Original Articles

Persistence of Serum Antibodies to 64,000-M_r Islet Cell Protein After Onset of Type I Diabetes

MICHAEL R. CHRISTIE, DENIS DANEMAN, PATRICIA CHAMPAGNE, AND TERRY L. DELOVITCH

Antibodies to an islet protein of 64,000 M_r (64K antibodies) were measured in 15 diabetic children who were followed prospectively for up to 3 yr after onset of type I (insulin-dependent) diabetes. Of the 15 children, 12 were positive for 64K antibodies at diagnosis. Those patients who were negative for these antibodies at onset remained negative throughout the study. Modest increases in 64K antibodies were observed in 7 patients within 1 mo of diabetes onset, concomitant with an increase in C-peptide concentrations. All antibody-positive patients were still positive at the end of the study, with no significant decrease in antibody levels relative to those at diagnosis, whereas C-peptide concentrations decreased between 3 and 24 mo after onset. Islet cell antibodies, measured by immunochemical staining on sections of rat pancreas, were detected in 9 of 15 patients at onset, whereas only 3 of 11 patients were still positive after 3 yr. In an additional group of 11 patients with diabetes for 6–7 yr, when basal and stimulated C-peptide concentrations were undetectable, 4 patients were still positive for 64K antibodies. These results demonstrate that levels of 64K antibodies persist during the first 3 yr of diabetes, despite declining β-cell function and decreased immune responses to other islet antigens, but decrease during the next 3–4 yr as the remaining functional β-cells disappear. *Diabetes* 39:653–56, 1990

The onset of type I (insulin-dependent) diabetes is associated with immunological abnormalities that implicate an autoimmune mechanism for the specific destruction of pancreatic β-cells in the disease (1). Studies to determine the nature of islet cell autoantigens associated with the disease have identified an islet cell protein of 64,000 M_r to which antibodies are found in 70–90% of newly diagnosed diabetic patients (2–4). Such antibodies (64K antibodies) are rarely detected in nondiabetic individuals, including healthy nondiabetic children, first-degree relatives of diabetic patients, and patients with other autoimmune disorders (3,4), suggesting that the immune response may be diabetes specific. The 64K antibodies have been shown to occur early in the progression to the diabetic state, appearing several years before onset and in some cases before antibody activities to other islet cell components detected by the islet cell antibody (ICA) assay (3). An analysis of the tissue specificity of the 64,000-M_r antigen revealed a predominant expression of the protein in the islet β-cells; in other endocrine or nonendocrine organs, including the non–β-cells, this protein was not detected, suggesting a specific expression in the β-cells of the pancreas (5). These characteristics are compatible with the protein being a primary target antigen of β-cell–directed autoimmunity in type I diabetes. However, because type I diabetes is associated with multiple immune abnormalities, the immune response to the protein may also be elicited secondary to the release of antigens from islets damaged by other factors.

The continued decline in circulating C-peptide concentrations after diabetes onset (6) and the fewer β-cells observed on histological examination of pancreases obtained from long-term diabetic patients (7) suggest that active β-cell destruction continues for several years after onset of the disease. Immune responses that are relevant to autoimmune β-cell destruction may be expected to persist over this time. However, several studies have demonstrated that activities of serum antibodies associated with type I diabetes frequently decrease rather rapidly, i.e., 1–2 yr after onset of the disease (for review, see ref. 8). In this study, we compared the magnitude and duration of 64K antibody and ICA responses during the first years of diabetes. Our data demonstrate that 64K-antibody activity may be detectable for as

From the Banting and Best Department of Medical Research and the Departments of Immunology and Pediatrics, University of Toronto and the Hospital for Sick Children, Toronto, Canada.

Address correspondence and reprint requests to Dr. M.R. Christie, Department of Clinical Biochemistry, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK.

Received for publication 8 August 1989 and accepted in revised form 19 January 1990.

long as 7 yr after diabetes onset, whereas ICA levels are significantly reduced by 3 yr.

