Clin Exp Immunol 1998; **111**:173–180

β_2-Glycoprotein I-dependent and -independent anticardiolipin antibody in non-obese diabetic (NOD) mice

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(Accepted for publication 4 October 1997)

SUMMARY

In the present study we investigate whether or not anticardiolipin antibody (aCL) is produced in NOD mice, which is a representative animal model of insulin-dependent diabetes mellitus (IDDM). We found that IgG class aCL appeared in 6·9% of non-diabetic NOD mice at weeks 5–15. The rates increased with age to 31·6% at weeks 16–25 and 71·9% at weeks 26–35. In addition, the titre and incidence of aCL were higher in diabetic mice than in non-diabetic mice. It was also found that aCL in NOD mice involved β_2-glycoprotein I (β_2-GPI)-dependent and -independent aCL, when β_2-GPI was added to the aCL assay system. The IgG subclass of both β_2-GPI-dependent and -independent aCL belonged exclusively to IgG2a. In addition, immunohistochemical studies revealed the predominant accumulation of IgG2a- or IgG3-positive B lymphocytes within insulitis. These results suggest that the autoimmunity in NOD mice may thus be associated with Th1 predominant immunological response. In conclusion, aCL with multiple antigenic specificity were produced in NOD mice along with the development of insulitis and diabetes. NOD mice should thus be added to the list of animal models possessing antiphospholipid antibody.

Keywords cardiolipin antiphospholipid antibodies insulin-dependent diabetes mellitus NOD mouse

INTRODUCTION

NOD mice show a characteristic progressive autoimmune response which specifically and selectively destroys pancreatic β-cells [1,2]. Autoimmunity to either the β-cell or β-cell products in NOD mice involves both the cellular [3,4] and humoral [5,6] immune response. Cell-mediated immunity is demonstrated by pathognomonic histological lesions, also termed insulitis, in which inflammatory/immune cell infiltrates are observed within the pancreatic islets [7]. Adoptive transfer experiments using NOD mice have firmly established that autoreactive T cells play a primary role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) [8]. The development of humoral immunity against the islets is also shown as the appearance of autoantibodies to β-cell membranes, β-cell components (anti-64-kD and/or anti-glutamic acid decarboxylase (GAD) autoantibody), or β-cell secretory products (anti-insulin autoantibody (IAA) and anti-carboxypeptidase H autoantibody) [5,9–12].

Anticardiolipin antibodies (aCL) are frequently detected in patients with either autoimmune disorders or infectious diseases [13–15]. Some of these antibodies are known to be associated with thromboembolic manifestations of cerebral or myocardial infarction, deep venous thrombosis, and intra-uterine fetal loss due to placental infarction [13–15]. In addition, the aCL found in patients with infectious disorders, such as syphilis, malaria, hepatitis A, tuberculosis, or infectious mononucleosis, did not either affect coagulation activity or induce thromboembolism [14–16]. The aCL in infectious diseases react with solid-phase and fluid-phase CL in the absence of β_2-glycoprotein I (β_2-GPI-independent aCL), while aCL in patients with autoimmune disorders, such as systemic lupus erythematosus (SLE) or other connective tissue diseases, do not directly react with the CL structure, since aCL binding requires the presence of β_2-GPI (β_2-GPI-dependent aCL), and thus these antibodies can be distinguished from aCL in infectious diseases [17–19].

We previously reported that aCL were transiently produced in AKR/J mice, which are used as a model animal of IDDM and demonstrate streptozocin-induced diabetes [20]. In addition, Lorini *et al*. reported that aCL were found in 24% of all IDDM patients [21]. However, these reports did not distinguish between β_2-GPI-dependent and β_2-GPI-independent aCL. In this study, we

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investigated whether or not NOD mice can produce aCL. Having found that aCL were produced in NOD mice, we thus also attempted to differentiate β_2-GPI-dependent from β_2-GPI-independent aCL in NOD mice.

MATERIALS AND METHODS

Animals

Our female NOD mouse colony was produced from a breeding stock obtained from Clea Japan (Tokyo, Japan). All animals were maintained and fed *ad libitum* at the Kyushu University Animal Centre. Female ICR mice, which is the strain from which NOD mice originated, served as the control and were also purchased from Clea Japan. Sera were collected from the animals at from 5 to 35 weeks of age and then were stored at −80°C. The female NOD mice and ICR mice were split into three groups according to age: group I, 5–15 weeks, group II, 16–25 weeks and group III, 26–35 weeks. In our colony, the NOD mice began to develop diabetes after 16 weeks of age and the diabetes became increasingly severe thereafter. Diabetes mellitus was diagnosed when the plasma glucose levels exceeded 14 mM.

Reagents

Cardiolipin suspended in ethanol was obtained from Sigma Chemical Co. (St Louis, MO). Human β_2-GPI was from the Yamasa Shoyu Co. Ltd. (Tokyo, Japan). Human recombinant insulin was from Eli Lilly and Co. (Indianapolis, IN). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (lot no. 91104039), anti-IgG1 (lot no. 90603261), anti-IgG2a (lot no. 80901829), anti-IgG2b (lot no. 90803434), anti-IgG3 (lot no.90803448) were from Zymed Labs Inc. (San Francisco, CA). In addition, rabbit anti-human β_2-GPI antibody was from Cedarlane Laboratories Ltd (Ontario, Canada), and the HRP-conjugated anti-rabbit IgG (lot no. AP158P) was purchased from Chemicon International Inc. (San Francisco, CA).

