Distinct Antibody Specificities to a 64-kD Islet Cell Antigen in Type 1 Diabetes as Revealed by Trypsin Treatment

By Michael R. Christie,*§ Geeta Vohra, Patricia Champagne,* Denis Daneman,† and Terry L. Delovitch*

From the *Banting and Best Department of Medical Research and Department of Immunology, University of Toronto, Ontario, Canada M5G 1L6; the †Department of Pediatrics, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8; and the §Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford, United Kingdom OX3 9DU

Summary

Type 1 diabetes is associated with antibodies that immunoprecipitate a 64-kD islet cell membrane protein from detergent extracts of pancreatic islets. In this study we have determined whether mild trypsin treatment of islet membranes can release fragments of the antigen that bind antibodies in the serum of Type 1 diabetic patients. Partial tryptic proteolysis of [35S]methionine-labeled 64-kD antigen immunoprecipitated from detergent extracts of rat islets resulted in the formation of 50-, 40-, and 37-kD fragments. Similar sized fragments were recovered when sera from diabetic patients were employed to immunoprecipitate polypeptides solubilized by mild trypsin treatment of a particulate fraction of radio-labeled rat islets. Of 27 diabetic patients, 22 possessed antibodies to the 50-kD polypeptide and 21 to the 40- and 37-kD polypeptides. A positive association was found between 64k antibodies and antibodies to the 50-kD fragment but not between 64k antibodies and antibodies to the 40- or 37-kD fragments. Some 64k antibody negative patients possessed antibodies that efficiently immunoprecipitated the latter fragments. Serum from 25 of 27 (93%) diabetic patients immunoprecipitated at least one of the three tryptic polypeptides. One of 20 nondiabetic controls immunoprecipitated a 50-kD polypeptide and all controls were negative for antibodies to 40- and 37-kD fragments. Thus, Type 1 diabetes is associated with the presence of at least two antibody reactivities to distinct determinants of the 64-kD antigen, and some patients may possess antibodies to a cryptic epitope on the detergent-solubilized molecule. These data suggest that the detection of antibodies (present in 93% of patients) to epitopes on tryptic polypeptides of the 64-kD antigen may be of even greater diagnostic value for the onset of Type 1 diabetes than analyses of antibodies reactive with the intact 64-kD antigen.

Insulin-dependent (Type 1) diabetes is the result of the specific destruction of insulin-secreting β cells of the pancreatic islet by a mechanism in which autoimmunity is implicated (1). Studies on autoantigens associated with the disease have identified a β cell-specific protein of Mr 64,000 (64-kD antigen) that is immunoprecipitated from detergent extracts of islets by antibodies in the serum of diabetic patients (2-5). Antibodies to the 64-kD antigen (64k antibodies) are present in ~80% of newly diagnosed diabetic patients but are rarely found in healthy individuals (2-4). The antibodies appear several years before clinical onset (3), and in many patients persist for as long as β cell function is detectable (6).

In earlier studies, enrichment of amphiphilic proteins by phase separation in Triton X-114 detergent has been used as a preliminary purification step before immunoprecipitation of the 64-kD antigen (3-5). However, a significant proportion of the protein is lost into the aqueous phase by this method (5). Further characterization of the 64-kD antigen, and of immune responses to it, requires efficient procedures to extract antigen from islet cell membranes. In this study we have determined whether mild tryptic proteolysis might release fragments of antigen that retain the ability to bind 64k antibodies in patients’ sera. Proteolytic treatment to release 64-kD antigen might have the additional advantage of forming fragments that have distinct epitopes binding diabetes-associated antibodies, allowing an analysis of heterogeneity in immune responses to the protein between diabetic patients. Our approach was to identify the major fragments formed on tryptic proteolysis of purified 64-kD antigen and determine whether similar sized fragments can be recovered by immunoprecipitation from extracts prepared by trypsin treatment of islet membranes. The results demonstrate the pres-
ence, in 93% of Type 1 diabetic patients tested, of at least two distinct antibody specificities that immunoprecipitate different tryptic fragments of the 64-kD antigen that may include antibody reactivities to an epitope that may be hidden on the detergent-solubilized 64-kD antigen. These data also identify a significantly greater diagnostic potential for onset of Type 1 diabetes by the analysis of distinct antibody specificities for epitopes detectable on tryptic polypeptides of 64-kD antigen compared with those on native antigen.

