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Research Article

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Cytotoxic Antibodies to Cloned Rat Islet Cells in Serum of Patients with Diabetes Mellitus

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ABSTRACT We have found complement-dependent cytotoxic antibodies in the serum of 8 of 24 patients with insulin-dependent diabetes mellitus using a ^{51}Cr cytotoxicity assay with monolayers of cloned rat islet cells (clones RINm 5F and RINm 14B). In contrast, complement-dependent cytotoxicity with ^{51}Cr release $>24\%$ was not found with sera from 34 controls or from 5 patients with polyglandular failure without diabetes, and was present in only 1 serum out of 12 from patients with insulin-independent diabetes mellitus. The prevalence of antibodies appears to decrease with duration of insulin-dependent diabetes, and in one patient studied, cytotoxic antibodies were present at the time of diagnosis of diabetes. Cytotoxicity is independent of insulin synthesis, as evidenced by the linear correlation of cytotoxicity of sera for the insulin-producing clone RINm 5F and the somatostatin-producing clone RINm 14B. The present study identifies nonspecies-specific cytotoxic antibodies in the serum of patients with diabetes mellitus, and the assay used should facilitate studies of humoral immunity in the pathogenesis of diabetes mellitus.

INTRODUCTION

Using indirect immunofluorescent techniques on frozen (1) or Bouin's fixed sections of human pancreas (2), autoantibodies to pancreatic islet cells have been found in the sera of patients with insulin-dependent diabetes. Recently, a subset of such antibodies has been shown to fix complement (3). These antibodies decrease in prevalence as a function of the duration of diabetes, and in a number of instances have been detected before the development of disease. It appears that such antibodies react with a cytoplasmic component of islet cells (3). How such antibodies reacting with the cytoplasm may be pathogenic is not clear,

though it is possible that such antibodies reacting with damaged islet cells may enhance inflammatory processes within islets. Serum from insulin-dependent diabetic patients also contains antibodies to the surface of islet cells. Using indirect immunofluorescence, MacLaren and co-workers (4) found antibodies to the surface of a cultured human insulinoma in 34 out of 39 diabetic patients. Lernmark et al. (5), using a similar technique, have reported the occurrence of antibodies directed against the cell surface of rat islet cells. Rittenhouse and co-workers (6) have described non-species-specific factors in the sera of patients with diabetes that are cytotoxic to guinea pig islets. These and other studies have contributed to the development of the hypothesis that autoimmune phenomena are of importance in the pathogenesis of insulin-dependent diabetes mellitus.

Studies of the immunochemistry of anti-islet antibodies and isolation of islet cell antibodies have been hindered by the difficulty in obtaining large quantities of islet cells. Permanent cell lines expressing relevant cell-surface antigens would facilitate such studies. Using monoclonal antibodies directed against cell-surface antigens expressed by the RINm rat islet cell line, we have found that these cultured cells express many antigens (including differentiation antigens) that are present on normal rat pancreatic islet cells (7). Furthermore, these cells, when grown in monolayer culture, can be used in a sensitive ^{51}Cr cytotoxicity assay. In the present study we have therefore assayed serum from patients with diabetes mellitus and from controls for the presence of cytotoxic antibodies to RINm cells.

METHODS

Cell culture. RINm cells, RINm clone 14B, and RINm clone 5F were obtained from Drs. Adi Gazdar, Herbert Oie, and W. Chick (8). RINm5F cells synthesize insulin, and RINm14B cells synthesize somatostatin. These cell lines were derived from the RISL-transplantable NEDH rat islet

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cell tumor. Before use in the cytotoxicity assay, cells were cultured as monolayers in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (56°C for 30 min) in T75 Falcon flasks. Monoclonal antibody F41 6B2 was synthesized by a hybrid cell line derived from the fusion of spleen cells of a mouse immunized with RINm cells and the P3x63 mouse myeloma cell line (7). This mouse antibody is cytotoxic to RINm cells, and large amounts of monoclonal antibody have been produced by growing the F41 6B2 cell line as ascites tumors in Balb/C mice.