Microtitre plates

Ninety-six-well microtitre plain polystyrene plates (Falcon 3915) were obtained from Becton Dickinson (Oxnard, CA), while carboxylated (MS-3796F), irradiated (MS-3596F), and plain (MS-3496F) polystyrene plates were purchased from the Sumitomo Bakelite Co. Ltd. (Tokyo, Japan).

ELISA

Anticardiolipin antibody assay. Anticardiolipin antibodies were detected by a standard ELISA test as previously described [22]. In brief, 2 μg of cardiolipin per well in 50 μl ethanol were dried on plain plates (Falcon 3915). PBS with 10% fetal calf serum (FCS) was used as the blocking agent. After blocking, the plates were washed with PBS containing 0·05% Tween 20 (PBS–T) and 50-μl aliquots of test samples at 1:80 dilution in PBS–T were added to each of the duplicate wells and incubated for 1 h at room temperature. To the plates were added HRP-conjugated rabbit anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3, diluted 1:1000 in PBS–T, and these were incubated for 1 h at room temperature. The plates were treated with 0·1 M citrate buffer pH 5, containing 0·002% H_2O_2 and 0·04% *o*-phenylenediamine (Sigma). The reaction was allowed to take place at room temperature in a dark chamber for 10 min and then was stopped by adding 50-μl aliquots of 4 N H_2SO_4 to each well. The absorbance in each well was measured at 492 nm using an Easy Reader (Laboscience, Tokyo, Japan). Test samples were reported as having raised aCL levels when their values exceeded that of the mean + 3 s.d. of the control ICR mice.

β_2-GPI-dependent and -independent aCL assay. We performed this ELISA test using human β_2-GPI, because it has been reported that the amino acid sequence deduced from the nucleotide sequence of mouse β_2-GPI revealed a 76·1% homology with that of human β_2-GPI [23].

The sample consisted of sera of NOD mice, and aCL-negative sera of ICR mice from 26 to 35 weeks old using the above standard ELISA test. The ELISA test used was a modification of the method of Matsuura *et al*. [24]. In brief, a CL-coated ELISA plate (Falcon 3915) was blocked with 50 μl of 10 mM HEPES, 150 mM NaCl containing 0·3% bovine serum albumin (HEPES–BSA) for 1 h at room temperature.

After washing with PBS–T, wells were incubated with 50 μl of β_2-GPI (30 μg/ml) in HEPES–BSA for 10 min at room temperature to measure the β_2-GPI-dependent aCL, or 50 μl of HEPES–BSA to measure the β_2-GPI-independent aCL. The wells were then incubated with 50 μl of diluted sera at 1:80 for 30 min at room temperature. After washing with PBS–T, wells were incubated with 50 μl of HRP-conjugated rabbit anti-mouse IgG for 30 min at room temperature. The colour was developed and the optical density (OD) measured as described above. According as the procedure of some authors [25,26] with slight modifications, we judged β_2-GPI-independent aCL and -dependent aCL to be positive when the OD of the test sample exceeded the mean OD + 3 s.d. of the seven control ICR mice.

Anti-β_2-GPI antibody assay. Matuura and coworkers reported that β_2-GPI-dependent aCL directly binds to the modified form of β_2-GPI immobilized on polystyrene plate oxidized to generate C-O and C = O moieties by irradiation, but not to the native forms of β_2-GPI immobilized on a plain polystyrene plate in SLE patients [27], thus suggesting that the carboxylation of the plates is required to detect β_2-GPI-dependent aCL in the ELISA test. In this study, we used carboxylated plates which are produced by oxidation to generate C = O moieties on the surface (Sumitomo Bakelite Co.) instead of irradiated polystyrene plates. We confirmed that the amounts of β_2-GPI bound to the carboxylated and that to the plain plates were the same when 10 μg/ml of β_2-GPI were added to the plates (data not shown), and we thus decided to use the plain plates as controls. The ELISA test for the antibodies directed to β_2-GPI essentially followed the procedure of Matsuura *et al*., with slight modifications [27]. The microtitre plain (MS-3496F) or carboxylated (MS-3796F) plates were coated with 50 μl of β_2-GPI (10 μg/ml dissolved in 10 mM HEPES, 150 mM NaCl, pH 7·4) overnight at 4°C. After incubation, the β_2-GPI-coated wells were washed three times with 200 μl of PBS–T, and then were incubated with 200 μl of HEPES buffer containing 3% skim milk (Difco Labs, Detroit, MI) for 1 h at room temperature. After washing in the same manner, the wells were incubated with 50 μl of diluted sera for 1 h at room temperature. The wells were again washed and incubated with 50 μl of HRP-conjugated rabbit anti-mouse IgG diluted 1:1000 for 1 h at room temperature. Colour was developed and the OD was measured as described above.

