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Autoantibody Patterns in Diabetes-prone NOD Mice and in Standard C57BL/6 Mice

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Autoantibodies are commonly found in healthy individuals and strains of mice that are not prone to autoimmunity. The present study was undertaken to identify self antigens recognized by serum autoantibodies from unimmunized mice of two strains: NOD mice prone to spontaneously develop autoimmune diabetes and C57BL/6 mice known to be relatively resistant to autoimmune disease. IgM and IgG autoantibodies detected in the sera of NOD and C57BL/6 mice manifested different patterns of reactivity. The IgM autoantibodies from C57BL/6 serum reacted with more self antigens and showed higher OD values than the IgM autoantibodies from NOD mice. In contrast, the IgG autoantibodies from NOD serum reacted with more antigens and displayed higher OD readings than did IgG autoantibodies from C57BL/6 mice. Among the antigens recognized by the autoantibodies, particularly of the IgG class, were self antigens known to induce experimental autoimmune diseases in NOD and C57BL/6 mice. In addition, IgG autoantibodies from NOD mice reacted with self antigens reported to mark the spontaneous autoimmune diabetes that characterizes this strain of mice. These results suggest that naturally occurring IgG autoantibodies reflect susceptibility to induction of specific autoimmune diseases. In addition, the results suggest that IgM autoantibodies may by associated with mechanisms that might prevent autoimmune disease.   © 2001 Academic Press

Introduction

Healthy humans and mice are naturally populated with antibodies to self-antigens such as insulin, DNA, myelin basic protein, thyroglobulin among others [1–4]. These autoantibodies are present across strains and species [5–7]. The detection of autoantibodies in cord blood of newborns suggests that their synthesis might be independent of stimulation by foreign antigens [8, 9]. Although some of the self antigens recognized by autoantibodies have been identified, many are unknown. Furthermore, the connections between natural autoantibodies and autoimmune disease need to be investigated.

To identify self antigens targeted by autoantibodies associated with health or disease, we studied two strains of mice that differ in their susceptibility to experimental autoimmune disease. The NOD/LtJ strain spontaneously develops insulin-dependent diabetes mellitus (IDDM) as a consequence of the autoimmune destruction of the insulin-producing β-cells of the pancreas [10]. In contrast, the C57BL/6 strain does not develop any spontaneous autoimmune

disease, but is susceptible to induction by immunization of two diseases: experimental autoimmune encephalomyelitis (EAE) [11] and experimental autoimmune myasthenia gravis (EAMG) [12]. The mice were raised under pathogen-free conditions, so their repertoire of autoantibodies could not have been caused by pathogenic infections. Moreover, the mice are inbred, and so lack individual variation due to differences in genetic background. In this study, we analyzed the IgG and IgM reactivity of spontaneous serum autoantibodies reactive to a panel of 54 defined self-antigens.

Materials and Methods

Mice

Female mice of the NOD/LtJ strain were raised and maintained under pathogen-free conditions in the Animal Breeding Center of this Institute from breeders kindly supplied by Dr E. Leiter of Jackson Laboratories. These mice manifest insulitis beginning at approximately 1 month of age, which progresses to overt hyperglycemia beginning at roughly 3 months of age. The cumulative incidence of IDDM rises to

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85% or greater by 6 months of age [13]. Female C57BL/6 mice were also raised here.

Blood samples were taken from the lateral tail vein of groups of five normoglycemic female mice of each strain, aged 2 months. The blood was allowed to clot at room temperature, and after centrifugation, the sera were pooled and stored at −20°C until used.

Antigens

The 54 antigens used in these studies are enumerated in Table 1. These antigens were chosen from among the proteins, nucleotides and phospholipids reported to interact with autoantibodies. The antigens are classified in different groups according to their cellular localization, tissue distribution or function in the organism. We included two bacterial antigens, PPD and LPS, to serve as positive controls.

