Circulating Anti-Immunoglobulin Antibodies in Recent-Onset Type I Diabetic Patients

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Human sera from 51 recent-onset insulin-dependent (type I) diabetic patients and 47 unrelated control subjects were screened for the possible presence of circulating factors reacting with several anti-pancreatic islet monoclonal antibodies (MoAb.ISL) in solid-phase radioimmunoassay methods (the original goal being the detection of anti-idiotypic islet cell antibodies and/or specific islet cell antigen–bearing immune complexes). MoAbs from the parental myeloma cell line and purified immunoglobulins (Igs) from different animal species were controls. Type I diabetic sera showed significantly increased binding to MoAb.ISL-coated wells compared with normal subjects ($P <$ .001). However, the same sera also tended to show a higher binding to the control (non–islet-related) MoAb. Sera from type I diabetic patients also reacted with horse, bovine, pig, rabbit, and goat IgG. Displacement of the binding has been obtained by $F(ab')_2$ and/or Fc fragments of IgG. Evidence has been obtained regarding a similar reaction with human IgM. All the sera were negative when tested for rheumatoid factor by nephelometry. The circulating antibodies described have been proven to be different from islet cell autoantibodies. An anti-Ig antibody is thus present in the sera of recent-onset diabetic patients and represents an additional immunological phenomenon with possible physiopathological and clinical significance. *Diabetes* 37:462–66, 1988

Antibodies to islet antigens (1–3) and to insulin antibodies (4), circulating immune complexes (5), and activated T-lymphocytes are found in the circulation before the appearance of clinical signs of diabetes mellitus (7) and during the early stages of insulin-dependent (type I) diabetes (8–10). There have been no conclusive data on islet or other antigens that are possibly released in the circulation during the tissue-damaging processes or on anti-idiotypic islet cell antibodies possibly induced in the early stages of type I diabetes. However, a library of monoclonal antibodies (MoAbs) that work against human pancreatic islet antigens has recently become available (11,12), and it will be a powerful tool for conducting systematic investigations in these research areas in diabetes mellitus.

We studied type I diabetic patients at an early stage of the disease in search of anti-idiotypic islet cell antibodies and/or specific islet cell antigen–bearing immune complexes. For this purpose, a methodological approach has been developed with anti-human islet MoAbs. As counter-evidence of the specificity of the reaction, non–islet-related MoAbs and immunoglobulins (Igs) from different animal species and from humans were the controls.

MATERIALS AND METHODS

PATIENTS

Sera from 51 type I diabetic patients and 47 normal controls were searched for the presence of factors binding to a pool of anti-islet MoAbs. Of the diabetic patients, 34 were newly diagnosed as diabetic (≤3 days), and 17 were diagnosed within the previous year. Their mean age at the time of the study was 21.7 ± 11.3 yr. The male-to-female ratio was 1.7:1. Normal controls had a mean age of 31.5 ± 9.6 yr and a male-to-female ratio of 1.7:1. In the subsequent experiments to characterize the circulating immune factors detected, many sera from these patients were tested individually for possible different binding to different targets. In one of these experiments, two sera from relatives of type I diabetic patients with severely decreased insulin response, who subsequently developed overt diabetes, were included as well (see RESULTS). After blood collection, sera were kept frozen at −20°C until required for testing.

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TABLE 1
Mouse monoclonal antibodies staining all the different subtypes of endocrine pancreas cells and control MoAb

Monoclonal antibody	Antigen source	Ig class
HISL 1	Human islets	IgG1
HISL 4	Human islets	IgM
HISL 5	Human islets	IgG1
HISL 8	Human islets	IgG1
HISL 14	Human islets	IgG1
HISL 19	Human islets	IgM
HISL 22	Human islets	IgM
4F2*	Malignant cell lines	IgG2a
A2B5	Neurons	IgM
3G5	Neurons	IgM
P3X63*	Parental myeloma cell line	IgG1

*Islets were not stained.