Materials and Methods

Subjects. Serum from a newly diagnosed diabetic patient shown to be strongly positive for antibodies to the 64-kD antigen (standard diabetic patient serum in reference 4) was used for initial characterization of tryptic fragments of 64-kD antigen and for a positive reference serum in subsequent antibody analyses. In addition, sera were obtained within 10 d of diagnosis from 27 children (aged 6–17 yr) with Type 1 diabetes attending the Hospital for Sick Children, Toronto. Sera from 8 normal healthy adult blood donors, 6 healthy children, and 12 children (aged 5–17 yr) attending the Hospital for Sick Children with problems unrelated to diabetes served as controls.

Islet Isolation and Labeling with [35S]Methionine. Pancreatic islets from neonatal (5–7 d old) Wistar rats were prepared by the method of Brundstedt et al. (7). Islets were allowed a period of recovery of 18–36 h in Hepes-buffered RPMI 1640 medium (Gibco Laboratories, Burlington, Ontario) containing 10% newborn calf serum (Gibco) before labeling with [35S]methionine (Amersham, Oakville, Ontario) as previously described (4). Labeled cells were stored at −70°C.

Preparation of Islet Extracts. Labeled islets (1,000–2,000) were homogenized on ice by 20 passes of a motor-driven Teflon/glass homogenizer in 1 ml of 0.25 M sucrose, 10 mM Hepes (pH 7.4), 0.5 mM t-methionine, 10 mM benzamidine, 0.1 mM p-chloromercuriphenylsulphonic acid and 0.5% (wt/vol) aprotinin. The homogenate was centrifuged at 10,000 g for 30 min at 4°C. Antigens from the particulate material in the pellet were solubilized either by detergent extraction or by treatment of the pellet with trypsin as described below.

For the preparation of an amphiphilic membrane protein extract, the islet particulate fraction was extracted in 2% (wt/vol) Triton X-114 detergent and subjected to temperature-induced phase separation as previously described (4). The detergent phase of the fractionation procedure was used in subsequent immunoprecipitations.

For trypsin treatments, the particulate fraction was resuspended in 200 μl of 10 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mM methionine, 10 mM benzamidine, 0.5% aprotinin, and was centrifuged at 10,000 g for 15 min. The pellet was resuspended in 400 μl of 10 mM Hepes (pH 7.4), 150 mM NaCl and centrifuged at 10,000 g for 15 min. The pellet was then resuspended in 10 mM Hepes (pH 7.4), 150 mM NaCl, trypsin was added at the concentrations indicated in the text and the suspension was incubated for 15 min on ice. Digestion was stopped by the addition of an equal volume of 10 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM benzamidine, 0.1 mM p-chloromercuriphenylsulphonic acid and 0.5% (wt/vol) aprotinin and the suspension was centrifuged at 10,000 g for 15 min at 4°C to remove particulate material.

Immunoprecipitation of Islet Cell Antigens by Human Sera. Protease or detergent-solubilized extracts of islets were initially pre-cleared by incubation with 50 μl of normal human serum, negative for antibodies to the 64-kD antigen, for 2 h at 4°C followed by binding to 100 μl of protein A-Sepharose (Pharmacia, Baie d'Urfe, Quebec) for 45 min at 4°C. A second pre-clearance was performed with 50 μl of serum for 18 h at 4°C and 100 μl of protein A-Sepharose for 45 min. Extracts from 200 islets per sample in 100 μl were incubated with 25 μl of test serum for 5 h at 4°C and immune complexes were isolated on 50 μl of protein A-Sepharose. Immunoprecipitates were washed five times in 1 ml of 10 mM Hepes (pH 7.4), 155 mM NaCl, 0.5% Triton X-114, 10 mM benzamidine, 0.5 mg/ml BSA, and once in 1 ml of water. Elution and electrophoresis on 10% SDS-polyacrylamide gels were performed as described (3). Radioactive polypeptides immunoprecipitated were visualized by autoradiography. Antigens immunoprecipitated by individual sera were quantified by densitometric scanning of autoradiograms and standardized by comparison with immunoprecipitates from the standard human diabetic serum run in each experiment (4).