RESEARCH DESIGN AND METHODS

Fifteen children with type I diabetes (aged 6–17 yr at onset) attending the Diabetes Clinic at the Hospital for Sick Children (Toronto) were followed prospectively from disease onset for 64K antibody, ICA, and C-peptide concentrations; 11 patients were followed for 3 yr, 2 for 2 yr, and 2 for 1 yr. All patients were maintained on semi- or biosynthetic human insulin. The 64K antibodies were measured in serum taken at diagnosis and 10 days and 1, 3, 6, 12, 24, and 36 mo after diabetes onset. Stimulated C-peptide concentrations were measured at all time points from 10 days to 24 mo. Titers of ICAs were measured in samples taken at diabetes onset and 36 mo later. Stimulated C-peptide and 64K-antibody concentrations were measured in an additional group of 11 patients (aged 9–16 yr) with diabetes of 6–7 yr duration.

The 64K antibodies in serum samples were measured by immunoprecipitation of the [^{35}S]methionine-labeled protein from amphiphilic membrane protein preparations of neonatal rat islets (4). Negative and positive control serums (from healthy nondiabetic individuals or a diabetic patient previously shown to be positive for 64K antibodies) were included in every experiment. The precipitated protein was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. A semiquantitative analysis of antibody levels was performed by densitometric scanning of the appropriate band on autoradiographs, comparing peak heights to those obtained with the standard positive 64K-antibody control serum (4). The interassay variation was 18%. Titers of ICAs were measured by end-point titration of serums on acetone-fixed sections of rat pancreas essentially as described (9), except that a peroxidase-conjugated second-antibody detection system was employed. End-point titers were converted to Juvenile Diabetes Foundation (JDF) units by comparison to a standard serum sample calibrated against the JDF workshop standard (10). Peak serum C-peptide concentrations in response to Sustacal (Mead-Johnson, Belleville, Canada) ingestion (7 ml/kg over 5–10 min) were measured as previously described (11).

The significance of differences between observations was tested by the Mann-Whitney *U* test. $P < 0.05$ was considered significant.

RESULTS

Immunological and metabolic parameters were measured in 15 diabetic children at disease onset and at defined time points during the first 3 yr after diagnosis. The 64K antibodies in serum from these patients were detected in 12 of 15 (80%) patients at onset, a frequency similar to that in earlier studies (2–4). The 64K antibodies did not appear at any time in those 3 children who were negative at onset. A representative autoradiogram illustrating proteins immunoprecipitated by serum samples obtained at different time points from a single patient and those precipitated by negative and positive 64K-antibody control serums is illustrated in Fig. 1. The 64K antibodies tended to persist for the first 3 yr of diabetes in those patients positive at disease onset (Figs. 1 and 2). Quantification of antibody activities by densitometric scanning of the 64,000-M_r band on autoradiograms indicated a

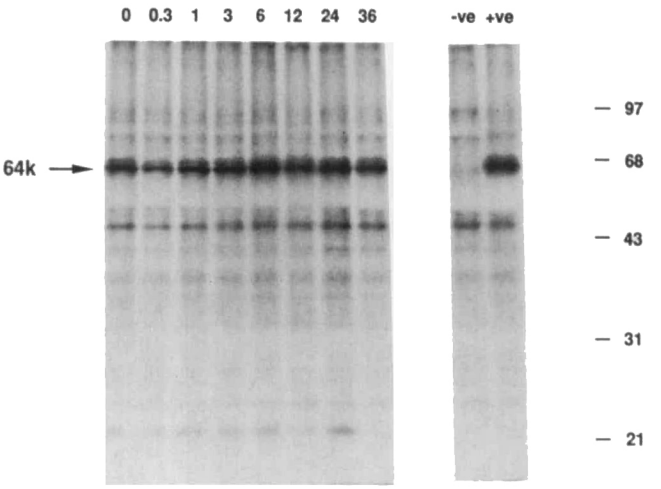

FIG. 1. Autoradiogram illustrating proteins immunoprecipitated from detergent-phase–purified extracts of [^{35}S]methionine-labeled rat islets by antibodies to islet protein of 64,000 M_r (64K antibodies) in serum samples from diabetic patient followed at time points up to 36 mo after onset of insulin-dependent diabetes (*left*). Proteins immunoprecipitated by negative and positive 64K antibody control serums tested in same experiment are shown at *right*. Mobility on gel of standard molecular-weight markers ($M_r \times 10^{-3}$) are indicated at *right margin*.

transient increase in 7 patients 1 mo after onset so that the mean antibody levels in the 64K-antibody–positive patients were significantly increased 1 mo after onset ($P < 0.05$, Mann-Whitney *U* test; Fig. 2).

