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Brain-reactive autoantibodies in BB/d rats do not recognize glutamic acid decarboxylase

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SUMMARY

The BB rat spontaneously develops insulin-dependent diabetes mellitus (IDDM) similar to that in humans. The most practical markers of β cell autoimmunity are circulating antibodies to islet cell components. In particular autoantibodies to the enzyme glutamic acid decarboxylase (GAD) are a common feature of IDDM development in humans. This study aims at investigating the prevalence and levels of autoantibodies in BB rats to antigens in a semipurified, GAD-enriched preparation from rat brain. Eighteen diabetes-prone BB/d rats (10 male and eight female) were tail bled weekly from age 28 days to 113 days and antibodies detected on the rat brain preparation by ELISA. Antibody levels were expressed as arbitrary units relative to a standard positive serum. Individual rats varied in the time and order of antibody appearance and IDDM onset, with the earliest occurrence being 42 days and 69 days, respectively. In some rats antibody production was maintained but declined in others. By 113 days 85% of diabetic rats had at some time been positive for autoantibodies to brain components, compared with 25% of non-diabetics ($P = 0{\cdot}09$ by Fisher's exact test). Immunoabsorption studies using recombinant rat GAD-65 or recombinant human GAD-67 failed to inhibit the binding of BB rat sera to the original rat brain preparation. A capture ELISA using GAD-6 MoAb to capture GAD-65 from rat brain preparation or from a preparation of recombinant rat GAD-65, failed to detect anti-GAD antibodies in BB rats. Immunofluorescent staining of tissue sections showed the autoantibodies to be brain-specific, but having distinct staining patterns to the anti-GAD antibodies of Stiff Man Syndrome serum. In conclusion, BB rats possess autoantibodies reactive with rat brain antigens which may be associated with IDDM. However, these are not directed against GAD.

Keywords insulin-dependent diabetes BB rats glutamic acid decarboxylase brain-reactive autoantibodies

INTRODUCTION

Type 1 insulin-dependent diabetes mellitus (IDDM) results from the autoimmune destruction of the insulin-producing β cells of the pancreatic islets. This destruction is T cell-mediated [1], but is also characterized by autoantibodies which, although thought not to be pathogenic, are a useful marker of the disease process. Disease onset is associated with a high prevalence of autoantibodies to islet cell components, particularly the classical islet cell antibodies (ICA), which are present in 80–90% of newly diagnosed individuals [2], antibodies to 64-kD islet cell antigens [3–5] and antibodies to insulin [6].

Animal models have provided valuable information about the pathogenesis of the human disease. One of the best available models for human IDDM is the diabetes-prone BB rat. Spontaneous diabetes appears between 60 and 120 days and exhibits physiological and biochemical features analogous to the human disease, including weight loss, ketonuria and insulitis ultimately leading to complete loss of β cells (reviewed in [7]). Diabetes-prone BB rats lack a population of T cells expressing the $RT6^+$ phenotype which are necessary to suppress diabetogenic effector cells. A disease-resistant subline of BB rats also exists, and depletion of $RT6^+$ T cells from these rats induces disease [7]. Disease can be prevented by the administration of antiserum against rat lymphocytes [8], by neonatal thymectomy [9] or treatment with immunosuppressive agents [10]. Adoptive transfer of $CD4^+$ T cells from diabetic BB rats can induce IDDM in non-diabetic disease-prone recipients [11], which would indicate a T cell-mediated pathogenesis.

A variety of autoantibodies have been identified in the BB rat with specificity for different tissues, e.g. thyroid, smooth

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muscle, islet and gastric parietal cells [12,13]. Antibodies directed against islet cell surface antigens are often present in the sera of BB rats 4–8 weeks before onset [14–16]. These antibodies are cytotoxic and provide indirect evidence that the humoral arm of the immune system contributes to the pathogenesis of diabetes in the BB rat. Two groups have shown that disease is associated with antibodies to a 64-kD rat islet cell protein [17,18]. These appear as early as 33–45 days of age, and in some animals are present for as long as 8 weeks before disease onset. ICA to cytoplasmic components have not been conclusively demonstrated in BB rats [11,12].

Glutamic acid decarboxylase (GAD) has been identified as the major 64-kD islet antigen in human IDDM [19], although diabetics do possess antibodies to a 64-kD pancreatic antigen distinct from GAD [5]. GAD catalyses the synthesis of γ-amino butyric acid (GABA), an important metabolite and major inhibitory neurotransmitter of the central nervous system. Autoantibodies to GAD have been found in 80–90% of newly diagnosed diabetic and pre-diabetic patients [19,20], in patients with polyendocrine autoimmunity [21] and in Stiff Man Syndrome (SMS), a rare neurological disorder with which diabetes is often associated [19,22]. Generally, IDDM sera have a much lower serum titre of anti-GAD than SMS or polyendocrine patients [19], and are unable to recognize partially denatured GAD on Western blots [19].

Two full length isoforms of GAD have been identified. The smaller membrane-bound amphiphilic form has a molecular weight of 65 kD (referred to as GAD-65), and the larger hydrophilic form with a molecular weight of 67 kD (GAD-67). The two forms share 65% sequence identity and 80% homology, with the greatest differences occurring within the amino terminal portion. Both forms are found in the GABA-secreting neurones of the central nervous system, and in rat islets [23]. GAD-65 is the main isoform expressed in human islets [24], although the GAD-67 gene has been cloned from human pancreas [25]. GAD-65 has been demonstrated to be the main immunogenic isoform in human IDDM [20,26], but SMS and polyendocrine patients may have antibodies against both isoforms [27,28].