SPECIFIC REAGENTS

The mouse anti-islet MoAbs, reacting with human islets and staining all cell subtypes of endocrine pancreas, are listed in Table 1 (13–16). All the MoAbs were precipitated with ammonium sulfate, and the concentration was calculated by the optical-density method. Non–islet-related MoAbs, obtained from the parental myeloma cell line (P3X63), were the controls after precipitation.

Goat anti-mouse Ig antibodies (4100 series, lot no. 53-19-02; Tago, Burlingame, CA) were isolated from serum by affinity chromatography with the appropriate Sepharose-bound antigen. The final purified antibody preparation showed a reactivity to human sera of <1.3%. The antibody was absorbed on immunobeads coated with human Ig to further minimize cross-reactivity. Animal Igs comprised horse, rabbit, bovine, goat, and pig (I 4631, I 5006, I 5506, I 5256, I 4381, respectively; Sigma, St. Louis, MO) IgG fractions. They were purified by fractionation and column chromatography and shown to be free of other serum proteins by immunoelectrophoresis and Ouchterlony double diffusion.

Goat anti-human Ig antibodies (4100 series, lot 03.14.06, Tago) were purified by affinity chromatography, and their reactivities to animal sera were as follows (%): bovine, <0.2; rabbit <2.5; horse, <6.4; mouse, <0.4; and human, 100. Antibody titers, as determined by the reverse Mancini method, were (mg/ml): anti-IgG, 0.86; anti-IgA, 1.10; and anti-IgM, 0.67. B6 is a human IgM produced by fusing circulating lymphocytes from a child with type I diabetes with a human myeloma cell line (17). The antibody was used after ammonium sulfate precipitation.

Goat anti-human IgG antibodies (4100 series, lot 00-46-02, Tago) were purified by affinity chromatography with minimal cross-reactivity with human IgM. $F(ab')_2$ and Fc fragments of goat immunoglobulins. (Cappel-Cooper, Malvern, PA) were purified chromatographically from pooled goat serum. Hydrophilic polyacrylamide beads were coated with goat Ig (Bio-Rad, Richmond, CA) with covalent bonds.

EXPERIMENT PROTOCOLS

The following experiments were performed in sequence.

Binding of circulating immune factors to pooled anti-islet MoAbs. Anti-islet MoAbs (200 μl at concn 10 μg/ml) were left for 1 h at room temperature and overnight at 4°C to coat highly adsorbent polystyrene microwells (Immulon, Dynatech, Alexandria, VA) precoated with goat anti-mouse Ig to increase the binding of MoAbs to plastic wells. Two hundred microliters of a 1:20 dilution of the serum sample to be tested were then incubated for 3 h at room temperature after being washed three times in phosphate-buffered saline (PBS) with Tween 20. After regular washings, 200 μl of ^{125}I-labeled goat anti-human Ig, at 20,000 counts/min in PBS with 1% of bovine serum albumin (BSA), was incubated for 2 h at room temperature. After washing, radioactivity was counted. Controls for aspecific binding were included in all the experiments. Each of the variables (anti-mouse antibody, MoAb, and serum) was substituted with 1% BSA in PBS in parallel experiments.

Binding to individual MoAbs. Each MoAb listed in Table 1 was left to coat microwell tubes, with the procedure described above, in a series of parallel experiments on pathologic and normal serum samples.

Binding to Ig of different animal species. Different animal Igs (horse, rabbit, bovine, goat, and pig), at a concentration of 10 μg/ml, coated plastic tubes. After washing, a 1:20 dilution of serum was added and incubated for 3 h at room temperature. The subsequent steps of the solid-phase assay were performed as described above.

Binding to human IgM. Human IgM, i.e., human MoAb B6, coated the microwell tubes. Serum was added according to the procedure described previously. ^{125}I-labeled goat anti-human IgG was then added, and radioactivity was counted after washing.