Antibodies

Secondary antibodies used in the ELISA assay were F(ab')2 rabbit anti-mouse IgG Alkaline Phosphatase (AP) and F(ab')2 rabbit anti-mouse IgM AP. Both were purchased from Jackson ImmunoResearch (West Grove, Pennsylvania, USA) and were used at a final dilution of 1:1500.

ELISA assays

Each well of 96-well ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) was coated overnight with 100 µl containing an antigen dissolved in carbonate buffer, pH 9.6. The plates were washed with PBS Tween 0.05 % (PBST), and blocked for 2 h with BSA 3%. Based on preliminary experiments, serum samples were tested at a 1/100 dilution in BSA 0.3%. Following 3 h of incubation at 37°C, the samples were removed and the plates were washed with PBST. Bound antibodies were detected with an appropriate alkaline phosphatase (AP)-conjugated antibody (Jackson Immuno-Research Labs, Inc.) incubated for 1.5 h at 37°C in a 1:1500 dilution in 0.3% BSA (Sigma, Rehovot, Israel). After washing, the plates were incubated with the substrate p-nitrophenyl/phosphate (P104, Sigma, Israel), and the OD was read using an ELISA reader at 405 nm.

The results represent the mean±SD of three independent assays; each assay was done in duplicate. The average variation in OD between assays of the same sera tested in different days was less than 10%. The background OD obtained in antigen-free wells incubated with serum or fractions was subtracted from each experimental value. The results are presented in two ways using Figures and Tables. The reactivities against the whole set of antigens are presented in Figures 1 and 2, as the mean OD±SD. Tables 2 and 3 focus on the more significant reactivities by tabulating

the OD values above chosen thresholds. We used thresholds or values of 0.3 and 0.4 for IgG and IgM, respectively.

Results

Serum IgG of NOD and C57BL/6 mice

Figure 1 shows that serum IgG from NOD mice reacts with more self-antigens than does the C57BL/6 serum, and also displays higher values of OD in the few cases of antigens recognized by both strains of mice (1.14 and 1.58 compared to 0.4 and 0.36 for MOG and AchR, respectively, antigens # 48 and 50). Table 2 lists the OD values above a threshold of 0.3 for serum IgG. The NOD serum harbored IgG autoantibodies against 15 different self antigens. Two of these antigens have been associated with the spontaneous diabetes of this strain: the 60 kDa heat shock protein (HSP60) and glutamic acid decarboxilase (GAD) (antigens # 45 and 46). We also detected autoantibodies to 13 other self antigens not linked to autoimmune diabetes. These include autoantibodies directed to antigens associated with cell structure (tubulin, collagen I, heparin, laminin and collagenase; antigens # 2, 7, 12, 13 and 14) and with the cell membrane (glucocerebroside; antigen #16). We also detected autoantibodies to the nuclear antigens histone IIA and single- and double-stranded DNA (antigens # 29–31), and to the complement protein C1 present in plasma (antigen # 38). NOD serum IgG also displayed reactivity against two antigens of the central nervous system, myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP), and reactivity to the acetylcholine receptor (AchR) (antigens # 48–50).

The autoantibody reactivities in both NOD and C57BL/6 sera would appear to be in the same range of magnitude as the IgG binding to the foreign antigen LPS (antigen # 56). Table 2 shows that the IgG autoantibodies from C57BL/6 serum bound to only two antigens at an OD greater than 0.3: MOG (antigen # 48) and AchR (antigen # 50).