Cytotoxicity assay. Serum was prepared for assay by diluting in Dulbecco's phosphate-buffered saline (PBS)¹ (1:1, vol/vol) heating at 56°C for 30 min and absorbing overnight with 1 mg of rat liver powder (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) per 400 μ l of diluted serum. Cells for assay were obtained by incubation (5 min at room temperature) with 5 ml of 0.025% trypsin (Worthington Biochemical Co., Freehold, N. J.) in Dulbecco's PBS. The resulting cell suspension was centrifuged at 500 g, the supernate discarded, and the cells resuspended in 10 ml of RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum ($2-5 \times 10^6$ cells/50 ml). 0.1 ml of the cell suspension was added to each well of a 96-well microculture plate and cultured for 48–96 h before assay. 4–24 h before the assay, medium was removed from each well, and 0.1 ml RPMI medium containing 10% heat-inactivated fetal calf serum and 75,000 cpm of ⁵¹Cr (counting efficiency 70%) was added. Immediately before assay, the plate was inverted onto a utility wipe and the wells were washed three times with 150 μ l of RPMI medium. For the cytotoxicity assay, 10 μ l of RPMI medium was added to each well followed by 50 μ l of a 1:1 (vol/vol) dilution (in PBS) of test serum. After incubation with serum at 37°C for 30 min 60 μ l of a 1:2 (vol/vol) dilution of rabbit complement in PBS with Ca and Mg was added, and after 30 min at 37°C, an aliquot of the medium was harvested with a micropipette or Titertek harvesting filter (Flow Laboratories, Rockville, Md.) and counted in a gamma counter. Total ⁵¹Cr incorporation was determined by adding 50 μ l of 0.3% Triton X-100. Release in the absence of antibody (PBS rather than serum) was <10% of total ⁵¹Cr release. Specific cytotoxicity was calculated as follows:

Specific cytotoxicity

$$= \frac{{}^{51}\text{Cr release with test serum} - {}^{51}\text{Cr release with PBS}}{{}^{51}\text{Cr release with Triton} - {}^{51}\text{Cr release with PBS}}$$

Patient population. Serum was obtained from non-hospitalized healthy individuals (control), patients with a history compatible with insulin-dependent diabetes mellitus (ketoacidosis, onset of diabetes mellitus less than age 40, usually less than age 20, receiving insulin), patients with a history compatible with insulin-independent diabetes mellitus (abnormal glucose tolerance test with fasting glucose > 140 mg%, no history of ketoacidosis, treatment with diet, or oral hypoglycemic agents for > 1 yr), and patients with polyglandular failure without diabetes (primarily Addison's disease and autoimmune thyroid disease [9]).

NH₄SO₄ precipitation. A 1:1 (vol/vol) dilution of serum was incubated in a 45% saturated NH₄SO₄ solution (pH 7.2) at room temperature for 30 min; the precipitate was washed with 45% saturated NH₄SO₄ and then redissolved in PBS.

¹ Abbreviation used in this paper: PBS, phosphate-buffered saline.

Insoluble material was removed by centrifugation at 1,000 g for 15 min (4°C) and the supernate was incubated with 40% saturated NH₄SO₄ for 30 min (room temperature). The precipitate was then washed once with 40% saturated NH₄SO₄, redissolved in PBS, and then dialyzed against PBS (50:1) three times.

Incubation of serum with anti-human immunobeads. Serum diluted in PBS (1:2, vol/vol) or the redissolved NH₄SO₄ precipitate was incubated with 2.2 mg/ml of rabbit anti-human IgG immunobeads (specific for γ -chains), rabbit anti-human IgM immunobeads (specific for μ -chains), or 1.1 mg/ml of both anti-IgG anti-IgM immunobeads (Bio-Rad Laboratories, Richmond, Calif.) for 60 min at 4°C, after which the immunobeads were removed by centrifugation and the supernates tested for cytotoxicity.