Trypsin Treatment of Immunoprecipitates. In experiments to identify tryptic fragments of the protein, immunoprecipitated 64-kD antigen was treated with trypsin before elution and electrophoresis. After washing of immunoprecipitates, the protein A-Sepharose pellets were resuspended in 100 μl of 10 mM Hepes (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, and 0.2 mg/ml trypsin and incubated for 30 min at 4°C on a rotary mixer. The protein A-Sepharose pellets were sedimented by centrifugation and washed in 1 ml of water. Matrix-bound polypeptides were eluted and analyzed by SDS-PAGE as previously described (3).

Statistical Analysis. The degree of association between antibody activities in serum samples was tested by linear regression analysis.

Results

Identification of Tryptic Fragments of the 64-kD Antigen. To analyze the major polypeptides formed on partial tryptic proteolysis of the 64-kD antigen, a standard serum from a Type 1 diabetic patient previously identified as having a high activity of 64-kD antibodies (standard diabetic serum in reference 4) was used to purify the protein from detergent extracts of neonatal rat pancreatic islets of Langerhans. The predominant protein immunoprecipitated from Triton X-114-detergent phase purified extracts of [35S]methionine-labeled rat islets by serum antibodies from this patient was the 64-kD antigen. This protein migrated as a doublet on SDS-PAGE (Fig. 1 a). Minor contaminating proteins in this immunoprecipitate were also recovered when sera from normal healthy control individuals were used and represent proteins carried nonspecifically through the immunoprecipitation procedure (8). The 64-kD antigen isolated as an immune complex bound to protein A-Sepharose was incubated with trypsin (0.2 mg/ml) for 30 min at 4°C before elution and analysis by SDS-PAGE and autoradiography. No [35S]methionine-containing polypeptides that were specific for immunoprecipitates with the standard diabetic patient's serum were released from the protein A-Sepharose matrix by the trypsin treatment (results not shown). However, subsequent elution of polypeptides remaining bound to diabetes-associated antibodies on the matrix resulted in the recovery of three major polypeptides of 50, 40, and 37 kD (Fig. 1 b). The absence of intact 64-kD antigen after trypsin digestion indicated an efficient digestion of the protein, even when bound to specific antibodies. The polypeptides detected were not seen on trypsin treat-
ment of immunoprecipitates with a control serum. Thus, these polypeptides represent the major methionine-containing fragments formed on partial tryptic proteolysis of the completed 64-kD antigen.

Serum antibodies from the same diabetic patient were used to determine whether similar sized fragments could be recovered from proteins solubilized by mild trypsin treatment of a particulate fraction of [35S]methionine-labeled rat islets. These antibodies immunoprecipitated 50-, 40-, and 37-kD polypeptides from extracts prepared at 0.5 mg/ml trypsin (Fig. 1 c) with electrophoretic mobilities identical to those generated by tryptic digestion of immunoprecipitated 64-kD antigen. Preparation of extracts at 5 mg/ml trypsin resulted in a reduced yield of the 50- and 37-kD polypeptides and additional fragments of 39 (not clear on figure) and 21 kD. Trypsin treatment can therefore solubilize fragments of membrane-bound 64-kD antigen that retain the ability to bind diabetes-associated antibodies.

**Trypsin Concentration Dependence of Release of Polypeptide Fragments.** Particulate fractions from [35S]methionine-labeled islets were treated with trypsin at concentrations ranging from 0.05–5 mg/ml and the release of the antigenic fragments was analyzed by immunoprecipitation with the standard diabetic patient’s serum. An estimate of quantities of antigen released was determined by densitometric scanning of bands on autoradiograms corresponding to the respective polypeptides. Optimum recovery of the 50-kD fragment was obtained at a trypsin concentration of 0.5 mg/ml, whereas peak recovery of the 37-kD component was at 0.1 mg/ml trypsin (Fig. 2). Higher concentrations of trypsin resulted in reduced yields of these two polypeptides. Recovery of the 40-kD fragment was relatively constant throughout the range of trypsin concentrations employed. At higher concentrations of trypsin (1–5 mg/ml), antibodies in the diabetic patient’s serum immunoprecipitated additional 39- and 21-kD polypeptide fragments.