Among the autoantibodies known to be associated with human IDDM, those directed against GAD are the ones which appear earliest and are present in the largest number of patients. It is therefore interesting to look for the presence of these antibodies in BB rats. Antibodies to GAD have been reported in BB/OK rats [29], although these studies only looked for the presence of antibodies at one or two time points. In this study we examined the humoral autoimmune response in BB/d rats from age 28 to 113 days and looked for the possible presence of anti-GAD antibodies in relation to disease onset.

MATERIALS AND METHODS

Preparation of semi-purified GAD from rat brain

Whole rat brains were homogenized in ice cold isotonic buffer (10 mM Tris–HCl pH 7·5 containing 100 μM PMSF) at 4°C and ultracentrifuged at 160 000 ***g*** for 90 min using a Sorval OTD 65 Ultracentrifuge. The supernatant was passed down a DEAE Sephacel column equilibrated with Tris–HCl pH 7·7 and eluted with increasing concentrations of NaCl. Fractions (50 ml) were collected and tested for the presence of GAD using sheep anti-serum to GAD in a dot blot assay (see below). The GAD-containing fraction was concentrated to 10 ml and further purified by passing down a Pharmacia K26/100 column containing S-300 Sephacryl. Fractions (10 ml) were collected and tested for the presence of GAD by a dot blot assay. Alternatively, the supernatant was passed down a Q Sepharose Fast Flow (QSFF) FPLC column (Pharmacia, Milton Keynes, UK) and 2-ml fractions were collected and tested for the presence of GAD using a dot blot assay. GAD-containing fractions were pooled. Total protein was determined by the Bicinchoninic Acid (BCA) assay (Sigma Chemical Co., Poole, UK) [30].

Monoclonal antibodies and antisera

GAD-6 MoAb with a specificity for GAD-65 [31] was purchased from Boehringer Mannheim UK Ltd. (Lewes, UK). Sheep anti-serum raised against purified rat brain GAD [32] and pre-immune sheep serum was a kind gift from Dr I. J. Kopin (National Institute of Health, Bethesda, MD). This anti-serum recognizes both GAD-65 and GAD-67. K2 is a rabbit anti-serum raised against feline GAD-67 [33] obtained from Dr Alan Tobin (Department of Biology, University of California). D. H. is serum obtained from a SMS patient with high titre anti-GAD antibodies (1 : 50 000) by ELISA.

Animals

Diabetes-prone BB/d rats were maintained under semi-barrier conditions in the Clinical Sciences Department, Leicester Royal Infirmary, UK. Rats were defined as diabetic if their blood glucose exceeded 12 mmol/*l* and insulin dosing was commenced. Rats were tail bled weekly from 4 weeks of age and serum separated and stored. In a preliminary experiment, six rats were selected and labelled as 1′, 2′, 3′, 4′, 5′ and 6′. A second larger experiment included 18 rats labelled as A1, A2, A3, A4, B1, B2, B3, C1, C2, C3, C4, C5, D1, D2, D3, D4, E1 and E2.

Transient expression of recombinant rat GAD-65

Escherichia coli transfected with pCDM8 vectors containing GAD-65 was a kind gift from A. Karlsson and O. Kampe (Department of Internal Medicine, University Hospital, Uppsala, Sweden) [34]. The pCDM8 vestor was originally obtained from Dr B. Seed (Massachusetts General Hospital, Boston, MA), and the rat cDNA clone which codes for GAD-65 was made available by Dr A. Tobin (Department of Biology, UCLA). pCDM8 plasmids containing rat GAD-65 were prepared from an overnight culture of *E. coli* and purified by caesium chloride/ethidium bromide extraction. Plasmid (10 μg) was transfected by electroporation, using a Biorad Gene Pulser, into COS-7 cells which had grown to 80% confluence. These cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). Following transfection, COS-7 cells were cultured for 48 h in Dulbecco's modified Eagle's Medium (Sigma) containing 2 mM glutamine, 1 mM HEPES and 10% fetal calf serum (FCS). Cells were lysed for 1 h at 4°C in 20 mM Tris–HCl pH 8·2, 20 mM NaCl containing 0·2% Triton X100, 2 mM PMSF and 1 μg/ml aprotinin. Control lysates were prepared from non-transfected COS-7. GAD-65 expression was confirmed using a dot blot assay (see below) and protein concentration determined by the BCA protein assay.

Recombinant human GAD-67

A cell lysate of *E. coli* containing human recombinant GAD-67 was a gift from Dr D. Kaufman (Department of Psychiatry,

University of California). A lysate of non-transfected *E. coli* was also available as a control.