Displacement of the binding by cold IgG fragments. To check the specificity of the binding of circulating factors to Ig-coated wells, aliquots of serum to be tested were diluted 1:20 with PBS, containing either Fc or $F(ab')_2$ fragments of goat IgG at a concentration of 10 μg/ml, and left to incubate for 2 h at room temperature. The mixtures were then added to goat Ig-coated tubes. Further steps were performed according to the method described above. An aliquot of the same serum was mixed with BSA at a concentration of 10 μg/ml, instead of cold IgG fragments, and assayed in parallel as a control.

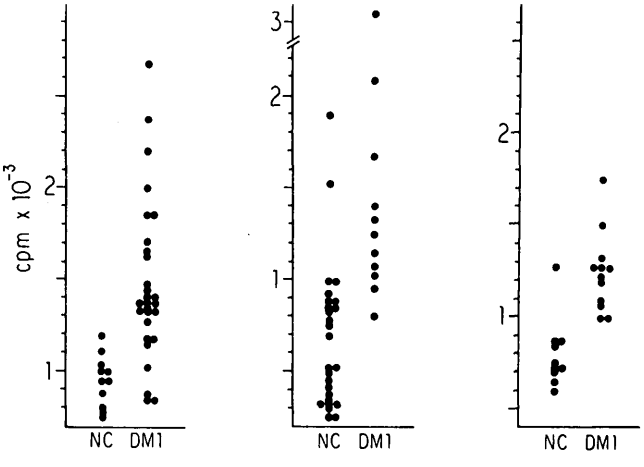

FIG. 1. Three consecutive experiments, performed according to 1st experiment protocol, indicating binding of serum factors [in counts/min (cpm) on *y*-axis] to islet monoclonal antibody–coated tubes. Total of 51 different sera from type I diabetic patients (DM1) and 47 from normal controls (NC) were tested.

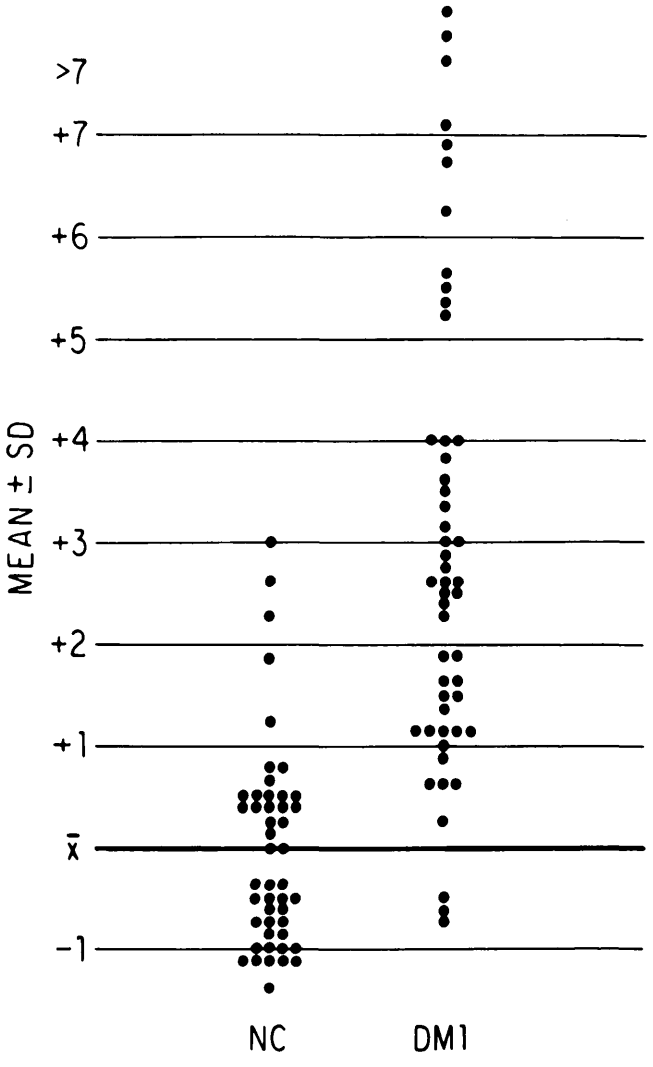

FIG. 2. Results of experiments in Fig. 1 recalculated as means ± SD of normal values.