RESULTS

Prevalence of cytotoxic antibodies. As illustrated in Fig. 1, strongly cytotoxic sera (⁵¹Cr release > 24%) were obtained almost exclusively from patients with insulin-dependent diabetes mellitus. Serum from one-third (8/24) of patients with insulin-dependent diabetes

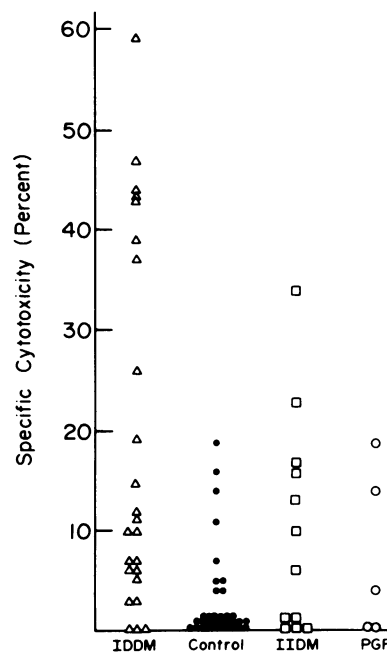


FIGURE 1 ⁵¹Cr release from RINm 5F monolayers (percent specific cytotoxicity) in the presence of rabbit complement and heat-inactivated absorbed serum from patients with insulin-dependent diabetes mellitus (Δ), normal individuals (\bullet), patients with insulin-independent diabetes (\square), and patients with polyglandular failure without diabetes mellitus (\circ). Specific cytotoxicity was calculated from ⁵¹Cr release as described in Methods. Mean cytotoxicity was significantly greater in the insulin-dependent diabetes mellitus group (19.0 ± 3.8) compared with controls (2.7 ± 0.9 , $P < 0.01$) and insulin-independent diabetes group (9.7 ± 3.1 , $P < 0.05$).

was strongly cytotoxic, whereas serum from 0 of 34 normal individuals, 0 of 5 patients with polyglandular failure without diabetes, and only 1 of 12 patients with insulin-independent diabetes released $>24\%$ of incorporated ^{51}Cr . In addition to the quantitative index of cytotoxicity provided by ^{51}Cr release, the killing of cells in monolayer culture was apparent with phase-contrast microscopy (Fig. 2). Cytotoxicity depended on incubation with both antibody and rabbit complement (Fig. 3). In the absence of complement there was essentially no cytotoxicity.

Fig. 4 plots the cytotoxicity of serum from patients with insulin-dependent diabetes mellitus vs. the duration of diabetes. All strongly cytotoxic sera were obtained from individuals within 6 yr of diagnosis of diabetes. Serum from one patient, which was strongly cytotoxic, was obtained at the time of initial diagnosis of diabetes.

Antibody characterization. Because cytotoxicity was rapid, complement dependent, and required small amounts of diluted serum, it seemed likely that the cytotoxicity of serum from patients with insulin-dependent diabetes was antibody mediated. To test this hypothesis, immunoglobulin from one cytotoxic serum was precipitated twice by ammonium sulfate precipitation (see Methods) and then redissolved in twice its original volume of PBS and dialyzed extensively. As shown in Table I, this partially purified antibody preparation was cytotoxic. Furthermore, a 90-min incubation with a combination of anti-human IgG and anti-human IgM immunobeads reduced specific cytotoxicity of this antibody preparation from 45 to 11% (Table I). The ability of incubation with immunobeads to reduce serum cytotoxicity was also tested with two cytotoxic serums and one noncytotoxic serum without prior ammonium sulfate precipitation (Table

