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Immunology

Murine monoclonal anti-DNA antibodies with an absolute specificity for DNA have a large amount of idiotypic diversity

(lupus erythematosus/clonal diversity/idiotype)

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ABSTRACT **The clonal heterogeneity of nine monoclonal antibodies with absolute specificities for deoxyribonucleic acid (DNA) was analyzed. These monoclonal anti-DNA antibodies were generated in three different fusion experiments using autoimmune (NZB × NZW)F_1 mouse spleen cells. Isoelectric focusing analyses demonstrated different isoelectric points within the IgG2a and IgG2b subclasses. Three anti-idiotypic antisera were prepared (one in a rabbit and two in mice) against two monoclonal anti-DNA antibodies. These antisera detected idiotypic determinants uniquely associated with homologous hybridoma anti-DNA antibodies. Two of these idiotypes could be detected at low levels in the sera of (NZB × NZW)F_1 mice. Anti-PME77 idiotypic antiserum had no effect *in vitro* on the total binding capacity of (NZB × NZW)F_1 sera. Taken together these results demonstrate that, in (NZB × NZW)F_1 mice, the anti-DNA antibody repertoire contains molecules that show similar antigen binding characteristics but are not structurally uniform.**

The autoimmune disease of (NZB × NZW)F_1 (B/W) mice is characterized by the spontaneous development of hypergammaglobulinemia, hypocomplementemia, and glomerulonephritis, and it resembles human systemic lupus erythematosus. These mice produce a variety of autoantibodies, including antibodies directed against deoxyribonucleic acid (DNA), which are thought to play a major role in the pathogenesis of the disease (1).

A detailed characterization of these autoantibodies is important to understanding the etiology of the disease and to studying autoimmune phenomena. More precisely, characterization would allow the definition of the homogeneous or, conversely, the heterogeneous nature of this antibody population, the determination of whether anti-DNA antibodies produced in murine systemic lupus erythematosus are identical to those produced by normal individuals, and the correlation of the presence of a particular family of anti-DNA antibodies with clinical manifestations.

Hybridoma technology provides a unique opportunity to analyze the anti-DNA antibodies produced *in vivo* in autoimmune mice (B/W, MRL/1, BXSB). We recently reported the production from B/W spleen cells of anti-DNA monoclonal antibodies (mAb) whose antigenic specificities were demonstrated to be identical and directed against the B helical form of double-stranded DNA (ds DNA) (2, 3).

To investigate further the heterogeneity of the B cell clones producing anti-ds DNA antibodies, we studied the isoelectric focusing and idiotypic properties of nine anti-ds DNA mAb. The present investigation demonstrates that anti-DNA mAb with an absolute specificity for DNA have a large amount of idiotypic diversity.

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MATERIALS AND METHODS

Experimental Animals. A/J, NZB, DBA/2, and C57BL/6 mice were obtained from the Centre de Sélection et d'Elevage des Animaux de Laboratoire–Centre National de la Recherche Scientifique (Orléans, France). NZW mice were generously provided by Pierre Verroust (Hôpital Tenon, Paris). B/W mice were the offspring of NZB females and NZW males. New Zealand White rabbits were obtained locally (La Clef des Champs, Orléans, France).

DNA-Binding Capacity. DNA-binding capacities of B/W mouse sera were measured by using a cellulose ester filter radioimmunoassay with ds [^{14}C]DNA from *Escherichia coli* (Amersham, Le Vésinet, France) (4).

Cell Fusion. The hybridomas secreting anti-DNA antibodies were obtained after fusions between a nonsecreting myeloma line (P3x63-Ag8653) and B/W spleen cells. The selection of hybrids producing anti-DNA antibodies, cloning and subcloning of the lines, and characterization of the classes and subclasses of the antibodies were all described in detail previously (2).