Adsorption of circulating immune factors on Ig-coated beads. Goat Ig-coated polyacrylamide beads, preincubated with normal serum to avoid aspecific binding, were mixed with the serum to be tested and incubated for 3 h at room temperature. A first supernatant was obtained after spinning for 20 min at 2500 rpm at 4°C. HCl-glycine buffer, pH 2.8, was added to the precipitate and mixed for 10 min. A second supernatant was obtained after spinning under the same conditions after the pH was neutralized. Anti-Ig antibodies and islet cell antibodies (ICAs) were measured in both the supernatants.

OTHER METHODS

Antibodies were labeled with ^{125}I according to the chloramine-T method (18). The ^{125}I-to-protein molecular ratio was 1:1.

The presence of rheumatoid factor, as detected by laser nephelometry, was assayed with a commercially available diagnostic kit (Rheumatoid Factor Reagent, Beckman, Brea, CA).

ICAs were measured by indirect immunofluorescence on cryostat sections of snap-frozen fresh human pancreatic tissue.

The Mann-Whitney test was used for the statistical evaluation of results.

RESULTS

Binding of circulating immune factors to pooled anti–islet MoAbs. Sera from type I diabetic patients showed a higher binding to MoAb.ISL-coated tubes than normal controls ($P < .001$; Fig. 1). The distribution of values for normal individuals was positively skewed. When the results obtained were calculated as means ± SD of normal values, >80% were above the 90th percentile of normal control levels (Fig. 2).

Binding to individual MoAbs. Eight serum samples (4 newly diagnosed diabetic subjects, 2 relatives of type I diabetic patients with decreased insulin response who subsequently developed diabetes, and 2 normal subjects) were tested against the various MoAbs coating the tubes. There was no significant difference in the binding of circulating human factors among the different anti-islet MoAbs, and the rank order of binding of individual sera reacting with them was similar (Fig. 3). Subject 6 had an insulin response <1st percentile and developed diabetes after a few months. Surprisingly, almost the same binding was shown by the non–islet-related MoAb derived from the parental myeloma cell line (P3X63).

Binding to animal Ig. Eleven serum samples (6 from the 51 type I diabetic patients and 5 from the 47 normal controls) were tested with horse, rabbit, bovine, goat, and pig Ig and again against MoAb.ISL with or without the precoating of tubes with goat anti-mouse Ig (Fig. 4). Diabetic sera tended to show similar binding to Ig in the solid phase regardless of the animal species of Ig.

Binding to human IgM. To investigate whether the circulating Igs were reacting with human Ig as well, the binding of IgG from six diabetic sera and five normal sera (the same used in the above experiment) to human IgM was evaluated. Four of six of the diabetic sera showed a higher binding than that of normal subjects.

Displacement of binding by cold IgG fragments. $F(ab')_2$ and Fc fragments of goat IgG were added to different aliquots of sera from 8 diabetic subjects known to be positive for anti-Ig antibodies and to aliquots of sera from 8 normal subjects; the sera were chosen from among the 51 diabetic and 47 control subjects. A significant displacement of binding (>30% when corrected for variations in normal subjects) was found in all the diabetic subjects with either Fc or $F(ab')_2$ fragments. In 4 of 8 patients, it was obtained with $F(ab')_2$ fragments; in 7 of 8, with Fc fragments.

Adsorption of binding by Ig-coated beads. Five diabetic sera (from those included in the study), positive for both anti-Ig antibodies and ICAs, were adsorbed with Ig-coated beads. In all five samples the first supernatant was positive for ICAs alone, whereas the second supernatant was positive for only the anti-Ig antibody.