Inhibition experiments

Inhibiting the binding of β_2-GPI-dependent aCL by fluid-phase β_2-GPI with or without cardiolipin. To measure the inhibition of β_2-GPI-dependent aCL by fluid-phase β_2-GPI or β_2-GPI–CL complex, the sera at a dilution giving 50% of maximal binding to β_2-GPI were preincubated with soluble β_2-GPI at various

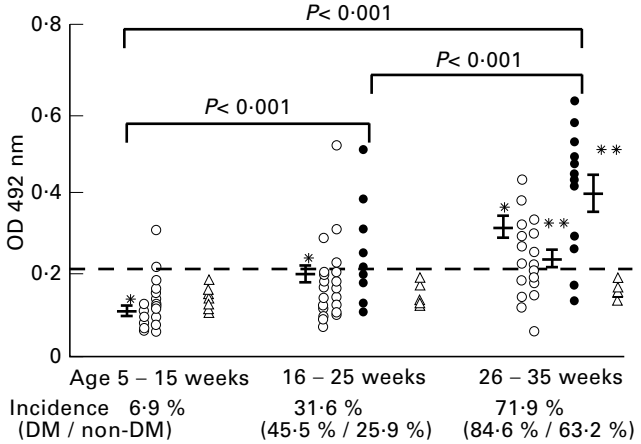

Fig. 1. Anticardiolipin antibody (aCL) in non-diabetic or diabetic NOD mice, and ICR mice. Each point indicates the absorbance of an individual serum: non-diabetic NOD mice (○); diabetic NOD mice (●); ICR mice (Δ). Groups are as indicated in Materials and Methods. Broken lines represent mean ± 3 s.d. of ICR mouse results. Bars represent means ± s.e.m. *The statistical evaluation applies to comparisons between NOD group I and groups II and III, respectively. **Diabetic NOD mice *versus* non-diabetic NOD mice in group III: $P < 0{\cdot}001$. Results are as percentage aCL-positive.

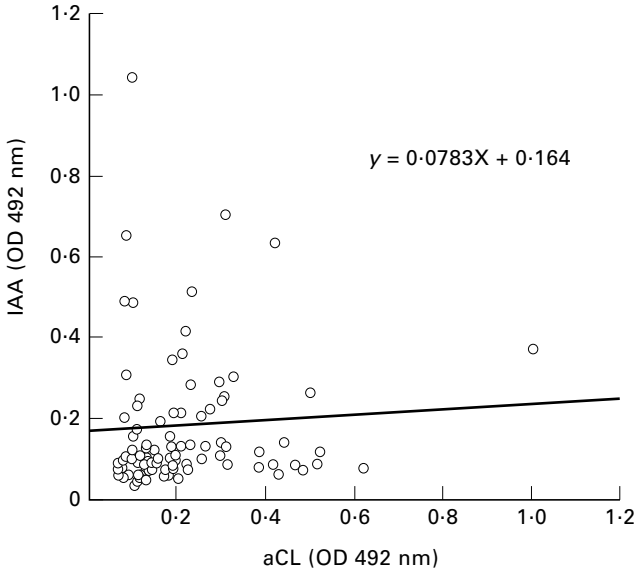

Fig. 2. Comparison of anticardiolipin antibody (aCL) and anti-insulin autoantibody (IAA) in NOD mice sera. Ninety-nine sera from either diabetic or non-diabetic NOD mice were tested in ELISA using cardiolipin or recombinant human insulin. There was no significant difference between the ELISAs using either cardiolipin or insulin.

concentrations (1·5, 6·0, 25, 100 μg/ml) either with or without cardiolipin (100 μg/ml). Cardiolipin was dried under argon, and then rehydrated in PBS for 10 min with intermittent vortexing before preincubation. The amount of free antibody in the serum–inhibitor mixtures was then measured in the anti-β_2-GPI ELISA test using a carboxylated plate. Serum incubated without the antigen was used as controls. The percentage of inhibition was calculated as follows:

$$\%\ \text{inhibition} = \left(1 - \frac{\text{OD 292 nm count in the presence of inhibitor}}{\text{OD 492 nm count in the absence of inhibitor}}\right) \times 100$$

Anti-insulin antibody assay

All 96-well microtitre plates (MS-3596) were coated with 0·5 μg of human recombinant insulin in 50 μl of 0·05 M bicarbonate buffer pH 9·6 overnight at 4°C. The wells were then saturated with PBS containing 1% BSA for 1 h at room temperature. This blocking solution was then removed and serum samples were added to insulin-coated wells for 1 h at room temperature. After washing three times with PBS–T, the plates were incubated at room temperature for 1 h with 50 μl of HRP-conjugated rabbit anti-mouse IgG. Finally the colour was developed and the OD was measured.

Immunohistochemical study

For the immunohistochemical study, the following antibodies were used: rat anti-mouse L3T4 (CD4) (clone GK1.5, lot no. J0419) which reacts with helper/inducer T lymphocytes, and rat anti-mouse Lyt-2 (CD8) (clone 53-6.7, lot no. F0115) which mainly reacts with suppressor/cytotoxic T lymphocytes, purchased from Becton Dickinson (Mountain View, CA), goat anti-mouse immunoglobulins (IgA + IgG + IgM) (lot no. 30162), purchased from Organon Teknika N.V.-Cappel Products (West Chester, PA), rabbit anti-mouse IgG1 (lot no. 90102332), IgG2a (lot no. 80801705), IgG2b (lot no. 7110218), IgG3 (lot no. 80801704), biotinylated goat anti-rat IgG, biotinylated goat anti-rabbit IgG purchased from Zymed Labs. To stain specific antigens, 5 μm thick cryostat sections of pancreas from 20-week-old female non-diabetic NOD mice were dried in room air for 15 min, fixed in acetone for 30 min and then again air-dried for 30 min. After a 10-min incubation with 10% normal serum, an appropriately diluted antibody for the specific antigen was overlaid for 60 min, then the preparations were washed three times in PBS and incubated with