**Antibodies to 64-kD Antigen and Tryptic Fragments in Diabetic and Control Individuals.** Analysis of the specificity of antibodies to tryptic fragments of the 64-kD antigen was extended to 27 diabetic children of recent onset and 26 nondiabetic individuals. Sera from the diabetic patients were analyzed for 64-kD antibodies by immunoprecipitation of the protein from Triton X-114 detergent phase–purified extracts of rat islets. Antibodies to the 64-kD antigen were detected in 22 (81%) of the 27 diabetic patients, a frequency consistent with that seen in earlier studies (2-4). For analysis of antibodies to tryptic fragments, sera were incubated with an extract of islets prepared using 0.5 mg/ml trypsin, immune complexes were isolated on protein A-Sepharose and analyzed by SDS-PAGE and autoradiography. The 50-, 40-, and 37-kD polypeptides were the major polypeptides immunoprecipitated by the diabetic patients’ sera (Fig. 3). There were clear differences between patients in the ability of their antibodies to recognize the individual polypeptides; patients were identified that immunoprecipitated all three tryptic fragments (lanes D–F), only the 40- and 37-kD fragments (lanes G, I, J) or solely the 50-kD polypeptide (lane H). Thus, sera
from diabetic patients contain distinct antibody specificities that bind to the different tryptic fragments. Antibodies to the 50-kD polypeptide were detected in 22 of 27 (81%) of the diabetic patients, whereas 21 of 27 (78%) were positive for antibodies to the 37- or 40-kD fragments. Since the antibody activities were distinct, 25 of 27 (93%) of the diabetic patients were found to possess antibodies that immunoprecipitate at least one of the polypeptide fragments. None of the control individuals had antibodies to the 40- or 37-kD components. Serum from one of the 26 controls, a 14-yr-old girl with juvenile rheumatoid arthritis who was persistently positive for antinuclear antibodies but negative for rheumatoid factors, possessed antibodies to the 50-kD polypeptide.

**Associations between Antibody Activities.** Densitometric scanning of bands on autoradiographs representing immunoprecipitated polypeptides was used to obtain a semiquantitative estimate of amounts of antigen immunoprecipitated by individual sera and allowed analyses of possible associations between antibody activities. Comparison of antibodies immunoprecipitating the 50-kD trypsin-solubilized polypeptide with 64k antibodies demonstrated a significant \( r = 0.692, p < 0.001 \) association between these two specificities (Fig. 4a). No significant association was found between 64k antibodies and antibodies to either the 40- or 37-kD trypsin-solubilized polypeptides (Fig. 4b and c). In particular, some patients who were negative or weakly positive for 64k antibodies were found to possess antibodies that immunoprecipitated efficiently the 37- and 40-kD trypsin-solubilized fragments.

A strong positive correlation was observed between antibodies to the 40- and 37-kD tryptic polypeptides (Fig. 4d; \( r = 0.889, p < 0.001 \)), suggesting that the antibodies may bind to epitope(s) common to both of these polypeptides. No association was found between antibodies to the 50-kD polypeptide and antibodies to either the 40- or 37-kD tryptic polypeptides (\( r = 0.124, p > 0.5 \) and \( r = 0.103, p > 0.6 \), respectively). The results demonstrate the presence of at least two distinct antibody activities in Type 1 diabetic patients that immunoprecipitate islet cell protein antigens.