Rheumatoid factor. Eleven sera (6 from diabetic patients and 5 from normal subjects, the same as were used in the experiments with individual MoAbs and with human MoAb B6) were screened for rheumatoid factor. None of the sera tested positive with the technique used.

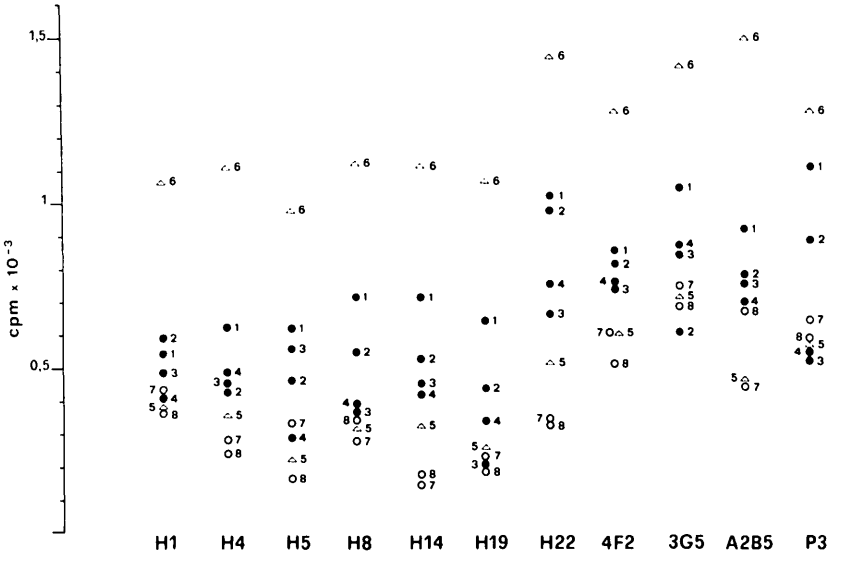

FIG. 3. Binding of serum factors to individual monoclonal antibodies listed in Table 1. H, human islets; cpm, counts per minute. ●, Type I diabetic patients; ○, normal controls; △, relatives of type I diabetic subjects with decreased insulin response shortly before they developed overt diabetes. Subjects were numbered 1–8.

DISCUSSION

The immunological basis for the islet β-cell destruction occurring in the most common form of type I diabetes is being increasingly substantiated. The effector humoral (specific islet cell autoantibodies) and cellular (self-reactive T-lymphocytes, macrophages, and other mononuclear cells) immune mechanisms are associated with an underlying genetic susceptibility governed by HLA-related immune-response genes (and possibly other non-HLA diabetogenic genes) (19,20).

Our study adds another facet to understanding the abnormal immune response and regulation of type I diabetes. In the sera of recent-onset type I diabetic patients there are circulating factors binding anti-islet MoAbs, other MoAbs, Igs from various animal species, and human Igs. These findings can be explained by the presence of anti-Ig antibodies in the circulation of type I diabetic patients. These anti-Ig antibodies are directed against the Fc and/or the $F(ab')_2$ fragments of the target Ig and differ from those autoantibodies reacting with islet cell antigens (including those known as ICA).

Anti-Ig antibodies are conceptually similar to those found in other human disorders, e.g., rheumatoid arthritis (21), but are not detected by the common routine methods used to measure the rheumatoid factor. On the other hand, as shown by this study, they are not a mere curiosity, i.e., an antibody reacting with antigen from other species, but are capable of reacting with human Ig, and therefore the in vitro binding described here most likely occurs in vivo as well.

Igs directed against other Igs may well participate in the complex immunologic interactions that take place in type I diabetes, perhaps modulating the physiopathological events with clinical significance (22).