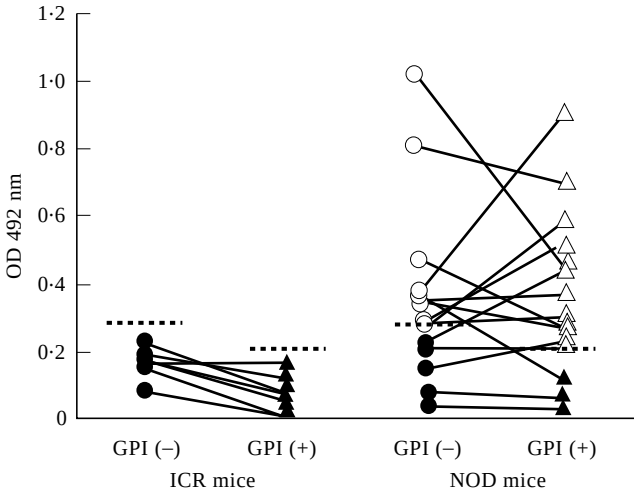

Fig. 3. β_2-glycoprotein I (β_2-GPI)-independent or -dependent anticardiolipin antibody (aCL) titres in NOD mice sera. Anticardiolipin antibody titres measured in absence and presence (30 μg/ml) of human β_2-GPI, indicated by β_2-GPI (–) (circles) and β_2-GPI (+) (triangles), respectively. ○,Δ, Samples exceed mean + 3 s.d. of the optical density (OD) values of ICR mice sera; ●,▲, samples below mean + 3 s.d. of the OD values of ICR mice sera. Broken lines represent mean + 3 s.d. of ICR mouse results.

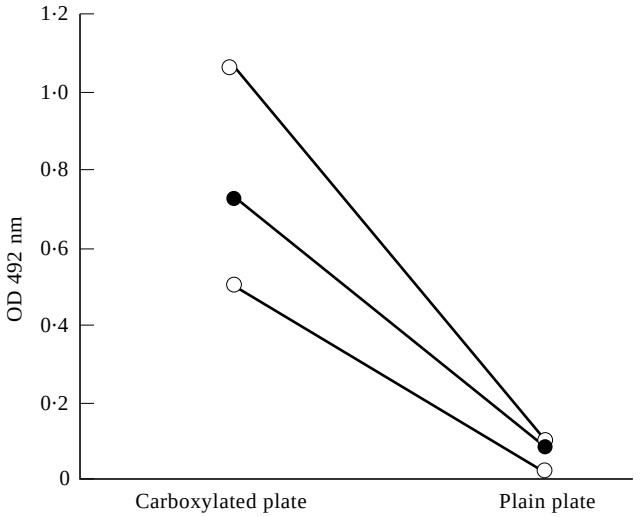

Fig. 4. Detection of autoantibodies to β_2-glycoprotein I (β_2-GPI) is dependent on the type of ELISA plate. ○, Samples exceed mean + 3 s.d. of ICR mouse results using both anticardiolipin antibody (aCL) assay system in the presence of β_2-GPI and in the absence of β_2-GPI; ●, sample is β_2-GPI-dependent aCL. Sera, diluted 1:80, were tested using the plain plate and the carboxylated plate.

biotinylated anti-IgG for 30 min. They were next washed three times in PBS and incubated with avidin and biotinylated horseradish complex for 30 min. After another washing in PBS, they were exposed to diaminobenzidin (DAB) and H_2O_2, and later counterstained with Mayer's haematoxylin.

Expression of results

The aCL and IAA activity was expressed as absorbance at 492 nm. The sample was defined as positive when the absorbance exceeded the mean of the normal controls + 3 s.d. Statistical analysis was performed using Student's *t*-test. $P < 0{\cdot}05$ was regarded as significant.

RESULTS

aCL in NOD mice

The sera from 75 non-diabetic NOD mice, 24 diabetic NOD mice and 17 control ICR mice were studied. Each of the three groups of NOD mice were tested only once. Figure 1 illustrates the representative IgG binding activity to CL in the NOD mice and control ICR mice sera by the standard aCL ELISA test using plain plates. Thirty-seven of 99 NOD mice showed aCL activity in their sera, while none of the control ICR mice produced any aCL. The incidence of aCL in NOD mice was 6·9% (2/29) in younger (group I at the age of 5–15 weeks), 31·6% (12/38) in group II at the age of 16–25 weeks and 71·9% (23/32) in group III at the age of

