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Immunology

Unexpectedly high occurrence of catalytic antibodies in MRL/*lpr* and SJL mice immunized with a transition-state analog: Is there a linkage to autoimmunity?

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ABSTRACT **Upon testing the ability of several strains of mice to elicit esterolytic antibodies after immunization with a *p*-nitrobenzyl phosphonate hapten, we have found that the occurrence of catalytic antibodies in SJL and MRL/*lpr* autoimmune mice is dramatically higher than in normal mouse strains (e.g., the wild-type MRL/++ or BALB/c). Fewer than 10 catalytic clones are usually obtained from a single fusion of lymphocytes taken from normal mice, whereas several hundred catalytic clones are obtained in SJL or MRL/*lpr* mice. Differences in the numbers of hapten-binding clones do not account for the high occurrences of catalytic clones in these strains. This phenomenon prevailed in the early responses; in both SJL and MRL/*lpr* mice a significant decline in the appearance of catalytic clones was observed after multiple immunizations. Esterolytic antibodies were not found in MRL/*lpr* mice immunized with haptens that do not mimic the transition state for the hydrolysis of the ester substrate (e.g., with a substrate analog). The catalytic antibodies manifest high specificity to the antigen and variability in their binding and catalytic properties. The use of autoimmunity-prone mice may greatly expand the repertoire of catalytic clones elicited against a transition-state analog hapten. More intriguing is the possible linkage between autoimmunity and the appearance of catalytic antibodies. These results suggest that there is normally a selection against the expression of certain variable genes encoding antibodies with catalytic activity.**

Antibodies elicited against haptens that resemble the transition state (TS) of a given reaction can exhibit catalytic activities. A variety of catalytic antibodies (abzymes) for more than 30 different chemical reactions have been prepared. In fact, antibodies are the most advanced enzyme mimics described thus far (for reviews see refs. 1 and 2). Are catalytic antibodies merely a laboratory creation or do they have any biological significance? Two examples of naturally occurring catalytic antibodies have thus far been reported: Paul *et al.* (3) described cleavage of a peptide (vasoactive intestinal peptide, VIP) by polyclonal antibodies isolated from an asthma patient; and Shuster *et al.* (4) identified DNA-hydrolyzing autoantibodies in the serum of a systemic lupus erythematosus (SLE) patient. In both cases, similar catalytic activity was not demonstrated in the sera of healthy individuals. More recently, we have noted a variable (V)-region sequence homology between groups of catalytic monoclonal antibodies obtained by immunization of normal mouse strains with a TS analog (TSA) hapten and binding antibodies having completely different specificities generated in autoimmune-prone mouse strains (5).

These sporadic indications, and the fact that antibodies with catalytic activity are usually rare among TSA-binding antibodies, raised the intriguing possibility of the existence of a regulatory mechanism which selects against the expression of