Direct ELISA for anti-GAD antibodies

Semi-purified rat brain GAD (100 μl/well) at 65 μg total protein/ml in 0·05 M carbonate/bicarbonate buffer pH 9·6 was coated on wells of Maxisorp ELISA plates (Nunc, Paisley, UK). Plates were incubated at 4°C overnight. Wells were washed three times in PBS containing 0·1% Tween-20 (PBS–T) and blocked with 2% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Control wells were blocked with 2% BSA only. Control and test rat sera from weekly tail bleeds (diluted 1:100 in 1% BSA PBS–T) were applied to GAD-coated and control blocked wells and incubated for 2 h at room temperature. Following three washes in PBS–T, goat anti-rat IgG alkaline phosphatase conjugate (Sigma), diluted 1:1000 in 1% BSA PBS–T, was added to all wells for 1 h at room temperature. Plates were washed three times in PBS–T and substrate (*p*-nitrophenyl-phosphate in diethanolamine buffer pH 9·8 (Sigma)) was added. Plates were read at 30 and 60 min at 405 nm on a Dynatech plate reader.

Positive control rat serum, 4′, was used to generate a standard curve for arbitrary levels of autoantibodies. A 1:25 dilution of serum represented 400 U of autoantibody, 1:50 represented 200 U, 1:100 represented 100 U, 1:200 represented 50 U and 1:400 represented 25 U. All rat serum samples were expressed relative to this standard curve, and considered positive if antibody levels were greater than or equal to 36 U (the value given by normal rat sera).

The GAD-enriched rat brain preparations produced either by DEAE-Sephacel/S300-Sephacryl or by QSFF FPLC fractionations were used as the antigen source, in each case the standard curve being calibrated with BB rat 4′ serum.

Capture ELISA for anti-GAD antibodies

ELISA plates were coated with GAD-6 MoAb diluted to 2 μg/ml in coating buffer and incubated at 4°C overnight. Plates were washed and blocked with 2% BSA in PBS for 1 h at room temperature. Following one wash, semi-purified rat brain GAD, recombinant rat GAD-65 or control lysate from COS cells was captured onto GAD-6 by incubating at room temperature for 2 h. Plates were washed three times in PBS–T and diluted serum applied for 2 h at room temperature. The assay was continued as described for the direct ELISA.

Dot blot assay

Rat brain GAD (1 μl), recombinant rat GAD-65, recombinant human GAD-67 or control lysates were spotted onto nitrocellulose strips and air dried for 5 min. Strips were blocked for 30 min in 5% dried milk powder in PBS. Blocking buffer was aspirated and replaced with BB sera (diluted 1:100 in 5% BSA in PBS) or control antisera (sheep anti-GAD 1:500, K2 1:1000, GAD-6 at 1 μg/ml or D.H 1:1000). Strips were incubated on a shaker at 37°C for 2 h. Strips were washed six times (5 min for each wash) in PBS and the appropriate anti-immunoglobulin alkaline phosphatase conjugate diluted 1:1000 in 5% BSA/PBS was added for 1 h at 37°C. Strips were washed as before and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate in buffer of 100 mM Tris–HCl, 100 mM NaCl and 5 mM $MgCl_2$ pH 9·5.

Immunoabsorption studies

Neat BB rat sera and control sera were subjected to four different pre-absorptions before testing in ELISA on semi-purified rat brain GAD: (i) preabsorption with 50 μg COS cell lysate containing recombinant rat GAD-65; (ii) preabsorption with 50 μg COS cell control lysate; (iii) preabsorption with 10 μl *E. coli* lysate containing human recombinant GAD-67; (iv) preabsorption with 10 μl control *E. coli* lysate. Preabsorptions were for 1 h at 37°C. Diluent of 2% BSA in PBS was added to give a final dilution of 1:100 for BB sera, 1:500 for D.H. and sheep anti-GAD and negative controls. Following microcentrifugation, supernatants were taken for testing on semi-purified rat brain GAD in direct ELISA.

Data for six rats, 3′, B2, C1, C4, D1 and E1, were pooled for each pre-absorption, and results are shown in Fig. 3. Comparisons were made between each condition and unabsorbed rat serum using the non-parametric Wilcoxon rank sum test for paired data. Significance was taken as $P < 0.05$.

Indirect immunofluorescence on frozen tissue sections

Frozen sections of rat kidney, liver, stomach, cerebellum and human thyroid were cut using a Jung CM3000 cryostat to thickness of 6 μm and transferred to poly-L-lysine-coated microscope slides. Slides were brought to room temperature