Peak serum C-peptide concentrations in response to Sustacal stimulation were determined as a measure of residual β-cell function. C-peptide concentrations were significantly increased 1 mo after onset (Fig. 2). Between 3 and 24 mo, there was a steady decline in C-peptide concentrations and

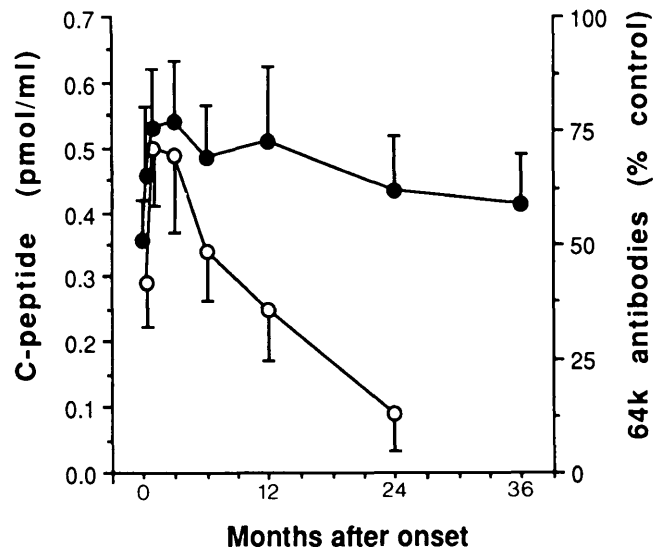

FIG. 2. Activities of antibodies to islet protein of 64,000 M_r (64K antibodies) relative to standard positive 64K-antibody control serum (●) and C-peptide concentrations in response to Sustacal ingestion (○) in 12 diabetic children who were positive for 64K antibodies at diabetes onset. At 1 mo, 64K antibodies were significantly increased relative to those at onset. C-peptide concentrations were significantly increased at 1 mo and decreased at 24 mo relative to concentrations 10 days after onset.

a significant reduction in concentrations at 24 mo compared with those at 10 days ($P < 0.05$, Mann-Whitney U test; Fig. 2).

Nine of the children analyzed at disease onset were ICA$^+$ as measured on sections of rat pancreatic tissue. No association was observed between 64K antibodies and ICAs. In contrast to the persistence of 64K-antibody activities, a significant reduction ($P < 0.01$, Mann-Whitney U test) in ICA activity was observed on analysis of samples taken 3 yr after diagnosis from 11 patients followed to this time, with only 3 patients still positive (Fig. 3).

A separate group of 11 patients with diabetes for 6–7 yr were also studied. Stimulated C-peptide concentrations were below the level of detectability of the assay (0.03 pmol/ml) in all 11 patients. The 64K antibodies were still detectable in 4 of these patients compared with 12 of 15 positive patients at onset and 9 of 11 positive patients at 3 yr (Fig. 3).

DISCUSSION

The 15 children with type I diabetes displayed the typical transient increases in C-peptide concentrations in the first 1–3 mo after diagnosis reported in earlier studies (6,12,13). It has been suggested that the partial recovery of β-cell function that follows initiation of insulin therapy is due to a relief of β-cell exhaustion caused by chronic hyperglycemia at the time of diagnosis (13). Opposing this recovery, there is likely to be continuing destruction of the remaining β-cells, because C-peptide concentrations steadily decline between 3 and 24 mo after diagnosis. In the group of patients with diabetes for 6–7 yr, stimulated C-peptide was undetectable, indicating a very low residual β-cell mass by this time.