10 of the diabetic patients were also analyzed for antibodies immunoprecipitating 39-kD and 21-kD fragments released on digestion of the islet particulate fraction at a trypsin concentration of 10 mg/ml. All patients positive for antibodies to the 50-kD polypeptide also immunoprecipitated the 39- and 21-kD fragments. Positive associations were found be-
between antibodies to the 50-kD polypeptide and both the 39-
kd fragment ($r = 0.869, p < 0.001$) and 21-kD fragment
($r = 0.696, p < 0.025$). No such association was found
between antibodies to the 39-kD fragment and either the 40-
kD ($r = 0.135, p > 0.7$) or 37-kD ($r = 0.215, p > 0.5$) polypeptides or between antibodies to the 21-kD fragment
and the 40-kD ($r = 0.104, p > 0.7$) or 37-kD ($r = 0.375, p >
0.2$) polypeptides. These results suggest that the 39-kD and
21-kD fragments may be the products of further tryptic pro-
teolysis of the 50-kD polypeptide.

Discussion

Previous studies have shown that the 64-kD antigen is the
only protein that is consistently and specifically immunopre-
cipitated from detergent extracts of islets by antibodies in
sera from Type 1 diabetic patients (3, 4, 8). The 64-kD an-
tigen is a relatively minor component of islets, representing
<0.02% of total islet protein, and migrates as two com-
ponents on two dimensional gel electrophoresis (8). As a con-
sequence of the low abundance, immunoprecipitates of 64-
kD antigen may be contaminated with background proteins
that represent major cellular proteins carried nonspecifically
through the immunoprecipitation procedure (8). Other pro-
teins recognized by individual sera (from both diabetic and
nondiabetic individuals) are occasionally found but these anti-
body activities appear to be specific to the individual rather
than associated with disease.

As an alternative to detergent extraction of the protein from
islet membranes, proteolytic treatment of the membranes
might efficiently release fragments of islet antigen containing
epitopes for diabetes-associated antibodies. The aim of this
study was to identify tryptic fragments of the 64-kD antigen
that are recognized by these antibodies. In our initial studies,
serum from a diabetic patient containing a high activity of
antibodies to the 64-kD antigen was used to immunoprecip-
itate the protein from detergent extracts of rat islets. The
protein immunoprecipitated by this serum migrated as a dou-
blet on the one dimensional gel system used. To identify the
major products of mild tryptic proteolysis, the 64-kD an-
tigen, isolated as an immune complex on protein A-Sepharose,
was incubated with trypsin for 30 min at 4°C. Under these
conditions, regions of a protein involved in antibody binding,
as well as regions inaccessible to protease as a result of the
conformational folding of the molecule, are protected from
proteolytic cleavage and relatively large fragments are gener-
ated (9). Trypsin treatment of immunoprecipitated 64-kD an-
tigen resulted in the recovery of three major radiolabeled polypeptides of 50, 40, and 37 kD.

In view of the rare occurrence of islet cell proteins other
than the 64-kD antigen that are immunoprecipitated by dia-
betic patients’ serum antibodies, we determined whether 64-
kD antigen-derived fragments could be recovered directly from
an islet extract prepared by treating an islet particulate frac-
tion with trypsin. Polypeptides of identical $M_r$ to those
generated by tryptic digestion of immunoprecipitated 64-kD an-
tigen were recovered from an islet extract prepared at 0.5
mg/ml trypsin. Comparison of antibody activities in individual
patients demonstrated a significant positive association be-
tween antibodies to the 64-kD antigen and antibodies to the
50-kD polypeptide. Antibodies immunoprecipitating 40- and
37-kD polypeptides were clearly a distinct activity, and were
not associated with antibodies to the detergent-solubilized
64-kD antigen or to the 50-kD tryptic fragment. An unex-
pected finding was that a number of patients who were nega-
tive or only weakly positive for 64k antibodies did possess
antibodies that immunoprecipitate efficiently the 40- and 37-
kD fragments from trypsin extracts. Antibodies in these sera
did not immunoprecipitate additional proteins from deter-
gent phase-purified extracts of islets. These results suggest
that antibodies to the 40- and 37-kD tryptic fragments bind
to epitopes that are not accessible to antibodies on the
detergent-solubilized precursor but that are exposed on pro-
teolytic cleavage. The identical pattern of bands observed after
trypsin treatment of the 64-kD antigen and after immuno-
precipitation of polypeptides from trypsin extracts of islets,
[together with the rare occurrence of diabetes-associated anti-
body responses to other islet cell proteins, is strong evidence
that the 50-, 40-, and 37-kD tryptic fragments are derived
from the 64-kD antigen, although conclusive evidence re-
quires sequence analysis of the respective components.