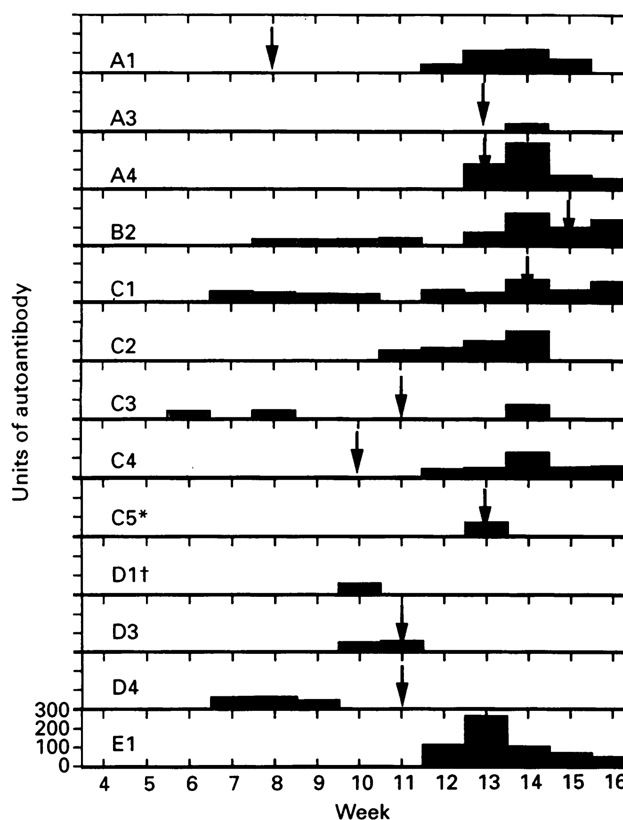

Fig. 1. Shaded boxes indicate the levels (in units) of autoantibodies in BB/d rats reactive with a glutamic acid decarboxylase (GAD)-enriched rat brain preparation. Only those values greater than or equal to 36 units, which are taken to be positive, are shown. Arrows indicate the onset of diabetes. * Rat died at 13 weeks. † Rat culled at 11 weeks. Five rats not included in this figure, A2, B1, B3, D2 and E2, were negative for autoantibodies, although B3 and D2 did become diabetic.

over a period of 30 min. Kidney, liver, stomach and thyroid sections were covered in sera from the final bleed outs from nine BB rats and control rat sera, 4′ and 6′ (all sera were diluted 1 : 20 in PBS). Rat brain cerebellum sections were incubated with BB rat serum (B2 or B3), SMS serum (D.H.) or normal human sera, diluted 1 : 20 in PBS. All slides were incubated for 30 min at room temperature in a humidity chamber. Slides were washed for 30 min in three changes of PBS, with a drop of Tween-20 included in the last 5 min of washing. Slides were returned to the humidity chamber and anti-rat IgG FITC conjugate (Sigma), diluted 1 : 40 in PBS, or anti-human IgG (Fc specific) FITC conjugate (Atlantic Antibodies, Incstar Ltd, Wokingham, UK), diluted 1 : 40 in PBS, was added to the appropriate sections. Slides were incubated for 30 min at room temperature and then washed again in three changes of PBS over 30 min. Slides were mounted in PBS-buffered glycerol/ DABCO mounting fluid.

RESULTS

Time course ELISA

In a preliminary experiment using direct ELISA it was possible to detect autoantibodies reactive with rat brain antigens in the semi-purified rat brain GAD preparation in 5/6 BB/d rats. Of these, four became diabetic. The rat showing the highest titre of autoantibody (rat 4′) was taken as a positive control for future assays, and the rat showing no measurable autoantibodies (rat 6′) was taken to be the negative control. Based on the findings of this preliminary experiment a larger scale study was set up to include 18 rats (10 males and eight females) which were screened weekly from the age of 4 weeks for autoantibodies reactive with the rat brain preparation (Fig. 1).

The rats varied in the time of diabetes onset and the time of appearance of autoantibodies. The earliest onset of diabetes was 69 days and the first appearance of autoantibodies was 42 days (see Fig. 1). For some rats (e.g. A1 and C4) autoantibodies appeared some time after disease onset, and for others (e.g. B2 and D4) autoantibodies preceded disease onset. Autoantibody levels were maintained in some rats to the end of the study (e.g. B2, C1), but in others peaked after onset and declined (e.g. A4, C4, E1). By 113 days 85% (11/13) of diabetic rats had at some time been positive for serum autoantibodies, compared with 25% (1/4) of non-diabetic rats ($P = 0{\cdot}09$ by Fisher's exact test). Rat D1 was culled and therefore not included. There was no significant difference in responses between male and female rats.

Based on these observations we attempted to determine whether these autoantibodies were truly directed against GAD or some other brain antigens.