Current evidence favors an autoimmune mechanism for β-cell destruction in type I diabetes (1). With rat pancreas as a source of antigens, autoantibodies assayed as ICAs were detected in 9 of 15 newly diagnosed diabetic patients. Antibodies immunoprecipitating a 64,000-M_r islet cell protein were detected in 12 patients. No significant association between antibodies detected by the two tests could be demonstrated, further emphasizing the heterogeneity in immune responses to islet cell antigens in the disease (8). Only one patient was negative by both tests. A significant decline in ICA titers was observed 3 yr after disease onset. Similar decreases in the titers and frequency of ICAs during the first 3 yr of diabetes have been reported in earlier studies (6,14). In contrast to these results, 64K-antibody activities tended to persist for at least the first 3 yr of diabetes. In addition, 7 patients demonstrated increases in antibody activity over a time course similar to the transient increases in C-peptide concentrations. This observation suggests that the recovery of β-cell function during the period immediately after initiation of insulin therapy may be associated with a greater availability of β-cell antigen to stimulate immune responses. Several studies have suggested that the functional state of the β-cell may influence autoimmune responses to islet cell antigens. Prophylactic insulin therapy, a treatment that may downregulate endogenous insulin secretion, has been shown to reduce the incidence of insulitis and diabetes in the spontaneously diabetic BB rat and nonobese diabetic mouse (15,16). In human type I diabetic patients, intensive insulin therapy during the first 2 wk after disease onset has been shown to prolong β-cell survival during a subsequent 1-yr period by a mechanism that may involve suppression of β-cell activity (17). In addition, the expression of the 64,000-M_r antigen (18) and β-cell antigens detected by specific monoclonal antibodies (19) has been shown to be influenced by the extracellular glucose concentration in vitro. However, other explanations for increases in 64K-antibody levels should be considered. Thus, the correction of insulin deficiency by insulin therapy may itself have a stimulatory effect on an ongoing immune response, as has been demonstrated in vitro and in vivo (8). Alternatively, the increased immune response to insulin that is initiated by the therapeutic injection of the hormone after disease onset may be associated with the release of factors capable of stimulating immune responses to other β-cell antigens.

To further define the progression of 64K antibodies after onset of diabetes, antibody levels were analyzed in a separate group of 11 patients with diabetes for between 6 and 7 yr. In this group of patients, 4 were positive. Although the antibody status of these patients at disease onset was not available, the frequency and antibody levels detected suggest that immune responses to the 64,000-M_r protein may decline in many subjects by 7 yr after onset of diabetes, when little endogenous β-cell mass remains. These results are consistent with the view that immune responses to the 64,000-M_r protein are dependent on the expression of the β-cell antigen, at least by the time clinical symptoms of the disease are apparent. However, 64K-antibody responses appear to be maintained for considerably longer than other immunological abnormalities such as ICAs, islet cell surface antibodies, and antibodies to lymphocytes, thyroid antigens, and gastric parietal cells (8). Antibodies to insulin that are continuously stimulated by the injection of exogenous insulin are an exception. Remarkably, 64K antibodies were still present in several patients 7 yr after onset of diabetes when C-peptide concentrations were no longer detectable. Immunocytochemical studies on pancreases from diabetic patients, together with measurements of circulating C-peptide concentrations, indicate that the residual β-cell mass in patients with diabetes for >1 yr is very low. Furthermore, anal-

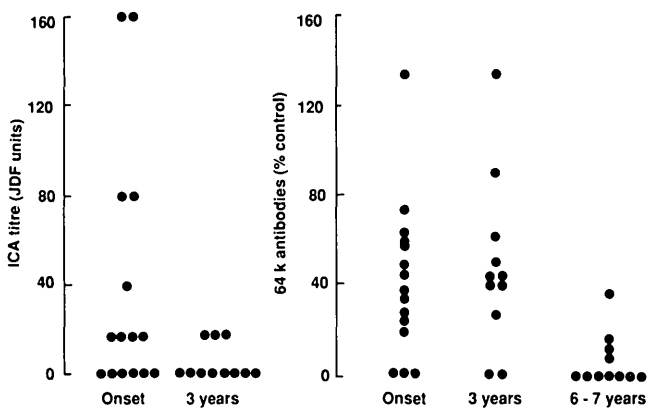

FIG. 3. Islet cell antibodies (ICAs) were measured in 15 diabetic children at diabetes onset and in 11 of these children 3 yr later (*left*). Titers of ICAs were significantly decreased at 3 yr. Antibodies to islet protein of 64,000 M_r (64K antibodies) were measured in 15 diabetic patients at onset, in 11 of these children 3 yr later, and in separate group of 11 patients with diabetes for 6–7 yr (*right*). JDF, Juvenile Diabetes Foundation.