Purification of Anti-DNA mAb. Culture media were collected from the established hybridoma cell lines. Immunoglobulins (Ig) were precipitated in 50% saturated ammonium sulfate and dialyzed in phosphate-buffered 0.15 M saline. The antibodies were purified by affinity chromatography as described (3).

Radioiodination of mAb. Thirty micrograms of purified F227, MA16, MA512, PME11, PMF79, PMF10, PMF50, and PME77 mAb were radioiodinated according to Hunter's protocol (5). The specific activity of the various ^{125}I-labeled reagents was 5–10 × 10^4 cpm/μg.

Isoelectric Focusing. Isoelectric focusing in agarose was performed according to the method described by Rosen and Aman (6). Sorbitol (10%) and agarose (0.8%) (agarose EF; LKB Instruments) were dissolved in double-distilled water. The pH 3.5–9.5 Ampholine carrier ampholyte solution (LKB) was added at a final concentration of 2%. The mixture was poured onto a Gelbond covered glass plate (LKB) and kept overnight at 4°C. Electrode strips containing 0.5 M NaOH or 0.5 M acetic acid were applied. Twenty-microliter samples of cell culture supernatants were placed onto sample application pieces. After focusing, pH was measured with a surface pH electrode. The gel was cut into 0.5-cm slices. The slices were incubated in 0.2 ml of phosphate-buffered saline for 24 hr at 4°C. The anti-DNA

Abbreviations: mAb, monoclonal antibodies; ds DNA, double-stranded DNA; Id, idiotype.

activities of the eluates were tested in a solid-phase radioimmunoassay (2). The Ig content was analyzed in a solid-phase radioimmunoassay using plastic plates coated with rabbit anti-mouse Ig as the adsorbing surface and ^{125}I-labeled rabbit anti-mouse Ig as a tracer (specific activity 10^6 cpm/μg).

Preparation of Anti-Idiotype (Id) Antisera. Anti-Id antisera were prepared against F227 and PME77 anti-DNA mAb, which were obtained in two separate fusion experiments. The production and the characterization of NZB and A/J mouse anti-Id antisera against purified F227 mAb have been described (7). Briefly, anti-Id antisera prepared in NZB and A/J mice were shown to recognize different idiotopes of F227 mAb. NZB anti-Id antibodies recognized non-ligand-modifiable idiotypic determinants, whereas A/J anti-Id antibodies recognized partially ligand-modifiable idiotopes. An anti-Id xenoantiserum against PME77 was prepared by immunizing rabbits according to the method described by Ju *et al.* (8). The 33% saturated ammonium sulfate precipitate of the rabbit antiserum was absorbed on affinity columns prepared with Sepharose-bound Ig isolated either from a pool of normal mouse sera or from NZB and NZW sera that lacked detectable DNA-binding capacity. Absorptions were repeated until the acid eluates from each column contained less than 0.001 A_{280} unit.

Idiotypic Binding Assays. Id binding and inhibition of Id binding were carried out as described (7). Briefly, disposable flexible polyvinyl chloride microtiter plates were coated with the anti-Id IgG fraction. The free binding sites on the plastic surface were saturated with 0.5% bovine serum albumin in phosphate-buffered saline. First, direct Id-binding studies were carried out by adding 15,000 cpm of either the ^{125}I-labeled homologous mAb or the various ^{125}I-labeled heterologous anti-DNA mAb. Second, the inhibition of binding of ^{125}I-labeled mAb to its homologous anti-Id was measured by incubating various dilutions of competitors with 15,000 cpm of ^{125}I-labeled mAb. After washing, the plates were cut into individual wells and the bound radioactive material was determined.

