum albumin

C: constant, as in C-region

CDR: complementarity determining region, also known as hypervariable region

C_H: constant region of the immunoglobulin heavy chain

CHEO: Children's Hospital of Eastern Ontario

C_L: constant region of the immunoglobulin light chain

cDNA: complementary deoxyribonucleic acid

Cl: curie

CIP: calf intestinal phosphatase

CIAA: competitive insulin autoantibody assay

cm: centimeter(s)

CSA: cyclosporin A

CV: coefficient of variation

°C: degree Celsius

D: diversity, as in D-region

DEPC: diethylpyrocarbonate

D_H: diversity region of the immunoglobulin heavy chain

DNA: deoxyribonucleic acid

EBV: Epstein-Barr Virus

EBV-PBL: peripheral blood lymphocytes immortalized with Epstein-Barr Virus

ELISA: enzyme-linked immunosorbent assay

F: Farad

FBS: fetal bovine serum

Fig.: figure

FR: framework region

γ: gamma

G418: geneticin sulfate

GMP: guanosine monophosphate

h: hour

H: heavy, as in immunoglobulin H chain

HAT: hypoxanthine, aminopterin, thymidine

HGPRT: hypoxanthine-guanine phosphoribosyl transferase

HLA: Human leukocyte antigen

humulin: Human recombinant insulin (Eli Lilly)

ICA: islet cell antibody (ies)

IA: insulin antibody (ies) (antibody directed towards insulin)

IAA: insulin autoantibody (ies) (antibody directed towards endogenous insulin)

Ig: immunoglobulin

IgA: immunoglobulin of the A isotype

IgG: immunoglobulin of the G isotype

IgM: immunoglobulin of the M isotype

IgM κ: immunoglobulin of the M isotype with a kappa light chain

IL-6: interleukin-6

IMDM: Iscove's Modified Dulbecco's Medium

IMP: inosine monophosphate

IPTG: isopropyl-β-D-thiogalactoside

J: joining, as in J-region

J_H: joining region of the heavy chain

J_L: joining region of the light chain

κ: kappa, as in kappa light chain

kb: kilobase

KGB buffer: 1 M potassium glutamate, 250 mM tris-acetate, pH 7.6, 100 mM magnesium acetate, 500 µg/ml BSA, 5 mM 2-mercaptoethanol

l: liter(s)

L: light, as in immunoglobulin L chain

LB: Luria-Bertania medium; as in LB agar plates

LPS: lipopolysaccharide

M: molar

MMLV: Moloney Murine Leukemia Virus

min: minute(s)

mol: mole(s)

MoCM: monocyte conditioned medium

μ: mu; as in μ-heavy chain

μ: micro; as in µl and µg

N insertions: random nucleotide additions

NIB: nonspecific insulin binding

nm: nanometer

NOD: nonobese diabetic; as in nonobese diabetic mouse

NP-40: nonidet P-40

OPD: o-phenylene dihydrochloride

p: probability

PAGE: polyacrylamide gel electrophoresis

PBL: peripheral blood lymphocyte

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PEG: polyethylene glycol

%: percent

PMSF: phenylmethanesulfonyl fluoride

R: replacement; as in replacement mutation

RIA: radioimmunoassay

RNA: ribonucleic acid

RNAse: ribonuclease

room T: ambient temperature

RT: reverse transcriptase

s: second(s)

S: silent; as in silent mutation

SD: standard deviation of the mean

SDS: sodium dodecylsulfate

SEM: standard error of the mean

SIB: specific insulin binding

Ti: hybridization temperature

TIB: total insulin binding

TE: Tris-chloride, ethylene diamine tetraacetate

TEMED: N,N,N',N'-tetramethylethylenediamine

Type I DM: insulin-dependent diabetes mellitus

U: unit; as in unit of insulin (1U of humulin = 36 μ g)

V: volt(s)

V: variable, as in V-region

V_H: variable region of the immunoglobulin heavy chain

V_L: variable region of the immunoglobulin light chain

v/v: volume per volume

X-GAL: 5-bromo-4-chloro-3-indolyl- β -D-galactoside

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1982

PUBLICATIONS:

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