The poor binding of certain antibody specificities to
detergent-solubilized antigen does not necessarily imply an
inability of antibodies to bind the native antigen on $\beta$ cell
membranes. Solubilization of the protein by detergent ex-
traction may alter the conformation of the molecule to hinder
access of antibody to particular epitopes. However, Triton
detergents are considered to be “mild” detergents causing min-
imal denaturation of proteins (10). Specific antibodies recog-
izing the appropriate epitope(s) on the antigen are required
to analyze binding on membranes or intact cells. If diabetes-
associated antibodies cannot bind epitopes on native antigen,
the immune response is presumably directed mainly to a
processed form of the antigen and it is hard to envisage a
direct role of such antibodies in the destruction of pancreatic
$\beta$ cells. Uptake and processing of the 64-kD antigen or its
fragments by infiltrating antigen-presenting cells, including
B lymphocytes that bear specific receptors for antigen and
antigen fragments, and subsequent presentation to antigen-
specific T helper cells may, nevertheless, be essential for sus-
taining an immune response to the $\beta$ cell antigen and gener-
ating cytotoxic T cells that may mediate islet $\beta$ cell damage.

Extraction of the islet particulate fraction at concentra-
tions of trypsin greater than 1 mg/ml resulted in reduced
yields of the 50- and 37-kD polypeptides on subsequent im-
munoprecipitation. At the higher trypsin concentrations ad-
tional polypeptides of 39- and 21-kD were recovered. Anal-
ysis of reactivity with patients’ sera suggested that both
polypeptides might be the result of further degradation of
the 50-kD polypeptide. No additional polypeptides with
similar immunoreactivity to the 37-kD polypeptide were de-
tected. Degradation of the polypeptide presumably results
either in the loss of antibody binding or in the formation of
fragments that do not contain methionine residues and,
consequently, are undetectable by the procedures used.

It is unclear whether the various fragments are derived from
the same polypeptide chain. Although the fragments are relatively large, they clearly do not share epitopes for the distinct antibody specificities detected. The polypeptides also have the different sensitivities to trypsin degradation; the 40-kD fragment being resistant to further trypsin digestion and the 37-kD fragment being the most sensitive. The different fragments are therefore likely to be derived from different components of the 64-kD antigen.

The significance of the different antibody specificities to fragments of the 64-kD antigen by diabetic patients is also unclear. Differences in immune responses to regions of the 64-kD antigen may be determined by the expression of different MHC-linked immune response genes. In view of the strong association of Type 1 diabetes with particular MHC haplotypes (11), it will be of interest to determine whether possession of particular antibody reactivities by diabetic patients correlate with the MHC haplotype of those individuals. The results of such an analysis may provide a link between diabetes susceptibility genes and specific immune abnormalities associated with the disease.

93% of the diabetic patients analyzed were found to have circulating serum antibodies to proteolytic fragments of the 64-kD antigen. This high frequency demonstrates the importance of antibodies to the protein as a marker for ongoing β cell destruction. The development of procedures that allow early identification of individuals who are destined to develop Type 1 diabetes will be critical for immunosuppressive therapies to be effectively used in disease prevention. Antibodies immunoprecipitating detergent-solubilized 64-kD antigen have been shown to appear early in the progression to Type 1 diabetes (3), and such antibodies are present at very low frequency in normal healthy individuals (2–4). However, one of the control group, a 14-yr-old girl with juvenile rheumatoid arthritis, had antibodies that immunoprecipitate a 50-kD tryptic fragment. This finding emphasizes the need to assess the frequency of antibodies in nondiabetic populations, particularly in individuals with other autoimmune abnormalities and individuals identified from family studies to be genetically susceptible to development of diabetes, to determine the value of these antibodies in disease prediction. Such studies will require the development of more specific, high capacity assays for antibodies to the 64-kD antigen. Purification and characterization of proteolytic fragments of the 64-kD antigen should facilitate the development of these tests.

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Address correspondence to Dr. M. R. Christie, Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford, United Kingdom OX3 9DU.

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