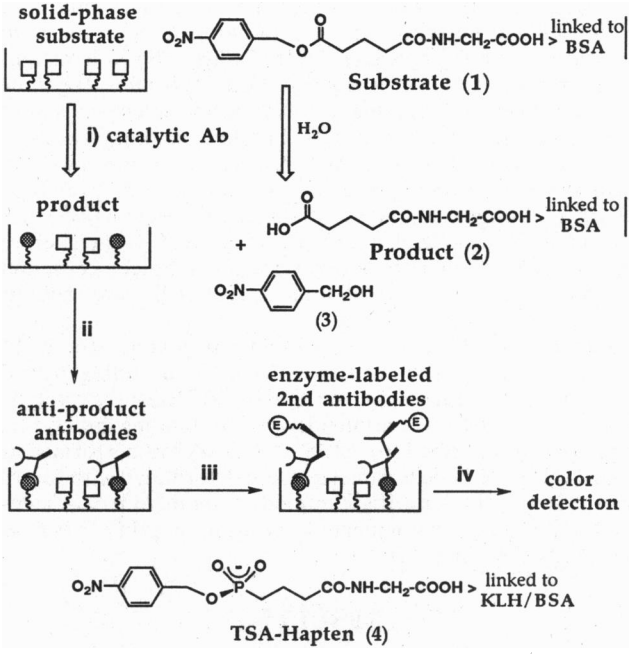

FIG. 1. Structures of ester substrate **1**; the products of its hydrolysis, acid **2** and *p*-nitrobenzyl alcohol (**3**); and the TSA hapten used for immunization, mono-*p*-nitrobenzyl phosphonate **4**. Amide substrate **5** [4-$NO_2C_6H_4CH_2NHCO(CH_2)_3CONHCH_2CO_2H$] is the amide analog of ester substrate **1**. KLH, keyhole limpet hemocyanin. Screening by a catalytic assay using ELISA (catELISA) is based on a "solid-phase" substrate [e.g., a bovine serum albumin (BSA) conjugate of ester **1** immobilized to microtiter plates]. Antibody-catalyzed conversion (step *i*) to a product (i.e., immobilized acid **2**) is detected by ordinary ELISA using binding anti-product antibodies (step *ii*) and an enzyme-labeled second antibody (step *iii*) which gives a signal with a chromogenic substrate (step *iv*).

V-region genes encoding antibodies with catalytic activity. We therefore set out on a comparative study in which catalytic antibodies were elicited in different strains of mice, including several autoimmunity-prone mice. In the studies presented here we examined antibodies raised against *p*-nitrobenzyl phosphonate **4**, which is a stable analog for the TS of the hydrolysis of the respective ester, **1** (Fig. 1). To screen the immortalized repertoire of monoclonal antibodies we exploited the advantages of catELISA (6), which allows the facile detection of all those hybrid clones secreting antibodies that catalyze the cleavage of *p*-nitrobenzyl ester **1**. We have found that early responses of MRL/*lpr* and SJL mice immunized with

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Abbreviations: TS, transition state; TSA, TS analog; V, variable; catELISA, catalytic assay using ELISA; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin.

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hapten **4** are characterized by the dramatically high occurrence of esterolytic antibodies.

MATERIALS AND METHODS

Preparation of Hapten and Substrates. The synthesis of substrates and haptens and the preparation of their protein conjugates were previously described (6, 7).

Immunizations and Fusions. BALB/c, C57BL/6, SJL/J, MRL/MPJ/++ (abbreviated MRL/++), MRL/MPJ-*lpr/lpr* (abbreviated MRL/*lpr*), NZW/LacJ (abbreviated NZW), and $NZBWF_1/J$ (abbreviated NZB×NZW) (from Jackson ImmunoResearch) 8- to 12-week-old female mice were immunized with the KLH conjugates of haptens **4** or **5** (hapten densities 15–20 per carrier molecule) by using a "short" or "long" protocol. For short immunizations, mice were first immunized by a foot-pad injection (50 μg of antigen per mouse in complete Freund's adjuvant). After 17 days, a boost in incomplete Freund's adjuvant was given. Four days later, the mice were sacrificed and draining lymph node and spleen cells were fused with NSO myeloma cells. For long immunizations, a boost, 14 days after the initial immunization, was given in incomplete Freund's adjuvant. Further boosts were given [i.p., 50 μg of antigen in phosphate-buffered saline (PBS) per mouse] in 3- to 4-week intervals. Three and 4 days before fusion two additional i.p. boosts were given. Spleen cells, and in some cases draining lymph node cells as well, were used for the generation of hybridomas.

Screening for Catalytic and Binding Antibodies. Ten to 14 days after fusion, the supernatants of the resulting hybrid clones were simultaneously screened by catELISA for catalytic antibodies and by conventional ELISA for binding antibodies, as previously described (6). ELISA for DNA was performed as described (8). Kinetic parameters were determined with staphylococcal protein A-purified antibodies obtained from ascites fluid and ester **1** (unconjugated) by using an HPLC assay as previously described (6).