Serum IgM of NOD and C57BL/6 mice

The results obtained with serum IgM contrasted with those obtained with serum IgG. Figure 2 shows autoantibodies of the IgM class of C57BL/6 and NOD mice, and Table 3 summarizes the OD reactivities above a threshold of 0.4. It can be seen that both strains of mice harbor IgM antibodies that recognize antigens related to cellular structure, cellular metabolism, the nucleus and specific tissues. In most of these groups, the IgM autoantibodies present in the C57BL/6 serum recognize more antigens than the NOD IgM autoantibodies. For example, in the group of cellular structure antigens, C57BL/6 IgM reacted with actin, myosin, vimentin, collagen I, heparin and collagenase (antigens # 1, 3, 5, 8, 12 and 14), whereas NOD IgM antibodies only reacted with heparin and

Table 1. *Antigens used*

Group	Function/structure	#	Antigen	Catalogue
Cellular structure	Cytoskeleton	1	Actin	A3653
		2	Tubulin	T4925
		3	Myosin	M6643
		4	Tropomyosin	T4770
		5	Vimentin	V4383
	Extracellular matrix	6	Fibronectin	F0895
		7	Collagen I	C7774
		8	Collagen II	C7806
		9	Collagen III	C4407
		10	Collagen IV	C7521
		11	Collagen V	C3657
		12	Heparin	H2149
		13	Laminin	L6274
		14	Collagenase	C9891
Cellular membranes	Phospholipids	15	Cardiolipin	C5646
		16	Glucocerebroside	G9884
		17	Phosphatidylethanolamine	P9137
		18	Cholesterol	C1145
Cellular metabolism	Glucose	19	Enolase	E0379
		20	Aldolase	A8811
		21	Acid phosphatase	P1774
	Apoptosis	22	Annexin 33 kDa.	A9460
		23	Annexin 67 kDa.	A2824
		24	Cytochrome P450C	C3131
	Monooxigenases	25	Catalase	C9322
		26	Peroxidase	P6782
		27	Tyrosinase	T7755
	Others	28	Ribonuclease	R4875
Nucleus	Protein	29	Histone II A	H9250
	DNA	30	Double stranded DNA	D1501
		31	Single stranded DNA	D1501
Plasma proteins	Carriers	32	Transferrin	T4132
		33	Fetuin	F2379
	Coagulation	34	Factor II	F5132
		35	Factor VII	F6509
		36	Fibrin	F5386
		37	Fibrinogen	F4883
	Complement	38	C 1	C2660
		39	C 1 q	C0660
Immune system	Cytokines	40	Interleukin 2	I2644
		41	Interleukin 10	I9276
		42	Interleukin 4	I4269
		43	Interferon-γ	I3265
	Cytokine receptors	44	TNFαR	(a)
Tissue antigens	Heat shock protein	45	HSP60	(b)
	Islet antigens	46	GAD	G2126
		47	Insulin	I0259
	CNS	48	MOG	(c)
		49	MBP	(d)
	Muscle and skeleton	50	AchR	(e)
		51	Myoglobulin	M6036
	Thyroid	52	Thyroglobulin	T1001
	Blood cells and platelets	53	Hemoglobin A	H0267
		54	Spectrin	S3644
Pathogens	Proteins	55	TB PPD	(f)
	Others	56	LPS	L3755