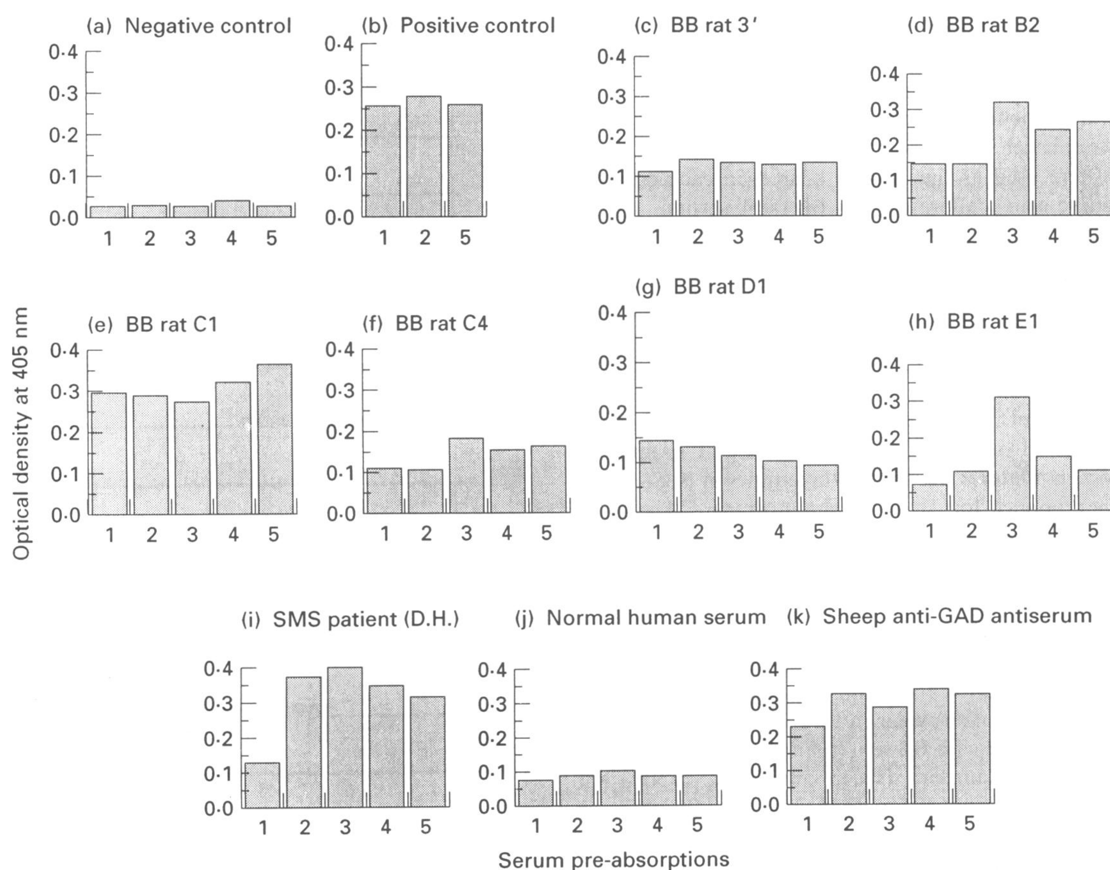

Fig. 2. Measurement of autoantibodies reactive with rat brain preparation by direct ELISA, following preabsorption of BB sera. Lane 1, preabsorption of sera with COS cell lysate containing recombinant rat glutamic acid decarboxylase (GAD)-65; lane 2, preabsorption of sera with COS cell control lysate; lane 3, preabsorption of sera with *Escherichia coli* lysate containing recombinant human GAD-67; lane 4, preabsorption of sera with control *E. coli* lysate; lane 5, non-absorbed sera. Positive control is BB rat 4′, negative control is BB rat 6′.

Preabsorption studies using recombinant rat GAD-65 or recombinant human GAD-67 to remove anti-GAD activity from BB rat and human sera

The binding of BB rat sera (taken at week 16) to crude rat brain antigens was not inhibited by pre-absorption with recombinant rat GAD-65 (Fig. 2a–h). There was no significant inhibition in binding to rat brain antigens when the serum was absorbed with COS cell lysate containing rat GAD-65 ($P = 0{\cdot}08$) or with COS cell control lysate ($P = 0{\cdot}156$) compared with unabsorbed serum (Fig. 3). Sera from SMS patient D.H., previously known to have activity to rat brain antigens and recombinant rat GAD-65, was inhibited from binding to rat brain antigens following preabsorption with rat GAD-65 (Fig. 2i). Sheep anti-GAD antiserum was marginally inhibited from binding to the rat brain preparation by absorption with recombinant rat GAD-65 (Fig. 2k). The results suggest that although BB rats contain antibodies to crude brain antigens, they are probably not directed against GAD-65.

Preabsorption of BB rat sera with *E.coli* lysate containing GAD-67 did not affect binding to the rat brain preparation in ELISA (Fig. 2a–h). There was no significant inhibition in binding to rat brain antigens when the rat serum was preabsorbed with *E. coli* lysate containing recombinant human GAD-67 ($P = 0{\cdot}218$) or serum absorbed with *E. coli* control lysate ($P = 0{\cdot}343$) compared with unabsorbed serum (Fig. 3). There was no inhibition of D.H. binding (Fig. 2i), and marginal inhibition of sheep anti-GAD (Fig. 2k). Results suggest that BB rats do not contain antibodies to human GAD-67, and that the majority of antibodies in the SMS patient's sample were directed against GAD-65.

Dot blot assay for measuring anti-GAD antibodies

Using *E. coli* lysate, containing recombinant human GAD-67 as the blotting antigen, BB rat sera showed no difference in binding from negative control rat serum, confirming the absence of anti-GAD-67 antibodies in these rats (Fig. 4a). Similarly using COS cell lysate containing recombinant rat GAD-65 as the blotting antigen, autoantibodies to GAD-65 in BB rats could not be detected (Fig. 4b).

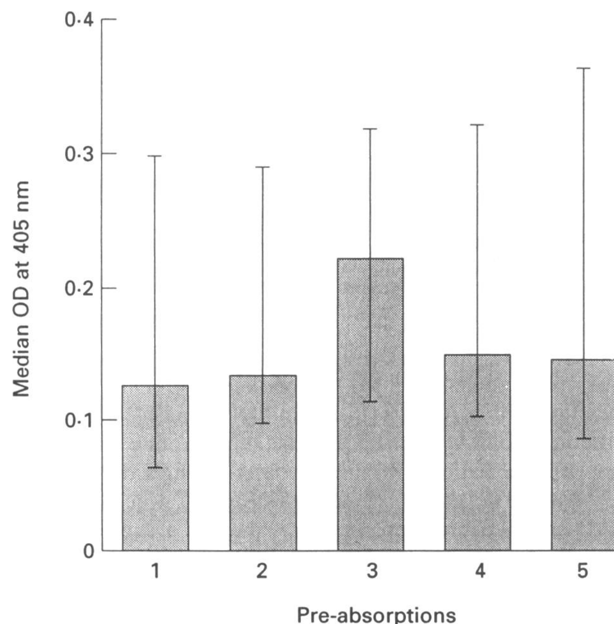

Fig. 3. The measurement of autoantibodies reactive with rat brain antigen preparation by direct ELISA following preabsorption of BB sera. Preabsorption conditions are as for Fig. 2. Results represent the median value and the range for six rats, 3′, B2, C1, C4, D1 and E1.