RESULTS

Results of Screening for Catalytic and Binding Antibodies. Lymphocytes taken from the spleen and draining lymph nodes of various strains of mice were fused with myeloma cells. The resulting hybrid clones were screened by catELISA for their ability to catalyze the hydrolysis of *p*-nitrobenzyl ester **1** and by ordinary ELISA for binding to the TSA hapten (i.e., to the BSA conjugate of **4**); Table 1 summarizes the results of these experiments. The initial attempts to generate esterolytic antibodies, using the experimental system described in Fig. 1, were all in normal inbred strains (see first four entries of Table 1 and ref. 6). The occurrence of catalytic antibodies—i.e., percentage of wells displaying catalytic activity among all wells containing hapten-binding clones—is generally low—i.e., up to 10 clones per fusion, or an average occurrence of <2%. A dramatic difference was observed when we screened hybridomas derived from lymphocytes taken from SJL or MRL/*lpr* mice (e.g., entries F6 or F9 and F18 in Table 1). The overall number of binding clones was generally higher than with normal mouse strains. However, the remarkable difference is both in the absolute number of catalytic clones [855 in SJL (F6) and 576 in MRL/*lpr* mice (F9)] and in their occurrence (75–87% of the hapten-binding clones). Not only is a higher number and occurrence of catalytic antibodies consistently observed in the *lpr* mice (Table 1, entries F9 and F18), but a normal occurrence of catalytic clones was found in the parental, wild-type MRL/++ mice (F11).

The marked increase of catalytic antibody expression in SJL and MRL/*lpr* mice (Table 1, entries F6 and F8) was not found in NZB×NZW mice [10 weeks old (entry F12) or 6 months old (entry F21)] or in NZW mice (entry F10). In both SJL and MRL/*lpr* mice a significant decline in the appearance of catalytic clones after further immunizations was observed [i.e., about 600 catalytic clones at the early response (21 days, entries F9 and F18) vs. 15 clones after a longer immunization period (65 days, entry F14)]. On the other hand, the number of anti-hapten clones was hardly changed (i.e., about 720 binding clones in the short immunizations vs. 828 after a longer immunization).

The generation of catalytic antibodies in both these mouse strains is undoubtedly antigen-selected. Esterolytic activity was not generated in nonimmunized mice (Table 1, entry F15), not in mice immunized with a TSA hapten that does not correlate with the TS for hydrolysis of ester **1** (entry F20), and

Table 1. Results of screenings for binding and catalytic clones

Entry	Mouse	Immunization	No. of clones			Catalytic/binding, %
			Total	Binding	Catalytic	
F1a	BALB/c	TSA **4**; long	1570	970	9	1
F1b	BALB/c	TSA **4**; long	960	ND	3	ND
F2a	C57BL/6 (3)	TSA **4**; short	576	60	1	1.3
F2b	BALB/c (3)	TSA **4**; short	864	32	0	0
F6	SJL (4)	TSA **4**; short	1200	1128	855	75
F8	SJL	TSA **4**; long	1728	162	10	6
F9*	MRL/*lpr* (4)	TSA **4**; short	768	640	576	87
F18*	MRL/*lpr* (4)	TSA **4**; short	1056	814	630	77
F11	MRL/++ (4)	TSA **4**; short	768	104	4	4
F14	MRL/*lpr*	TSA **4**; long	2208	828	15	1.8
F15	MRL/*lpr* (4)	Nonimmunized	1440	9	0	0
F19	MRL/*lpr*	Amide **5**; short	1104	1000	0	0
F20	MRL/*lpr* (4)	DPA 52;† short	768	ND	0	0
F10	NZW (4)	TSA **4**; short	672	252	3	1.2
F12	NZB×NZW (4)	TSA **4**; short	672	91	2	2.2
F21‡	NZB×NZW (4)	TSA **4**; short	664	315	2	0.6

Details of the immunization protocols are given in *Materials and Methods*. The following periods (in days, d) and number of injections (inj) were used in those fusions that followed a long immunization: F1a, 45 d, 4 inj; F1b, 93 d, 5 inj; F8, 106 d, 6 inj; F14, 65 d, 4 inj. One mouse or a few mice (for those cases, the number is indicated in parentheses) were used for fusion. The numbers of binding and catalytic clones represent results of the initial screening experiments. At this stage, hybridoma growth was apparent at 80–100% of the seeded wells. ND, not determined.