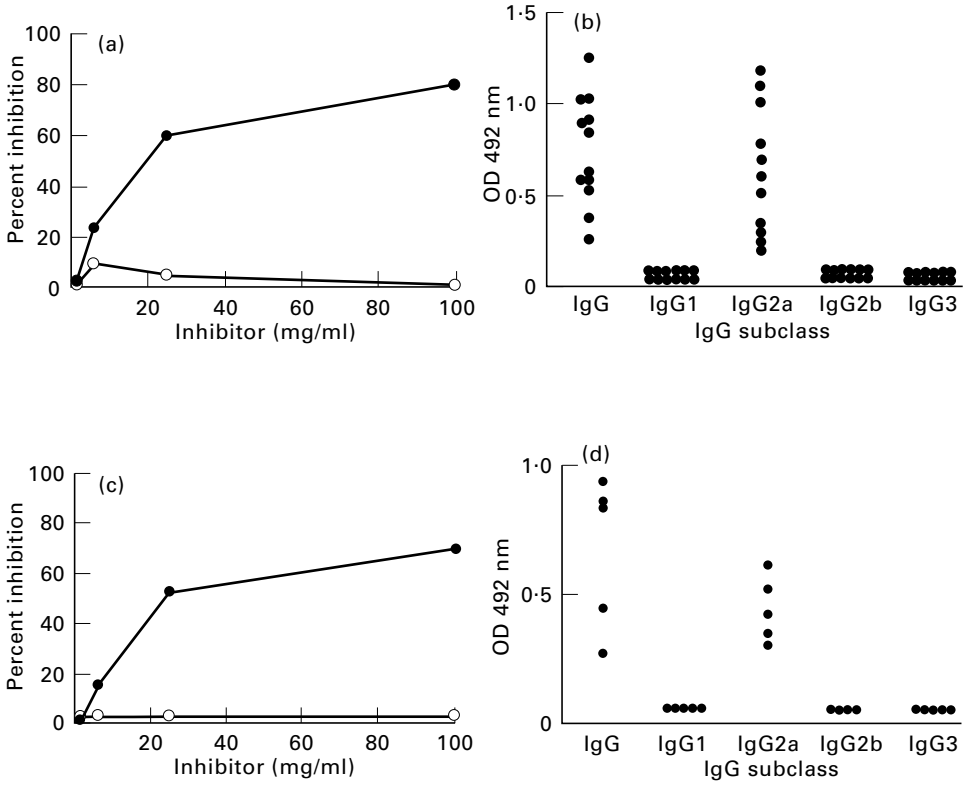

Fig. 5. Inhibition test and IgG subclass distribution of β_2-glycoprotein I (β_2-GPI)-dependent and -independent anticardiolipin antibodies (aCL). (a) Inhibition of autoantibody to β_2-GPI by the fluid-phase complex of β_2-GPI and CL. The β_2-GPI-dependent aCL-positive serum was preincubated with varying amounts of either β_2-GPI in the presence or absence of CL: β_2-GPI (○), β_2-GPI + CL (●). (c) Inhibition of autoantibody to CL by CL or β_2-GPI. The β_2-GPI-independent aCL-positive serum was preincubated with varying amounts of either CL or β_2-GPI: CL (●), β_2-GPI (○). (b,d) IgG subclass distribution of β_2-GPI-dependent aCL (b) and β_2-GPI-independent aCL (d) in the NOD mice sera by an ELISA using mouse monoclonal IgG subclass-specific antibodies. Each point indicates the absorbance of an individual serum.

26–35 weeks. The age dependence of aCL production in NOD mice was also indicated by the significantly higher incidence of aCL-positive sera. Similar differences appeared when considering the means of aCL serum levels as defined by absorbance at 492 nm, $0{\cdot}110 + 0{\cdot}008$ (mean ± s.e.m.) in group I, $0{\cdot}196 \pm 0{\cdot}016$ in group II and $0{\cdot}305 \pm 0{\cdot}025$ in group III, respectively ($P < 0{\cdot}001$). The diabetic NOD mice both had a higher incidence (84·6%) and higher titre ($0{\cdot}403 \pm 0{\cdot}040$) of aCL than the non-diabetic NOD mice (incidence 63·2% and titre $0{\cdot}238 \pm 0{\cdot}021$) in group III, respectively ($P < 0{\cdot}001$).

Correlation between aCL and IAA

The sera from the NOD mice were analysed for IAA and aCL. These data indicated that there was no association ($r = 0{\cdot}0783$) between the presence of aCL and IAA (Fig. 2).

β_2-GPI-independent and -dependent aCL

We attempted to determine whether or not sera contain GPI-dependent antibodies according to the procedure of Matsuda *et al*., with slight modifications [25,26]. In brief, we judged β_2-GPI-independent and -dependent aCL to be positive when the OD of the test sample exceeded the mean OD ± 3 s.d. (0·304 and 0·198, respectively) of the seven control ICR mice in the GPI (−) assay or the GPI (+) assay (Fig. 3). The OD of nine sera exceeded 0·304 in the GPI (−) assay, and one of them was below 0·198 in the GPI (+) assay (○–▲), while the other exceeded 0·198 (○–Δ) in the same assay. As a result, it thus suggested that the former (1/9) contains β_2-GPI-independent aCL alone, while the latter (8/9) contains both β_2-GPI-independent and -dependent aCL. On the other hand, the OD of five sera were below 0·304 in the GPI (−) assay, and three of them exceeded 0·304 in the GPI (+) assay (●–Δ), while the other two were below 0·304 (●–▲) in the same assay. The former (3/5) were thus suggested to contain β_2-GPI-dependent aCL alone, while the latter (2/5) contained neither β_2-GPI-independent nor -dependent aCL. We speculate that the β_2-GPI might partially block the epitope of antigen for β_2-GPI-independent aCL, or the conformation change of the epitope of CL might have been induced. We further examined whether or not the sera, which reacted both with β_2-GPI (−) and β_2-GPI (+) in CL-coated plate, contained both β_2-GPI-dependent and -independent aCL, or β_2-GPI-independent aCL alone.