Table 1. Immunofluorescent detection of autoantibodies to parietal cell, smooth muscle and thyroid follicular cells in BB rats

Rat	Immunofluorescent staining for autoantibodies			Autoantibodies to GAD-enriched rat brain preparation*
	Parietal cell	Smooth muscle	Thyroid	
A2	–	–	–	–
B1	–	–	–	–
B2	–	–	–	+
B3	–	–	–	–
C1	–	–	–	+
C2	–	–	–	–
C4	–	–	–	+
E1	+	+	–	+/–
E2	–	–	–	–
4′	–	–	–	+
6′	–	+	–	–

* Sera from the final bleed out were also tested for autoantibodies to glutamic acid decarboxylase (GAD)-enriched rat brain preparation by ELISA. +, Strong positive; +/–, weak positive; –, negative.

Capture ELISA

The capture ELISA provides a highly sensitive method for detecting antibodies to GAD. However, using recombinant rat GAD-65 as the capture antigen we were unable to detect antibodies to GAD-65 in 5/5 rats which were known to be positive for antibodies to the GAD-enriched brain preparation in conventional ELISA. Similar results were obtained when the GAD-enriched brain preparation was used as the capture antigen.

Immunofluorescent staining of tissue sections

Liver and kidney sections were used for the evaluation of smooth muscle antibodies, stomach for the evaluation of parietal cell antibodies and thyroid for the evaluation of thyroid follicular cell antibodies. The results of the immunofluorescent staining are summarized in Table 1. Autoantibodies to the GAD-enriched rat brain preparation in BB sera from the final bleed out are also included. All rats were negative for thyroid follicular cell antibodies. One rat, E1, was positive for parietal cell and smooth muscle antibodies, and was weakly positive for autoantibodies to the GAD-enriched brain preparation. Rat 6′, the negative control in ELISA, was positive for smooth muscle antibodies. There was no association between autoantibodies to the GAD-enriched brain preparation and autoantibodies to the tissues, since those rats which were strongly positive in ELISA (e.g. B2, C1 and 4′) were negative for parietal cell, smooth muscle and thyroid autoantibodies.

The evaluation of brain-specific autoantibodies by immunofluorescence is shown in Fig. 5. SMS serum stained a proportion of neurones in the granular layer of the cerebellum, with some staining of ascending fibres in the molecular