ysis of the expression of the 64,000-M_r antigen in isolated islet cells has indicated that the protein is only a minor component of the pancreatic islet cells, representing <0.02% of the total trichloroacetic acid–precipitable radiolabeled protein (2,20). Thus, the immune system maintains a high reactivity to the 64,000-M_r antigen even at very low concentrations of the protein. Reasons for the maintenance of immune responses to the 64,000-M_r antigen, despite a decline in both endogenous β-cell function and immune responses to other islet cell antigens, are not clear. The immune response could potentially be stimulated by the 64,000-M_r antigen or proteins bearing cross-reactive epitopes to the 64,000-M_r antigen expressed in tissues other than the pancreatic β-cell. However, in an analysis of the tissue specificity in the 64,000-M_r antigen, no protein was specifically immunoprecipitated by serums from type I diabetic patients from extracts of tissues other than pancreatic β-cells (5). Furthermore, the apparent decline in 64K-antibody levels between 3 and 7 yr after diabetes onset, during a period when endogenous β-cell activity is exhausted, suggests that the stimulus for the immune response may indeed reside within the pancreatic β-cell. Immunohistochemical studies have shown that insulin-containing β-cells can be found in the pancreas of a few patients as long as 23 yr after onset (7). β-Cell antigens could potentially be present for many years after the appearance of clinical symptoms of diabetes, albeit at low concentrations. The efficient uptake and presentation of antigen released from remaining β-cells, possibly by B lymphocytes bearing specific receptors for the antigen together with the local release of cytokines by islet-infiltrating lymphocytes, may be sufficient to sustain the immune response even at low concentrations of antigen. Differences in the kinetics of disappearance of anti-islet antibodies may reflect differences in the levels of expression of the respective autoantigen or differences in mechanisms of immune stimulation.

The finding that immune responses to islet cell antigens persist for considerably longer than has previously been recognized may have relevance to transplantation therapy, particularly because HLA-identical twin-twin pancreas transplants have been shown to fail shortly after grafting as a result of disease recurrence (21). Whether immune responses to the 64,000-M_r protein are relevant to immune-mediated β-cell destruction in type I diabetes remains to be determined. However, our observation that the 64K-antibody response persists for at least as long as, if not longer than, endogenous β-cell function is detectable emphasizes the need to further define the role of immune responses to this autoantigen in the pathogenesis of the disease.

ACKNOWLEDGMENTS

M.R.C. was the recipient of a postdoctoral research fellowship from the Charles H. Best Foundation. This work was supported by a grant from the Juvenile Diabetes Foundation International.

We thank the University of Toronto Banting and Best Diabetes Centre Core Laboratory for measurements of islet cell antibody titers in the patients studied.

This work was presented at the 49th annual meeting of the American Diabetes Association, Detroit, Michigan, 1989.