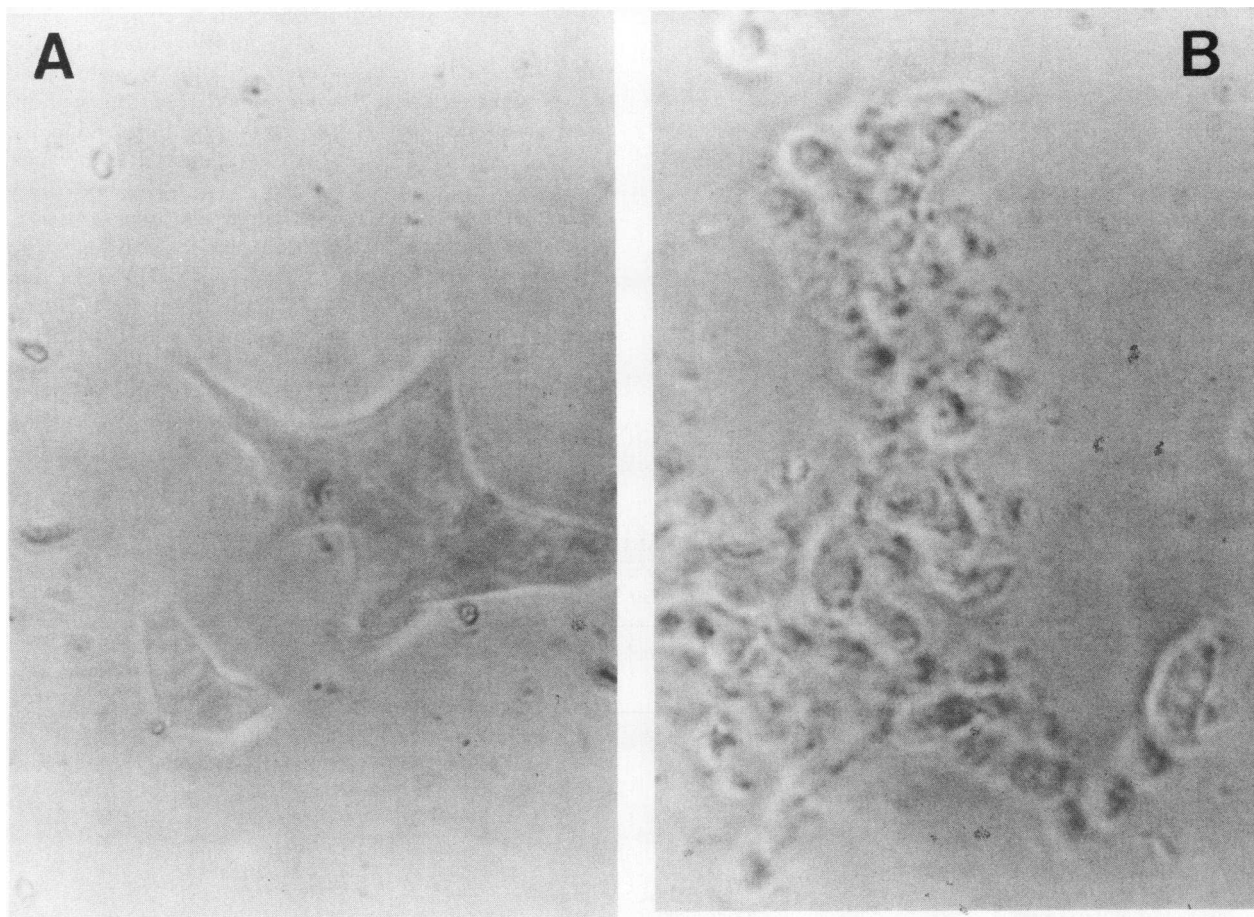


FIGURE 2 Phase-contrast photomicrograph of a monolayer of RINm5F cells at the completion of a cytotoxicity assay. Cells were incubated with a positive serum (^{51}Cr cytotoxicity assay) from a patient with insulin-dependent diabetes (panel B) or serum from a patient with insulin-dependent diabetes negative by the ^{51}Cr cytotoxicity assay (panel A).

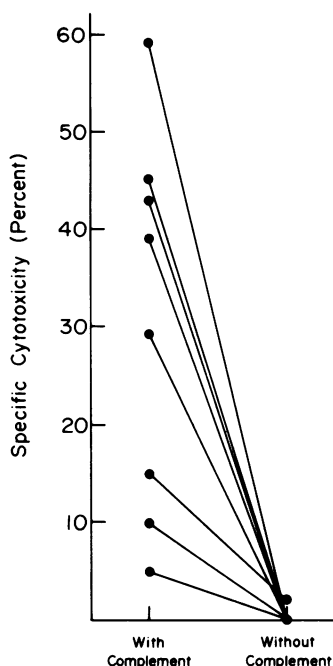


FIGURE 3 Specific cytotoxicity of serum from patients with insulin-dependent diabetes mellitus in the presence or absence of rabbit complement.