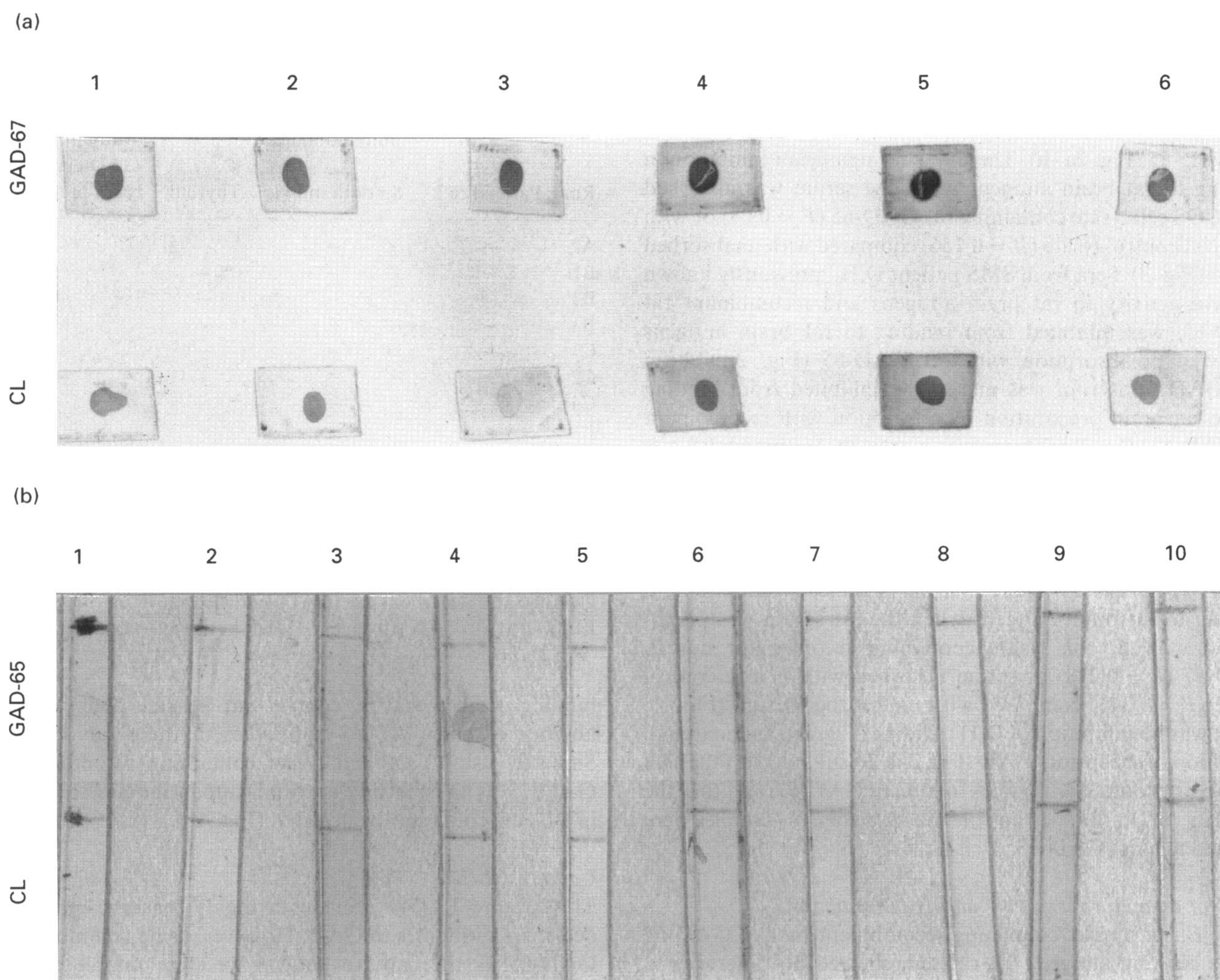

Fig. 4. Dot blot for detection of anti-glutamic acid decarboxylase (GAD) antibodies. (a) 1, Positive control rat 4′; 2, BB rat D1; 3, sheep anti-GAD anti-serum; 4, D.H. (Stiff Man Syndrome (SMS) serum); 5, K2 (rabbit anti-GAD); 6, negative control rat 6′. GAD-67, *Escherichia coli* lysate containing recombinant human GAD-67; CL, *E. coli* control lysate. (b) 1, Anti-human conjugate control; 2, anti-rat conjugate control; 3, normal human serum; 4, D.H.; 5, BBE1; 6, BBC4; 7, BBC1; 8, BBB2; 9, BB6′; 10, BB4′. GAD-65, COS cell lysate containing recombinant rat GAD-65; CL, control COS cell lysate.

layer (Fig. 5a). This staining pattern is similar to that previously described for anti-GAD in SMS [35]. Rat B2 (positive for autoantibodies to the GAD-enriched rat brain preparation) and rat B3 (negative for autoantibodies) shows some staining of neurones in the granular layer of the cerebellum, with some astroglial staining. There was also some staining of cells in the molecular layer (Fig. 5b). BB sera did not show staining patterns similar to that of SMS serum.

DISCUSSION

The BB rat is an established model of type 1 diabetes. We examined the humoral autoimmune response in these animals with particular attention to the possible presence of anti-GAD-antibodies in relation to disease onset.

Autoantibodies have proved to be very useful markers in predicting onset of human IDDM. ICA, the best validated risk markers, are currently used as a complementary test for the diagnosis of type 1 diabetes. ICA are present in approximately 90% of recent onset diabetics [2]. They appear several years before disease onset, and have a high predictive value in first degree relatives [36], but ICA are not a strong predictor in the general population [37]. Autoantibodies to insulin occur in 50% of patients at onset. Alone they have a poor predictive value, but if used in association with ICA the predictive value is nearly 100% [2].

Antibodies to 64-kD islet cell protein/GAD may precede other antibody markers [4]. Antibodies to 64-kD antigen have been reported in BB rats [17,18] and NOD mice [38], as well as antibodies to GAD in BB/OK rats [29], although these do not show an association with disease onset. Using a direct ELISA we were able to detect antibodies in BB/d rats which bound to a GAD-enriched brain preparation. There is a suggestion that these antibodies may be associated with IDDM because at 113 days of age 85% of diabetic rats were, or had been, positive for autoantibodies, compared with 25% of non-diabetics, although a larger group of animals would be required to show statistical significance. The earliest appearance of these autoantibodies was 42 days, and earliest onset of diabetes was 69 days. In some instances autoantibodies appeared after disease onset, which