REFERENCES

1. Eisenbarth GS: Type I diabetes mellitus: a chronic autoimmune disease. *N Engl J Med* 314:1360–68, 1986
2. Bækkeskov S, Nielsen JH, Marner B, Bilde T, Ludvigsson J, Lernmark Å: Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. *Nature* (Lond) 298:167–69, 1978
3. Bækkeskov S, Landin M, Kristensen JK, Srikanta S, Bruining GJ, Mandrup-Poulsen T, de Beaufort C, Soeldner JS, Eisenbarth G, Lindgren F, Sundquist G, Lernmark Å: Antibodies to a 64,000-M_r human islet cell protein precede the clinical onset of insulin-dependent diabetes. *J Clin Invest* 79:926–34, 1987
4. Christie M, Landin-Olsson M, Sundkvist G, Dahlquist G, Lernmark Å, Bækkeskov S: Antibodies to a M_r-64,000 islet cell protein in Swedish children with newly diagnosed type I (insulin-dependent) diabetes. *Diabetologia* 31:597–602, 1988
5. Christie MR, Pipeleers DG, Lernmark Å, Bækkeskov S: Cellular and subcellular localization of a M_r-64,000 protein autoantigen in insulin-dependent diabetes. *J Biol Chem* 265:376–81, 1990
6. Marner B, Agner T, Binder C, Lernmark Å, Nerup J, Mandrup-Poulsen T, Walldorff S: Increased reduction in fasting C-peptide is associated with islet cell antibodies in type I (insulin-dependent) diabetic patients. *Diabetologia* 28:875–80, 1985
7. Gepts W, De Mey J: Islet cell survival determined by morphology: an immunocytochemical study of the islets of Langerhans in juvenile diabetes mellitus. *Diabetes* 27 (Suppl. 1):251–61, 1978
8. Drell DW, Notkins AL: Multiple immunological abnormalities in patients with type I (insulin-dependent) diabetes mellitus. *Diabetologia* 30:132–43, 1987
9. Dib SA, Colman PG, Dotta F, Tautkus M, Rabizadeh A, Eisenbarth GS: Expression of cytoplasmic islet cell antigens by rat pancreas. *Diabetes* 36:982–85, 1987
10. Bonifacio E, Lernmark Å, Dawkins RL: Serum exchange and use of dilutions have improved precision of measurement of islet cell antibodies. *J Immunol Methods* 106:83–88, 1987
11. Clarson C, Daneman D, Drash AL, Becker DJ, Ehrlich RM: Residual β-cell function in children with IDDM: reproducibility of testing and factors influencing insulin secretory reserve. *Diabetes Care* 10:33–38, 1987
12. Madsbad S, Krarup T, Regeur L, Faber OK, Binder C: Insulin secretory reserve in insulin dependent diabetic patients at the time of diagnosis and first 180 days of insulin treatment. *Acta Endocrinol* 95:359–63, 1980
13. Sochett EB, Daneman D, Clarson C, Ehrlich RM: Factors affecting and patterns of residual insulin secretion during the first year of type I (insulin-dependent) diabetes mellitus in children. *Diabetologia* 30:453–59, 1987
14. Kolb H, Dannehl K, Zielasek J, Bertrams J, Hubinger A, Gries FA: Prospective analysis of islet cell antibodies in children with type I (insulin-dependent) diabetes. *Diabetologia* 31:189–94, 1988
15. Gotfredsen CF, Buschard K, Frandsen EK: Reduction of diabetes incidence of BB Wistar rats by early prophylactic insulin treatment of diabetes-prone animals. *Diabetologia* 28:933–35, 1985
16. Atkinson MA, Maclaren NK, Luchetta R, Burr I: Prophylactic insulin therapy prevents insulitis and insulin dependent diabetes (IDD) in NOD mice (Abstract). *Diabetes* 38 (Suppl. 2):87A, 1989
17. Shah SC, Malone JI, Simpson NE: A randomized trial of intensive insulin therapy in newly diagnosed insulin-dependent diabetes mellitus. *N Engl J Med* 320:550–54, 1989
18. Kämpe O, Andersson A, Björk E, Hallberg A, Karlsson FA: High-glucose stimulation of 64,000-M_r islet cell autoantigen expression. *Diabetes* 38:1326–28, 1989
19. Aaen K, Buschard K, Josefsen K, Petersen H, Horn T, Rygaard J: Antigen expression in beta cells, as detected by monoclonal antibodies, is dependent on the functional state of the cells (Abstract). *Diabetes* 38 (Suppl. 2):71A, 1989
20. Bækkeskov S, Warnock G, Christie M, Rajotte RV, Larsen PM, Fey S: Revelation of specificity of 64K autoantibodies in IDDM serums by high-resolution 2-D gel electrophoresis: unambiguous identification of 64K target antigen. *Diabetes* 38:1133–41, 1989
21. Sibley RK, Sutherland DER: Recurrent diabetes mellitus in the pancreas iso- and allograft: a light and electron microscopic and immunohistochemical analysis of 4 cases. *Lab Invest* 53:132–44, 1985