I). For patient 2, both anti-IgM and anti-IgG immunobeads reduced cytotoxicity, though anti-IgG beads were more effective. For patient 3, only anti-IgG beads reduced cytotoxicity. As expected, ^{51}Cr release of a negative serum and PBS controls were not significantly altered by prior incubation with immunobeads. The cytotoxicity of the mouse monoclonal

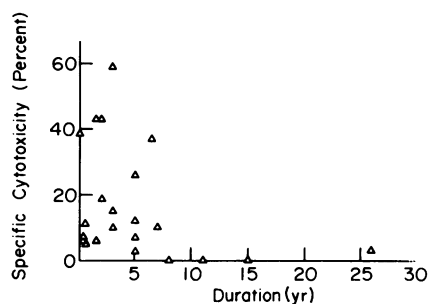


FIGURE 4 Specific cytotoxicity of serum from 22 patients with insulin-dependent diabetes mellitus vs. the duration of recognized diabetes.

antibody 6B2, which does not react with anti-human immunobeads, was unaffected by the immunobeads. Inhibition of cytotoxicity in some patients by both anti-IgG and anti-IgM immunobeads suggests that the cytotoxic antibodies are polyclonal.

Because both a somatostatin- and insulin-producing subclone of RINm cells were available, we were able to test whether the cytotoxic effect of serum was dependent on insulin synthesis by the cultured cells. Fig. 5 plots specific cytotoxicity against RINm clone 5F cells (x -axis, insulin-producing) vs. cytotoxicity against RINm clone 14B cells (somatostatin-producing). The cytotoxicities against the two cell lines were significantly correlated ($r = 0.65$, $P < 0.01$). Sera that resulted in $> 24\%$ release of ^{51}Cr from the 5F cell line released $> 20\%$ of clone 14B-incorporated ^{51}Cr . To determine whether serum from diabetic patients is cytotoxic to nonislet tumors, we tested the effect of three positive sera and two negative sera on the rat GH_3 pituitary cell line and the neuroblastoma-glioma

TABLE I
Inhibition of the Cytotoxicity of Serum from Patients with Insulin-dependent Diabetes Mellitus by Rabbit Anti-human Immunobeads

Serum	No additions	Anti-IgG (2.2 mg/ml)	Anti-IgM (2.2 mg/ml)	Anti-IgG and anti-IgM (1.1 mg/ml)
Patient 1 (NH_4SO_4 precipitation)	4,743 \pm 178*	3,958 \pm 508	3,903 \pm 124‡	1,895 \pm 213‡
Patient 2	9,486 \pm 168	2,343 \pm 823‡	6,023 \pm 247‡	5,095 \pm 868‡
Patient 3	2,176 \pm 251	1,301 \pm 225‡	2,421 \pm 469	1,811 \pm 12
Patient 4 (negative)	1,191 \pm 162	1,109 \pm 202	869 \pm 66	872 \pm 203
PBS	977 \pm 72	907 \pm 67	789 \pm 153	774 \pm 56
Mouse monoclonal antibody F41 6B2				
Diluted 1:100	6,248 \pm 1061	5,597 \pm 572	5,877 \pm 1355	5,713 \pm 559
Diluted 1:1,000	2,953 \pm 350	2,937 \pm 139	3,078 \pm 2	3,511 \pm 90

Results are the mean \pm SEM of triplicate determinations of ^{51}Cr release expressed in counts per minute.

* In this experiment, Triton X-100 released 8,367 \pm 117 cpm.

‡ $P < 0.05$ relative to no additions.