Anti-β_2-GPI antibody assay

We attempted to determine whether or not the sera which contain β_2-GPI-dependent aCL alone, directly bind to the β_2-GPI using both the carboxylated and the plain plates (Fig. 4). Although no binding to a plain plate was observed, autoantibody to β_2-GPI was detectable when the β_2-GPI was coated on the carboxylated plate, thereby confirmed that these β_2-GPI-dependent aCL directly bound only to the modified form of β_2-GPI immobilized on carboxylated plate. Second, we used two sera which were positive in both the GPI (−) and the GPI (+) assays, and these ODs rose in the GPI (+) assays relative to the GPI (−) assays, and thereafter we examined whether or not these sera bound to the β_2-GPI-coated carboxylated plate. We observed them to react with a β_2-GPI-coated carboxylated plate but not with a plain plate, thus suggesting that these sera contain not only β_2-GPI-independent aCL but also β_2-GPI-dependent aCL.

Inhibition test

The inhibition test was performed to confirm the specificity of β_2-GPI-dependent aCL for native soluble β_2-GPI or for complex of β_2-GPI and CL by the anti-β_2-GPI antibody assay system using carboxylated plates. As shown in Fig. 5a, the fluid-phase complex of β_2-GPI and CL inhibited β_2-GPI-dependent aCL binding to β_2-GPI immobilized on the carboxylated plate, but native soluble β_2-GPI alone did not inhibit these antibodies binding to β_2-GPI. Then a second test was performed to confirm the specificity of β_2-GPI-independent aCL for CL or for soluble β_2-GPI by the standard aCL

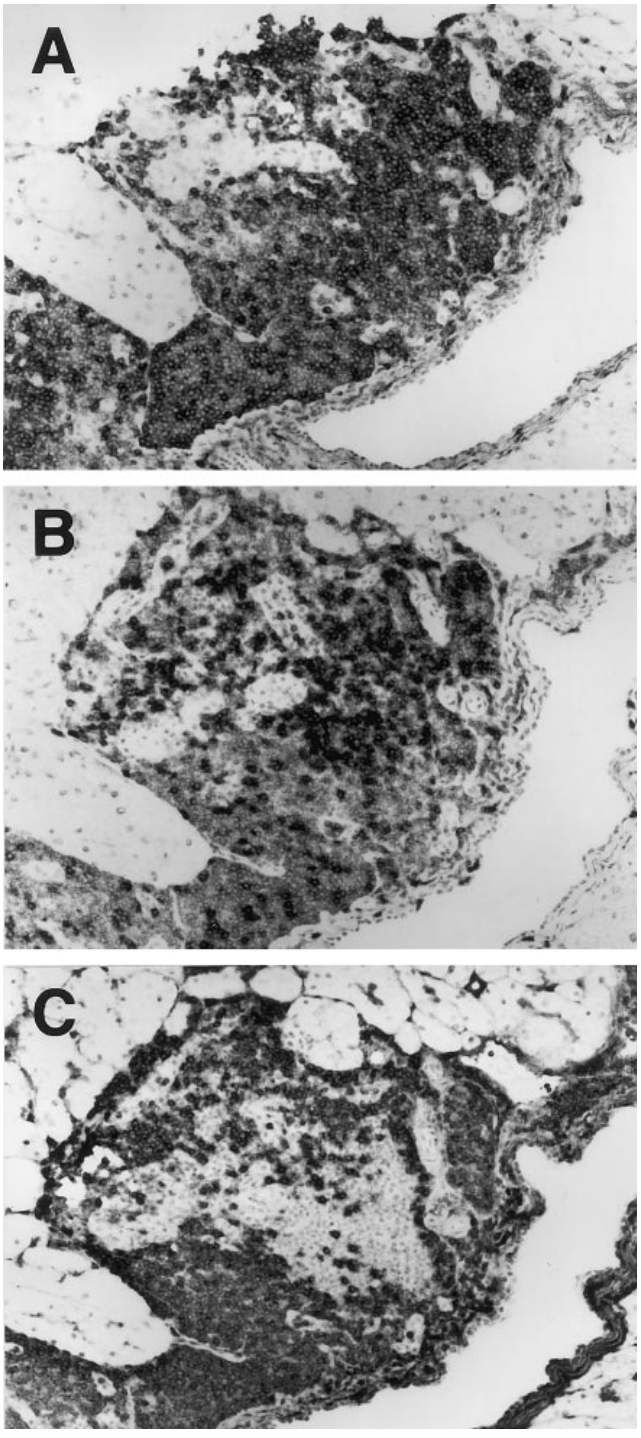

Fig. 6. Immunoperoxidase staining of cryostat pancreas sections of a 20-week-old NOD female mouse using different MoAbs defining $CD4^+$ T lymphocytes (A), $CD8^+$ T lymphocytes (B), sIg^+ B lymphocytes (C).