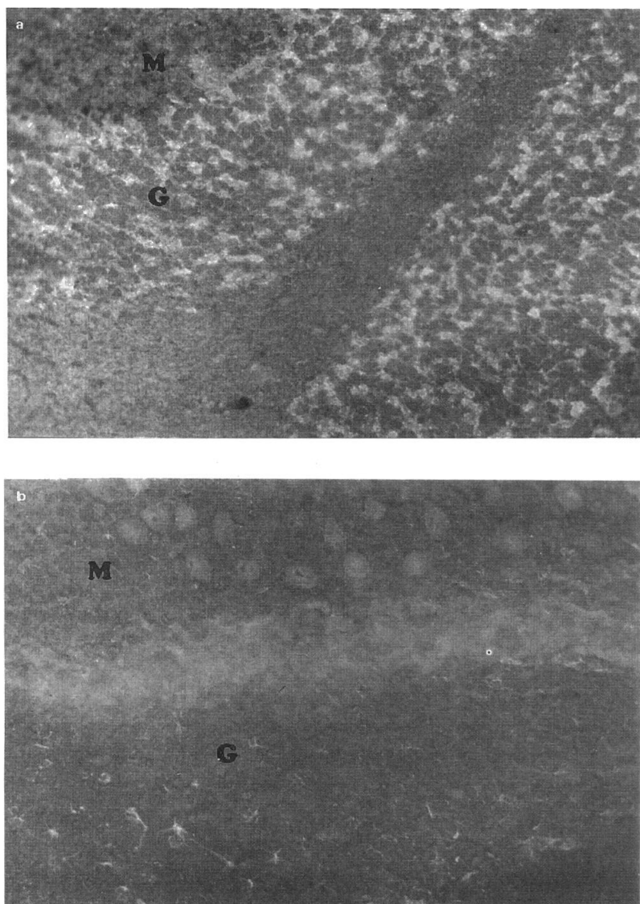

Fig. 5. Immunofluorescent detection of autoantibodies to rat brain cerebellum. (a) Cerebellum staining with Stiff Man Syndrome (SMS) serum. (b) Cerebellum staining with B2 (positive for autoantibodies to glutamic acid decarboxylase (GAD)-enriched rat brain preparation. M, Molecular layer of the cerebellum; G, granular layer.

indicates that they are not very predictive of future onset of diabetes in BB rats.

In order fully to characterize this autoantibody response a number of pre-absorption studies were performed. Pre-absorption of BB rat sera using recombinant rat GAD-65 or recombinant human GAD-67 was unable to remove activity to the GAD-enriched rat brain preparation, which would suggest that the antibodies are probably directed against other antigens present in the brain and not GAD. Despite the fact that human GAD-67 was used as an immunoabsorbant, this should still remove any anti-GAD-67 activity from BB sera, since rat and human GAD-67 share 98% sequence homology. A capture ELISA using GAD-6 to capture GAD-65 from rat brain preparation, or from recombinant rat GAD-65 COS cell lysate, was developed and found to be highly sensitive for detection of anti-GAD-65 in SMS sera, but failed to detect anti-GAD-65 antibodies in BB sera.

SMS serum, known to be positive for anti-GAD, was unable to block the binding of BB sera to rat brain GAD preparation in ELISA, and similarly BB sera were unable to block the binding of SMS serum (results not shown). Although these results are consistent with the absence of anti-GAD antibodies in BB sera, they are not definitive, as rat and human antibodies could recognize different non-cross-reactive epitopes on GAD.

It is unclear why our results contrast with those of others [29] who detected antibodies to GAD in BB/OK rats with a mean age of 120 days. However, the lack of anti-GAD antibodies in our rats is consistant with the lack of ICA in BB rats [12,13] and NOD mice [39]. Immunoprecipitation of *in vitro* translated, radiolabelled rat GAD-65 failed to detect autoantibodies to 64-kD/GAD in our BB rats (M. Christie, personal communication). Indeed, the ELISAs employed in the study of Ziegler *et al.* [29] do not definitively prove the occurrence of antibodies to GAD. They detected autoantibodies using semi-purified GAD from rat brain in a direct and sandwich ELISA. For the latter assay serum from a patient with SMS was used to capture GAD from the rat brain preparation. However, the autoantibodies in SMS serum may bind components of rat brain in addition to GAD, and it could be to these components that autoantibodies in BB rats were reacting.

The presence of anti-GAD autoantibodies in NOD mice is also controversial [40]. Although they possess antibodies which precipitate a 64-kD islet protein [38], there have been few reports published since the human 64-kD antigen was identified as GAD [41]. More recently two groups failed to detect anti-GAD antibodies in NOD mice by immunoprecipitation [42,43], and the expression of GAD-65 in the mouse islet could not be detected [42], although rat islets do express this isoform [40,42]. T cell responses to GAD-65, and marginally to GAD-67, have been detected in NOD mice [42], which suggests that the two GAD isoforms are T cell autoantigens in NOD mice which may be involved in early processes leading to the development of diabetes.

A variety of other autoantibodies have been reported in BB rats with a specificity for different tissues [12,13]. The appearance of anti-parietal cell antibodies seems to correlate with onset of diabetes [12]. Anti-smooth muscle and anti-thyroid colloid antibodies are present with greater frequency in diabetic animals [13], but their presence is not necessarily associated with diabetes. Using immunofluorescence we were able to detect autoantibodies to smooth muscle and parietal cells in only one BB rat, which had a low titre of autoantibodies to the GAD-enriched rat brain preparation. Rat 6′, negative on the brain preparation, had autoantibodies to smooth muscle. It seems likely, therefore, that we were detecting autoantibodies to a novel autoantigen, since those rats which showed strong reactivity to antigens in the GAD-enriched rat brain preparation were negative for other tissue-specific autoantibodies. The staining of brain tissue by BB sera showed a distinct pattern from SMS serum, suggesting that BB autoantibodies were recognizing an autoantigen(s) with a different biochemical composition and histological location to GAD. Preliminary ELISA data using BB sera on ion exchange and sizing fractions of the GAD-enriched rat brain preparation indicate a difference from GAD in isoelectric point and molecular weight of autoantigens recognized.

Although the spontaneous autoimmune diabetes which occurs in BB rats shows some strong similarities to human IDDM, there are also clear differences, e.g. the lymphopenia and the nature of the pancreatitis in BB rats. Our finding that BB rats do not appear to develop antibodies to GAD, whereas these autoantibodies are a prominent feature of the

development of human IDDM, highlights the need for caution in extrapolating from the rat to the human condition.

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