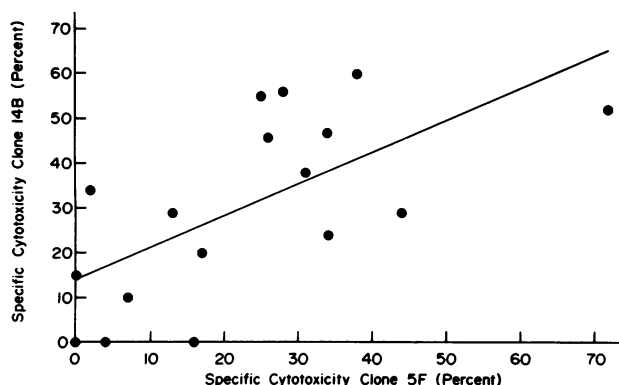


FIGURE 5 Specific cytotoxicity of individual sera from patients with insulin-dependent diabetes mellitus for RINm clone 14B (somatostatin-producing) and RINm clone 5F (insulin-producing).

hybrid cell line 108-15. None of the sera were significantly cytotoxic (specific cytotoxicity < 6% for all sera).

DISCUSSION

A major hindrance to detailed study of anti-islet antibodies in patients with insulin-dependent diabetes mellitus has been the difficulty in obtaining adequate quantities of islet cells. Several recent studies, particularly studies employing monoclonal antibody reagents, have revealed that continuous cell lines often express cell-surface differentiation antigens which are present on the normal cells from which the cell line or tumor originated (10, 11). Our own studies of monoclonal antibodies to RINm cells indicate that these cells express many antigens present on normal islet cells (7). The purpose of the present study was to determine whether serum from patients with insulin-dependent diabetes mellitus contains antibodies cytotoxic to these RINm cells. We have found that sera from one-third (8/24) of patients with insulin-dependent diabetes mellitus are strongly cytotoxic to RINm cells. We do not know whether the absence of cytotoxic antibodies in some patients with insulin-dependent diabetes reflects the fact that our assay detects only species-nonspecific antibodies. Several lines of evidence indicate that the cytotoxic factor(s) are antibodies: cytotoxicity is rapid, complement dependent, resistant to heat inactivation at 56°C for 30 min, precipitable by 40% NH_4SO_4 , nondialyzable, and most importantly, removed by anti-human immunobeads. Normal individuals, patients with polyglandular failure without diabetes, and patients with insulin-independent diabetes mellitus have a much lower prevalence of cytotoxic antibodies to RINm cells.

The duration of insulin-dependent diabetes mellitus appears to influence the prevalence of antibodies. All positive sera detected thus far have been obtained from patients with a duration of diabetes of < 6 yr.

Initial characterization of the cytotoxic antibodies to RINm cells suggests that they are polyclonal (reaction with both anti-IgM and anti-IgG immunobeads). Cytotoxicity appears to be independent of insulin synthesis by the cell lines. Both a somatostatin-(RINm clone 14B) and an insulin-producing (RINm 5F) clone of RINm cells are killed by serum from patients with insulin-dependent diabetes mellitus. Cytotoxicity of individual sera for the two cell lines is positively correlated. This lack of specificity for insulin-containing cells is similar to the antibodies described by Bottazo and co-workers (1, 3), in that all islet cells appear to react with these antibodies even though in insulin-dependent diabetes mellitus only beta cells are destroyed. The cytotoxic antibodies we are studying may similarly lack specificity for beta cells. It is possible, however, that the somatostatin-producing clone 14B, though it does not synthesize insulin, carries antigens expressed primarily on beta cells on its surface.

It is not known whether the antibodies we have detected in the serum of patients with insulin-dependent diabetes mellitus are pathogenic. The existence of these antibodies, and of anti-islet antibodies detected by other methods, may be a reflection rather than a cause of beta cell damage. Nevertheless, the cytotoxic nature of these antibodies certainly raises the possibility that they contribute to beta cell damage. In addition to direct complement-mediated cytotoxicity, such antibodies may be important for antibody-dependent cell-mediated cytotoxicity. The ready availability of these islet cell lines coupled with the speed and quantitative nature of the monolayer ^{51}Cr assay should facilitate studies to answer this question.

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