Absorption of B/W Mouse Sera on a Column of Anti-PME77 Id Antiserum Conjugated to Sepharose 4B. Five hundred microliters (B/W3) and 350 μl (B/W9) of two B/W mouse sera inhibiting the PME77 Id–anti-Id reaction were absorbed on an affinity column as follows. The Ig fraction of the rabbit anti-PME77 Id antiserum was attached to preactivated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The flow-through fractions were collected and pooled. The elutions were performed with 0.1 M glycine·HCl, pH 2.3. The eluted materials were buffered to neutrality with Tris·HCl, pH 9. The DNA-binding capacities of the unabsorbed B/W mouse serum and its corresponding flow-through fractions were compared in the cellulose ester filter radioimmunoassay. The presence of anti-DNA antibodies in the acid eluates was tested in a solid-phase radioimmunoassay using DNA-coated plastic plates as the adsorbing surface and ^{125}I-rabbit anti-mouse Ig as the tracer.

RESULTS

Isoelectric Focusing of Hybridoma Autoantibodies. After isoelectric focusing was completed, the eluates of gel slices were tested for their Ig contents and DNA-binding capacities. Fig. 1 is an example of the isoelectric focusing patterns of IgG2a (MA16) and IgG2b (PME45) mAb that were obtained from the culture fluid of hybridoma cell lines. MA16 and PME45 mAb gave sharp single DNA-binding peaks at pH 5.5 and 6.25, respectively, corresponding exactly to their Ig content peaks. Table 1 indicates the isoelectric points of anti-DNA mAb. IgG2a mAb had isoelectric points between 5.5 and 8, and IgG2b mAb, between 5.5 and 7. Thus, isoelectric focusing of anti-DNA mAb

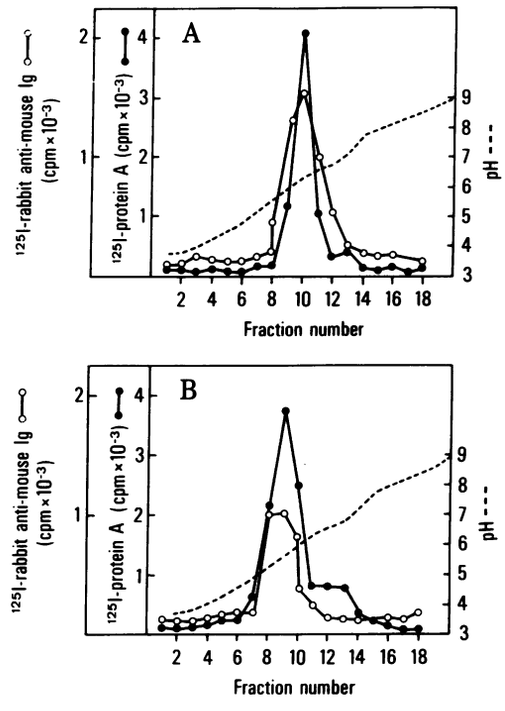

FIG. 1. Isoelectric focusing of IgG2a (MA16) (*A*) and IgG2b (PME45) (*B*) anti-DNA mAb. Focusings were carried out in the pH range 3.5–9.5. The gel was sliced and proteins were eluted. The anti-DNA activity (●) was tested in a solid-phase radioimmunoassay using DNA-coated plastic plates as the adsorbing surface and ^{125}I-labeled *Staphylococcus aureus* protein A as the tracer. The Ig content (○) was tested in a solid-phase radioimmunoassay using plastic plates coated with rabbit anti-mouse Ig as the adsorbing surface and ^{125}I-labeled rabbit anti-mouse Ig as the tracer.

demonstrated a large array of isoelectric points.

Specificity of Absorbed Rabbit Antisera for Idiotypic Determinants. Evidence has been presented elsewhere for the anti-Id specificity of the NZB and A/J mouse antisera prepared against F227 mAb (7). The data in Table 2 provide similar evidence for the rabbit antiserum directed against PME77 mAb. Small amounts of serum from the B/W mouse whose spleen was used in the fusion that subsequently produced the PME77 mAb strongly inhibited the binding of ^{125}I-labeled PME77 to its rabbit anti-Id antibodies. Large amounts of normal mouse sera and of a mixture of NZW and NZB mouse sera that lacked detectable DNA-binding capacity failed to inhibit the Id–anti-Id reaction. The reaction was strongly inhibited by bacteriophage λ ds DNA but not by ribonucleic acid (RNA).