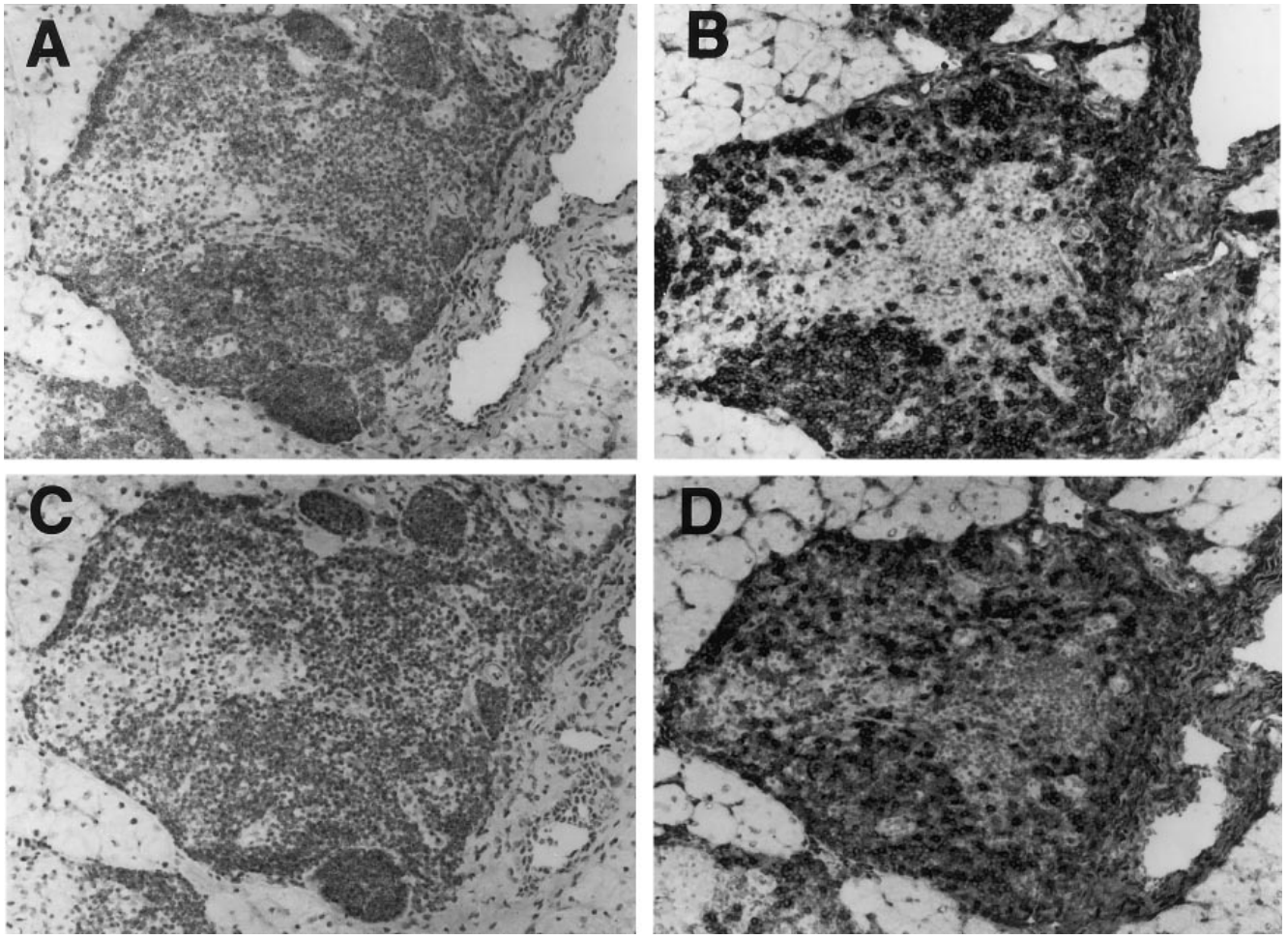

Fig. 7. Immunoperoxidase staining of cryostat pancreas sections of a 20-week-old NOD female mouse using different MoAbs defining IgG1 (A), IgG2a (B), IgG2b (C) and IgG3 (D)-positive B lymphocytes.

assay system using plain plates. As shown in Fig. 5c, the cardiolipin fluid phase inhibited aCL binding to CL immobilized on the plain plate. No inhibition was observed with similar concentrations of β_2-GPI. Similar results were obtained in other NOD mice sera with β_2-GPI-dependent aCL.

Immunoglobulin subclasses of aCL

The IgG subclass reactivity of β_2-GPI-dependent and β_2-GPI-independent aCL in the NOD mice sera with high β_2-GPI-dependent and β_2-GPI-independent aCL titres was determined by an ELISA test. The IgG subclass of both β_2-GPI-dependent and β_2-GPI-independent aCL exclusively belonged to IgG2a (Fig. 5b,d).

Immunohistochemical analysis of infiltrated lymphocytes in and around the islets

On week 20 in non-diabetic NOD mice, islets with insulitis were immunohistochemically examined for subsets of the infiltrated lymphocytes. The lymphocytes which infiltrated in and around the islets were mainly $CD4^+$ (helper/inducer T lymphocytes) (Fig. 6A), but only a few lymphocytes that reacted with anti-CD8 (cytotoxic/suppressor T lymphocytes) were observed (Fig. 6B). The surface immunoglobulin (sIg)-positive cells (B lymphocytes) also accumulated around these T lymphocytes (Fig. 6C). The subsets of the infiltrated B lymphocytes consisted mainly of IgG2a (Fig. 7B), while a few IgG3-positive cells were also observed (Fig. 7D).