The name, number, catalogue number of Sigma (Rehovot, Israel) and source of each of the 56 antigens used are indicated.
(a) Extracelullar domain of the TNF-α receptor was kindly provided by Prof. David Wallach, (b) purified as described [13], (c) kindly provided by Prof. Avraham Ben Nun, (d) kindly provided by Dr. Felix Mor, (e) kindly provided by Prof. Sara Fuchs, all of The Weizmann Institute of Science, Israel; (f) produced at the Statens Seruminstitut, Copenhagen, Denmark.
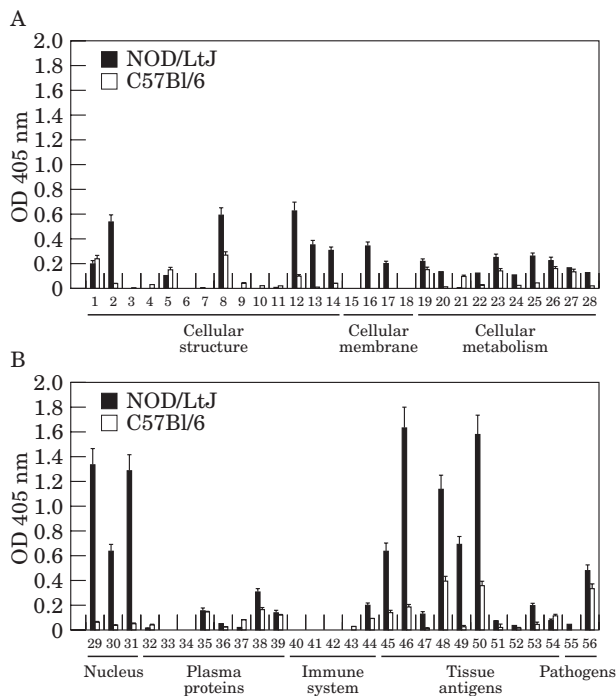
Figure 1. Serum IgG autoantibodies from NOD and C57BL/6 mice.
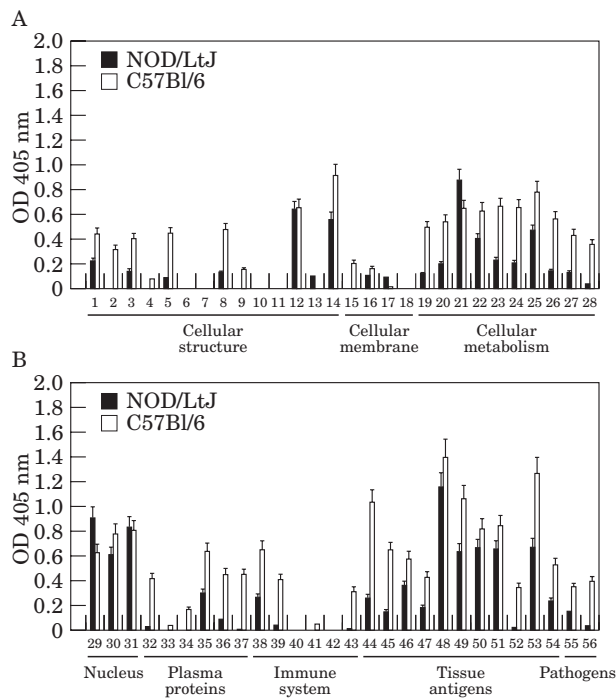

Figure 2. Serum IgM autoantibodies from NOD and C57BL/6 mice.

collagenase. For most of the groups of antigens, the reactions seen with C57BL/6 IgM antibodies showed equal or greater OD values than those seen with the NOD IgM autoantibodies. Only the nuclear antigens histone IIA, double-stranded DNA and single-stranded DNA showed no differences in OD between the strains (antigens # 29–31).

Some groups of antigens recognized by IgM auto-antibodies in C57BL/6 serum were not represented at all among the antigens bound by NOD autoanti-bodies. This was the case for most of the plasma proteins (antigen # 32–39) and for the extracellular domain of the TNF-α Receptor (antigen # 58). It is remarkable that NOD autoantibodies of the IgM class do not display significant reactivity to antigens involved in IDDM, like HSP60, GAD or insulin (anti-gen # 45–47); these antibodies were clearly detected in C57BL/6 serum. Thus, most of the autoreactivity in the NOD serum was IgG and most of the auto-reactivity in the C57BL/6 serum was IgM.

Discussion

The results presented here illustrate a number of points: (a) Autoantibodies, both of the IgG and IgM isotypes, are detectable in the sera of NOD and C57BL/6 mice; (b) the IgG autoantibodies in the NOD mice are reactive at higher OD values and to more self antigens than are the IgG autoantibodies of C57BL/6 mice; (c) the IgM autoantibodies in the C57BL/6 mice are reactive at higher OD values to more self-antigens than are the IgM autoantibodies of NOD mice; and (d) the specificities of the autoantibodies, particularly of the IgG autoantibodies, include self antigens which are known to be able to induce experimental autoim-mune diseases in NOD and C57BL/6 mice. The NOD mice also manifested IgG antibodies to self antigens reported to mark the autoimmune diabetes that develops spontaneously in these mice.