Idiotypic Similarity Among Hybridoma Anti-DNA Antibodies. Inhibition of binding of the labeled F227 and PME77 mAb to their homologous anti-Id antisera by other purified mAb was

Table 1. Isoelectric points (pI) of anti-DNA mAb obtained in three different fusion experiments

Fusion	mAb	Ig class	pI
A	F227	IgG2a, κ	8
B	MA16	IgG2a, κ	5.5
	MA512	IgG2a, κ	6.5
C	PME11	IgG2a, κ	6.5
	PMF79	IgG2a, κ	5.5
	PME45	IgG2b, κ	6.25
	PME77	IgG2b, κ	7
	PMF10	IgG2b, κ	5.5
	PMF50	IgG2b, κ	5.75

Table 2. Idiotypic specificity of rabbit antiserum directed against PME77 mAb

Unlabeled inhibitor		Binding, cpm per plate	Inhibition, %
None		5,515 ± 340	—
NMS*	10	4,905 ± 474	<20
	20	5,104 ± 340	<20
	40	5,753 ± 200	<20
NZB + NZW†	10	4,888 ± 320	<20
	20	5,240 ± 104	<20
	40	5,432 ± 132	<20
B/W PME77‡	10	2,978 ± 87	47
	20	3,639 ± 137	34
	40	4,081 ± 241	26
PME77 mAb, ng	300	338 ± 33	100
	100	773 ± 115	86
	30	2,498 ± 249	55
	10	3,583 ± 115	35.1
	3	4,712 ± 88	20
DNA, μg	100	1,477 ± 127	74.3
	30	1,891 ± 241	63.8
	10	3,168 ± 808	42.6
	3	4,203 ± 126	23.8
RNA, μg	100	4,868 ± 189	<20
	30	4,931 ± 134	<20
	10	4,675 ± 231	<20
	3	4,940 ± 342	<20

Idiotypic specificities were assessed by inhibition of the binding of PME77 mAb to the anti-PME77 Id antiserum.
* Normal mouse serum; amounts are reciprocal dilution.
† Mixture of young NZB and NZW sera that lacked detectable DNA-binding capacities; amounts are reciprocal dilution.
‡ Serum from the mouse whose spleen was used in the fusion that subsequently produced the PME77 mAb; amounts are reciprocal dilution.

carried out to determine the Id interrelatedness or uniqueness of hybridoma anti-DNA antibodies (Table 3). The results are expressed as the amount of antibody required for 50% inhibition. The homologous unlabeled anti-DNA mAb were always potent inhibitors of Id binding. The three anti-Id antisera NZB anti-F227, A/J anti-F227, and rabbit anti-PME77 recognized different idiotypic determinants uniquely associated with homologous hybridoma anti-DNA antibodies as indicated by the lack of detectable inhibition by any other anti-DNA mAb. Moreover, direct Id binding studies showed that none of the three anti-Id antisera bound ^{125}I-labeled heterologous anti-DNA mAb. Thus, one may conclude that some hybridoma antibodies specific for DNA do not share idiotypic determinants.

Table 3. Idiotypic interrelationships among hybridoma anti-DNA antibodies

	Homologous Id–anti-Id interaction		
	A/J anti-F227	NZB anti-F227	Rabbit anti-PME77
Polynucleotide*			
DNA	40	<5	85
RNA	<5	<5	<20
Poly(I)	<5	<5	<20
Poly(A)	<5	<5	<20
mAb†			
F227	3	4	–
MA16	–	–	–
MA512	–	–	–
PME11	–	–	–
PMF79	–	–	–
PME45	–	–	–
PME77	–	–	25
PMF10	–	–	–
PMF50	–	–	–

Interrelationships were assessed by inhibition of the binding of the labeled F227 and PME77 mAb to their homologous anti-Id antisera.
* Polynucleotides were used at 100 μg. Results are expressed as percent inhibition of Id binding.
† Results are expressed as ng required for 50% inhibition; a – indicates 50% inhibition not achieved at 200 μg.