Our findings, although giving evidence of the presence of anti-Ig antibodies, allow us only to speculate about their significance. The increased prevalence and elevated levels

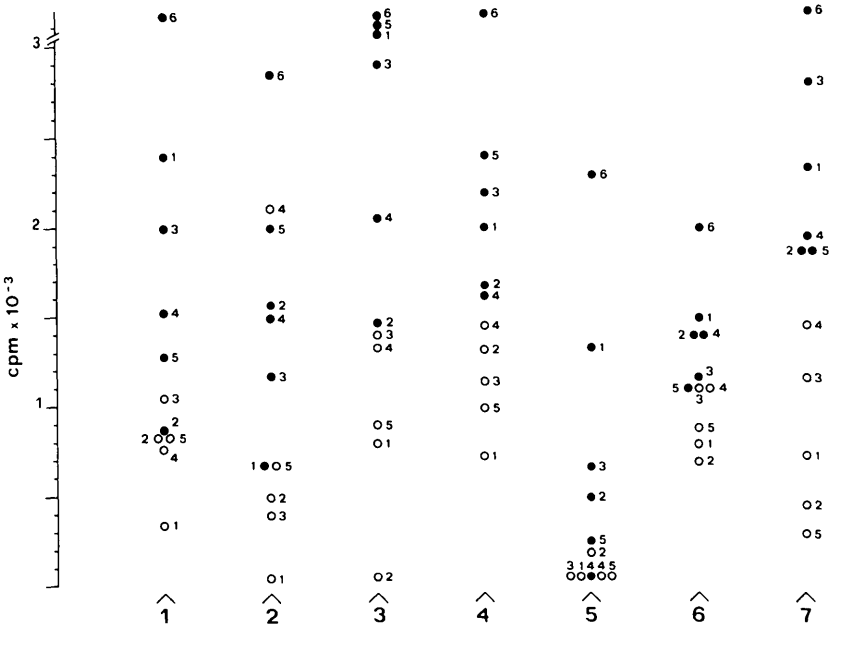

FIG. 4. Binding of serum factors to animal immunoglobulins. *Column 1* illustrates results obtained with horse IgG, *2* with rabbit, *3* with bovine, *4* with goat, *5* with porcine, *6* with islet monoclonal antibodies, and *7* with goat anti-mouse antibody followed by islet monoclonal antibodies. ●, Type I diabetic patients (1–6); ○, normal controls (1–5).

of anti-Ig antibodies in type I diabetic subjects compared with normal controls may reflect the heightened immune responsiveness and high-responder status that underlie the basic autoimmune diathesis of type I diabetes and related disorders. In this context, it is important to evaluate whether anti-Ig antibodies segregate with certain HLA alleles/haplotypes or Ig heavy-chain allotypes (Gm, Km) in families with both type I diabetic and normal members. Other examples of such non–organ-specific autoantibodies reported to be increased in type I diabetes include antibodies to DNA, lymphocytes, and albumin (23–25).

In this study the anti-Ig antibodies were found to be non–species specific and to have a broad reactivity with many Ig classes and subclasses. Thus, the original hypotheses that immune complexes comprised islet antigens or that anti-idiotypic antibodies were detectable by anti-islet MoAbs were not confirmed. Because these anti-Ig antibodies may interfere in the detection of anti-idiotypic antibody in the method used, the presence of idiotypic/anti-idiotypic complexes cross-reacting with other species cannot be definitively excluded.

The antibodies described here may react with epitopes shared by different Igs, e.g., carbohydrate components of the molecule. Because these glycoconjugates are the antigenic determinants likely to be recognized by the spontaneously occurring anti-cytoplasmic antibodies in type I diabetes (26), it is possible that antibodies specifically directed toward pancreatic antigens and present in diabetes may cross-react with carbohydrate-sharing Igs.

Carefully controlled, specifically designed clinical and epidemiological studies are necessary to address questions relating to the possible pathological significance of these anti-Ig antibodies both in the early stages of the disease and, theoretically, in long-term diabetic angiopathic complications.

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