DISCUSSION

To our knowledge, this is the first study to show the production of aCL in NOD mice. We have already reported that in AKR/J mice, in which multiple low doses of streptozocin caused insulitis and diabetes, aCL was transiently produced with hyperglycaemia and insulitis [20]. When the diabetes developed with the loss of islet cells, aCL disappeared. Therefore we suggested that the production of aCL might be induced by the damage of islet β-cells with insulitis rather than that of other organ cells. In contrast, as shown by this study, aCL was persistently produced along with the development of insulitis and diabetes in NOD mice. The detection of several autoantibodies to β-cell antigen, such as insulin, GAD, carboxypeptidase H, is decreased after the diabetic stage [10], because few antigens derived from β-cells remain in diabetic mice. We analysed IAA in the serum of NOD mice, but no correlation was observed between the presence of aCL and IAA. It was thus suggested that the mechanism of production of aCL in NOD mice may be different from β-cell-specific antibodies, such as IAA.

Anionic phospholipids, such as cardiolipin, are not expressed in the cell wall, but damage to the cell wall did result in their outer expression. The aCL in NOD mice were persistently produced after the onset of diabetes and loss of islet β-cells, so that we speculate that the production of these antibodies might be induced not only by the damage of islet β-cells but also by the destruction of other organ cells in NOD mice. The polyglandular autoimmune reactions observed in NOD mice also support this hypothesis.

The β_2-GPI-dependent aCL directly bind to the modified form, but not to be the native form of β_2-GPI in SLE patients [27]. The β_2-GPI-dependent aCL in NOD mice directly bound to the modified form of β_2-GPI immobilized on the carboxylated plate, but not to the native form of β_2-GPI on the plain plate. In addition, we also showed that β_2-GPI-dependent aCL bound to complex of β_2-GPI and CL, but not to the native soluble β_2-GPI alone based on an inhibition test. Therefore, these findings suggest that the epitopes which are recognized by these antibodies are expressed on a modified form of β_2-GPI by a conformational change which occurs when β_2-GPI interacts with an oxygen-substituted solid-phase surface, or with cardiolipin. Alternatively, it is possible that the epitope includes the linkage of the β_2-GPI with the oxygen-substituted surface or with CL.

The exact pathogenic role of aCL in NOD mice remains uncertain. Recently, it was reported that phospholipid autoantibodies occur frequently in the sera of patients with IDDM and also correlate with the extent of both neuropathy and retinopathy [28], and that the presence of a high aCL level is an independent risk factor for either myocardial infarction or cardiac death [29,30]. In addition, the β_2-GPI binds to anionic phospholipids and exhibits anticoagulant properties *in vitro* [31]. For example, β_2-GPI has been shown to inhibit the contact phase of intrinsic blood coagulation [32], interfere with adenosine diphosphate (ADP)-dependent platelet aggregation, and inhibit the prothrombinase activity of platelets [33,34], which are all anticoagulant properties. It was reported that the interaction of β_2-GPI-dependent aCL and β_2-GPI could be relevant to thrombotic disease associated with aCL in SLE patients [35]. Accordingly, we are tempted to speculate that the β_2-GPI-dependent aCL in NOD mice sera might play a new and significant role as a model of the autoantibodies associated with pathogenesis and/or the progression of diabetic micro- and macro-angiopathy in IDDM. Several animal models such as NZB, MRL-lpr/lpr or NZB/BXSB F_1 mice have been reported to produce aCL with concomitant development of thrombosis [36]. Based on these findings, NOD mice should also be added to the list of animal models of anti-phospholipid syndrome. β_2-GPI-independent aCL has been reported to be detected in patients with syphilis [25,26], but the pathological role of these antibodies in NOD mice remains unclear.

Our data also showed that the immunoglobulin subclasses of both of the β_2-GPI-dependent and -independent aCL in NOD mice have been found to belong mainly to IgG2a. The regulation of immunoglobulin subclass expression has been attributable to T cell control of antibody response, especially the nature and amount of cytokines produced [37,38]. It was reported that in mice at least three $CD4^+$ subsets exist: Th1, Th2 and Th0. Th1 cells secrete IL-2, interferon-gamma (IFN-γ) and tumour necrosis factor (TNF), and support macrophage activation, DTH responses and immunoglobulin isotype switching to IgG2a and IgG3. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13, and provide efficient help for B cell activation, for both switching to the IgG1 and IgE isotypes, and for antibody production. Th0 cells are characterized by the production of cytokines of both the Th1 and Th2 types, and are thought to be obligatory precursors of Th1 and Th2 cells [39,40]. Recently, it has been proposed that the development of diabetes in NOD mice is controlled by the Th1 *versus* Th2 phenotype of autoreactive Th cells: Th1 cells promote diabetes, whereas Th2 cells may actually help protect against disease [41,42]. The immunoglobulin subclass of both β_2-GPI-dependent and -independent aCL belonged to IgG2a, though autoantibodies possessing IgG3 subclass specificity were not detected in NOD mice sera. We are looking for the target antigens reactive with IgG3 autoantibodies. In addition, immunohistochemical studies revealed the predominant accumulation of IgG2a- or IgG3-positive B lymphocytes within insulitis. These results, taken together, suggest that autoimmunity in NOD mice may be associated with the Th1 predominant immunological response both locally and systemically.

Although the appearance of β_2-GPI-dependent and -independent aCL in NOD mice was clearly demonstrated in this study, further studies are still called for to elucidate the exact role of aCL in the immunological mechanism.

ACKNOWLEDGMENTS

We would like to thank Eiji Matsuura of the Department of Biochemistry, Hokkaido University School of Medicine, for his numerous valuable suggestions, discussions, and technical assistance. This work was supported by a Grant-in-aid no. 07770350 from the Ministry of Education, Science and Culture, Japan, and by the Foundation for the Advancement of Clinical Medicine.

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