At the outset, one might question whether the autoreactivities detected here represent specific autoantibodies or merely non-specific binding. We believe, however, that the autoantibodies are probably specific; the sera were diluted 1:100, and the OD magnitudes of the autoreactivities were at least as great as those detected for the foreign bacterial anti-gens LPS or PPD. Moreover, it is not likely that the autoantibodies, particularly the IgG autoantibodies, were 'polyspecific' antibodies such as those described in the literature [14, 15]. Many of the autoantibodies bound discrete self-antigens unlike the fibrillar pro-teins bound by polyspecific antibodies. Finally, many of the autoantibodies bind self antigens shown to induce specific experimental autoimmune diseases in the NOD and C57BL/6 strains. Thus we believe we cannot simply dismiss these autoreactivites as mean-ingless artifacts; the meaning of these autoantibodies is a worthy subject for investigation.

Important questions relate to the different patterns of the specificities of the IgG and IgM autoantibodies and the possible relationships of the autoantibodies to autoimmune disease. Comparing the patterns of IgG and IgM autoantibodies in the NOD and C57BL/6 strains might suggest that susceptibility to a specific autoimmune disease can be positively associated with specific IgG and negatively associated with specific IgM autoantibodies. Many, if not most experimental autoimmune diseases involve pathogenic T cells

Table 2. *IgG autoantibodies from NOD and C57BL/6 mice*

	Antigen	#	NOD/LtJ	C57BL/6
Cellular structure	Tubulin	2	0.54	—
	Collagen I	7	0.60	—
	Heparin	12	0.63	—
	Laminin	13	0.35	—
	Collagenase	14	0.30	—
Cellular membrane	Glucocerebroside	16	0.34	—
Nucleus	Histone II A	29	1.33	—
	DS DNA	30	0.63	—
	SS DNA	31	1.29	—
Plasma proteins	C 1	38	0.31	—
Tissue antigens	HSP60	45	0.64	—
	GAD	46	1.64	—
	MOG	48	1.14	0.40
	MBP	49	0.69	—
	AchR	50	1.58	0.36
Foreign antigens	LPS	56	0.48	0.34

Significant reactivities detected in serum IgG from NOD and C57BL/6 sera. Only reactivities above the thresholds are shown (OD≥0.3). Reactivities below threshold are indicated as (—).

Table 3. *IgM autoantibodies from NOD and C57BL/6 mice*

	Antigen	#	NOD/LtJ	C57BL/6
Cellular structure	Actin	1	—	0.45
	Myosin	3	—	0.41
	Vimentin	5	—	0.45
	Collagen I	7	—	0.48
	Heparin	12	0.64	0.65
	Collagenase	14	0.56	0.91
Cellular metabolism	Enolase	19	—	0.49
	Aldolase	20	—	0.54
	Acid phosphatase	21	0.87	0.64
	Annexin 33	22	0.40	0.63
	Annexin 67	23	—	0.66
	Cytochrome C	24	—	0.65
	Catalase	25	0.47	0.78
	Peroxidase	26	—	0.56
	Tyrosinase	27	—	0.43
Nucleous	Histone II A	29	0.91	0.63
	DS DNA	30	0.61	0.78
	SS DNA	31	0.83	0.81
Plasma proteins	Transferrin	32	—	0.42
	Factor VII	35	—	0.64
	Fibrin	36	—	0.45
	Fibrinogen	37	—	0.45
	C 1	38	—	0.66
	C 1 q	39	—	0.41
Immune system	TNFαR	44	—	1.03
Tissue antigens	HSP60	45	—	0.65
	GAD	46	0.36	0.58
	Insulin	47	—	0.43
	MOG	48	1.16	1.40
	MBP	49	0.64	1.06
	AchR	50	0.67	0.82
	Myoglobulin	51	0.66	0.84
	Hemoglobin A	53	0.67	1.27
	Spectrin	54	—	0.53

Significant reactivities detected in serum IgM from NOD and C57BL/6 sera. Only reactivities above the thresholds are shown (OD≥0.4). Reactivities below the threshold are indicated as (—).