Presence of F227 and PME77 Id in Sera from Individual B/W Mice. We have already shown that F227 idiotopes recognized by A/J antiserum were found in all B/W sera tested and that, conversely, F227 idiotopes recognized by NZB anti-Id antiserum were not detected in B/W sera. Similarly, rabbit anti-PME77 Id antiserum was used to screen a variety of B/W mouse sera to study the presence of idiotypic determinants associated with the PME77 individual hybridoma mAb. Twenty-one B/W sera were examined for their ability to inhibit the PME77 Id–anti-Id binding. Nineteen out of the 21 B/W sera tested inhibited the Id–anti-Id reaction (Table 4). No correlation was observed between the PME77 Id content and the DNA-binding capacity of sera from individual B/W mice. For instance, B/W female 9 serum with a low DNA-binding capacity (1.8 μg of DNA/ml) gave a strong inhibition of the PME77 Id–anti-Id reaction. Conversely, B/W female 19 serum, with a high DNA-binding capacity (4.5 μg/ml) caused no inhibition of Id–anti-Id binding.

Effect of Anti-PME77 Antiserum on the DNA-Binding Capacities of B/W Mouse Sera. Two B/W sera strongly inhibitive of the PME77 Id–anti-Id reaction were passed through an affinity chromatography column of anti-PME77 Id antiserum conjugated to Sepharose 4B (Table 5). The absorption of these sera did not reduce their DNA-binding capacities, as assessed by the cellulose ester filter radioimmunoassay. The fractions,

Table 4. DNA binding capacities and PME77 Id content in individual B/W sera

Serum source	Inhibition given by serum diluted 1:10,* %	DNA-binding capacity,† μg/ml
BW 20	68	1.5
BW 3	60	1.7
BW 14	32	1.7
BW 9	85	1.8
BW 4	30	1.8
BW 7	30	1.8
BW 23	47	1.8
BW 24	45	1.8
BW 25	52	1.8
BW 5	27	2
BW 2	0	2
BW 8	51	2
BW 26	50	2
BW 14‡	46	2.2
BW 13	34	3
BW 28	40	3
BW 22	50	3.6
BW 30	55	3.6
BW 1	50	4
BW 19	0	4.5
BW 21	65	5

* Inhibition of the binding of the labeled PME77 mAb to the rabbit anti-Id antiserum.
† DNA-binding capacities were measured by using a cellulose ester filter radioimmunoassay.
‡ Serum from the mouse spleen was used in the fusion that subsequently produced the PME77 mAb.

Table 5. Absorption of two BW sera on an anti-PME77 Id-conjugated affinity chromatography column

Serum	Capacity of unabsorbed serum,* ng	Anti-PME77 Id-Sepharose 4B: Capacity of flow-through fractions,* ng	Anti-PME77 Id-Sepharose 4B: Activity of eluted fractions,† cpm
BW 3	20	25	2,166
BW 9	25	25	311

*DNA-binding capacities were measured with a cellulose ester filter radioimmunoassay. Results are expressed as the amount of ^{14}C-labeled *E. coli* DNA bound per mg of protein.

†The anti-DNA activities of the eluates were tested in a solid-phase radioimmunoassay using DNA-coated plastic plates as the adsorbing surface and ^{125}I-labeled rabbit anti-mouse Ig as the tracer. Results are expressed as the difference in cpm between the DNA-coated wells and the uncoated wells.

eluted at pH 2.3 and concentrated, bound to DNA, demonstrating that the material retained on the column contained anti-DNA antibodies.