[2, 16]; this would explain the connection between disease susceptibility and the prevalence of IgG autoantibodies dependant on active T cell help. The IgG autoantibodies might herald a relatively high frequency of activated autoimmune T cells available for expansion and differentiation by active immunization with specific self-antigens. Indeed, Fathman and co-workers demonstrated that self reactive T cells directed to endogenously processed and presented self antigens can be elicited in NOD mice [17]. NOD mice would seem to have a high frequency of self-reactive T cells [18]. Whether this increased self-reactivity is linked to the particular features of the NOD MHC class II IA^{g7} molecule remains unclear [19–21]. However, it seems that under appropriate conditions spontaneous self-reactivity can be translated into active autoimmune disease. Indeed, both the NOD and the C57BL/6 strains are susceptible to active induction of the experimental diseases to which they show IgG autoantibodies; this is particularly clear for the C57BL/6 strain in which their two natural IgG autoantibodies, anti-MOG and anti-AchR, represent the two experimental autoimmune diseases most readily inducible in these mice: EAE via MOG [11] and EAMG via AchR [12].

The association of IgM autoantibodies with the generally greater resistance of the C57BL/6 strain is less understandable. If the IgM autoantibodies were merely neutral, one might expect the NOD serum to show a similar degree and scope of IgM autoreactivity. Thus it is conceivable that the IgM autoantibodies prevalent in C57BL/6 mice might reflect some regulatory mechanism that protects C57BL/6 mice, but is lacking in NOD mice. The possible functions of IgM autoantibodies in autoimmune regulation need further experimentation [22].

The findings presented here are compatible with the idea that the regularity of the self antigens involved in experimental autoimmune diseases in rodents is connected in some way to an underlying regularity of natural autoimmunity that precedes the induction and development of the specific autoimmune disease. This idea, embodied in the concept of the immunological homunculus [2–4], suggests that specific autoimmune disease involves the dysregulation of natural autoimmunity to specific target self-antigens. Susceptibility to EAE [11] and EAMG [12] in C57BL/6 mice would seem to be forecast by the natural IgG autoimmunity to MOG and AchR. Likewise, the susceptibility of NOD mice to the induction of murine lupus [23] and to EAE [11] would go along with the specific IgG autoantibodies in NOD serum. The presence of natural IgG anti-AchR in NOD mice suggests that this strain would be susceptible to experimental autoimmune myasthenia gravis.

The problem, of course, is to understand the factors that promote or block the transition from benign to pathogenic autoimmunity. Note that the realization of a potential for a certain disease can be influenced by seemingly non-specific factors: the spontaneous diabetes of NOD mice can be aborted by infection [24–26] or by the administration of an oligonucleotide containing the CpG motif [13] that interacts with the innate tlr-9 receptor [27]. NOD diabetes can also be prevented by inducing murine lupus [23]. This suggests that the expression of a spontaneous autoimmune disease can be regulated by manipulation of other seemingly unrelated autoreactivities present in the immunological homunculus. For example, Lewis rats are susceptible to active induction of EAE or adjuvant arthritis [28]; but both diseases cannot be induced simultaneously in the same rat—EAE always dominates (Mor and Cohen, unpublished observations). Of course, patterns of autoimmunity inherent in inbred rodents raised free of pathogens are only an introduction to the problem of defining disease susceptibility in outbred humans exposed to the real world. Can the patterns of autoantibodies in humans be used to anticipate susceptibility to a specific autoimmune disease?

Acknowledgements

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