DISCUSSION

The clonal diversity of nine anti-DNA antibody-producing hybridomas obtained from three different fusion experiments using B/W spleen cells was analyzed. These anti-DNA mAb were previously demonstrated to be uniquely specific for DNA and to recognize the B helical form of ds DNA (3). Thus, these anti-DNA mAb were shown to be homogeneous in terms of antigenic specificity. It still remained to be determined whether this subgroup of anti-DNA antibodies arises from the same clonal family or from independent B cell clones.

The isoelectric focusing study demonstrated a large spectrum of isoelectric points for both IgG2a (5.5–8) and IgG2b (5.5–7) anti-DNA mAb. Because all these hybridoma-produced anti-DNA antibodies were shown to contain the same species of light chain (κ) (2), one may conclude that the different isoelectric points observed within the same subclass are generated by differences in the amino acid sequences of the variable regions, by posttranslational modifications, or by differences in the carbohydrate compositions. The large differences between the mAb isoelectric points favor the first hypothesis. Thus, the isoelectric focusing study supports the assumption that most of the nine anti-ds DNA mAb are clonally heterogeneous. This supposition was confirmed by the analysis of these anti-DNA mAb by means of anti-idiotypic antisera.

Three polyclonal anti-Id antisera were obtained. Two of them were prepared in mice (NZB and A/J) against the F227 anti-DNA mAb. These sera detected different idiotopes on the F227 molecules (7). None of these idiotopes was present on the nine other purified anti-ds DNA mAb. Similar results were observed with the rabbit anti-Id antiserum prepared against the PME77 mAb, highlighting the uniqueness of the F227 and PME77 molecules. F227 and PME77 idiotopes were detected in B/W mouse sera, demonstrating that F227 and PME77 anti-ds DNA mAb truly reflect components of the serum Id and do not constitute an aberration resulting from the fusion procedure. Experiments on inhibition of the Id–anti-Id reactions showed that F227 and PME77 idiotypic determinants were present at low levels in B/W mouse sera and represented minor recurrent Id among the anti-DNA antibody populations produced *in vivo*. This was confirmed by the absorption of B/W sera onto anti-Id affinity chromatography columns, which did not reduce their DNA-binding capacities. Taken together, these results demonstrate that anti-DNA antibodies produced in B/W mouse sera with absolute specificities for DNA have a large amount of idiotypic diversity and arise from different B-cell clones.

The restricted antigenic specificity and the large amount of idiotypic diversity of the anti-DNA mAb reported in this study are at variance with the properties of anti-DNA mAb obtained by other groups. Andrzejewski *et al.* (9) analyzed anti-DNA mAb-secreting hybridomas generated from fusion experiments using different MRL/1 spleen cells. Most mAb had unique patterns of antigenic specificity, could bind to several different nucleic acids, and shared idiotypes. Moreover, they showed that one anti-Id antiserum reacted with two-thirds of the anti-DNA mAb and detected recurrent idiotopes (up to 5 mg/ml) in all MRL/1 sera (10). Similar observations were made by Marion *et al.* (11), who analyzed anti-DNA mAb generated from a single B/W mouse.

On the other hand, the characteristics of our anti-ds DNA mAb are similar to those of the B/W anti-RNA antibodies studied by Eilat *et al.* (12, 13). These authors demonstrated that two anti-RNA mAb as well as the totality of anti-RNA antibodies in B/W sera had similar antigen-binding characteristics. Rabbit anti-idiotypic antibodies prepared against the two RNA mAb recognized private idiotypes on their cognate antibodies and had little effect on the RNA binding capacities of the totality of Ig from B/W sera (13).

Finally, the anti-DNA antibody population analyzed in the present study may contribute to the extension of the heterogeneity of the anti-DNA antibodies produced in autoimmune mouse strain, which, so far, according to previous studies (10, 11) were considered as originating from a restricted number of clone families.

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