*In F9, lymphocytes for fusion were taken from draining lymph nodes, whereas in F18 both spleen and lymph node lymphocytes were fused.

†DPA52 is an amino phosphinate hapten [Ph-P(=O)(-OH)-CH_2-L-Pro-Gly] which is structurally unrelated to phosphonate hapten **4** or ester substrate **1**.

‡F21 was performed with 6-month-old NZB×NZW immunized mice.

not even in mice immunized with amide **5** [*N*-(4-nitrobenzyl)-*N'*-carboxymethylpentanediamide; entry F19], an analog of ester substrate **1** rather than of the TS of the reaction.

Characterization of the Catalytic Clones. Randomly chosen positive wells from each of the fusions described in Table 1 (1–20, in proportion to the total number of catalytic clones) were subcloned, and the binding and catalytic properties of the resulting monoclonal antibodies were studied in more detail. A set of validation experiments proved that the catELISA signal indeed resulted from an *antibody*-catalyzed hydrolysis of ester **1** (Fig. 2). These experiments indicated the following: (*i*) none of the selected clones scored on catELISA when immobilized substrates other than *p*-nitrobenzyl ester **1** were used [e.g., *p*-nitrobenzyl amide **5** or *N*-(benzylglutaryl)glycine, $PhCH_2OCO(CH_2)_3CONHCH_2COOH$; (*ii*) inhibition of the catELISA color development was observed when the added rabbit anti-product antibodies were preincubated with the free, unconjugated, product **2**; (*iii*) the same affinity patterns of the catalytically active clones were obtained by competitive inhibition of esterolytic activity, as measured by catELISA with ester **1** or by hapten binding, as measured by ordinary ELISA with hapten **4** (Fig. 3). These data exhibited the pattern of specificity as expected for an antibody that was elicited against hapten **4** (i.e., hapten **4** > a "short" hapten > amide substrate **5** > *p*-nitrobenzyl alcohol, **3**). Moreover, inhibition patterns of both binding and esterolytic activities were identical for each individual clone (see for example, Fig. 3 *C* and *D*). This correlation provides strong evidence that catalysis is indeed taking place in the antibody active site and is not due to extraneous enzymic impurities in the assayed supernatants. In addition, several hybridomas from SJL mice (F6) were propagated as ascites and the monoclonal antibodies were purified by protein A affinity chromatography. The esterolytic activity of these antibodies was confirmed by using the free, unconjugated, ester **1** and an ordinary HPLC assay (data not shown).

The isolation of individual hybrid clones after fusion and subcloning does not necessarily guarantee that they in fact originate from different B lymphocytes or that they secrete *different* monoclonal antibodies. Characterization of the antibodies for their relative affinities to the hapten (**4**) and its derivatives (i.e., methyl *p*-nitrobenzyl phosphonate), the substrate (**1**), and the product of its hydrolysis (*p*-nitrobenzyl alcohol, **3**) clearly indicates a clonal variability. Representative data for three randomly selected clones isolated from MRL/*lpr* mice (F9) are given in Fig. 3.

The catalytic efficiency of esterolytic antibodies derived from SJL or MRL/*lpr* mice was compared to that of antibodies elicited against the same hapten in BALB/c mice. Rate accelerations by antibodies isolated from SJL or MRL/*lpr* mice after a short

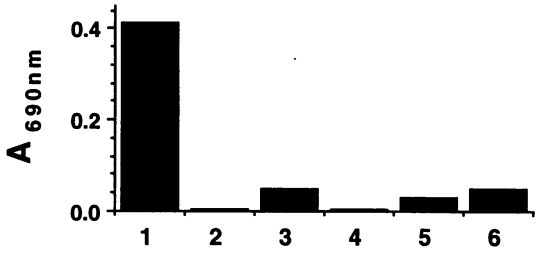

FIG. 2. Catalytic activity of antibody F9.1 was assayed by catELISA using the following substrates and conditions: bar 1, with *p*-nitrobenzyl ester **1**; bar 2, with *p*-nitrobenzyl amide **5**; bar 3, the antibody was incubated with ester **1** in the presence of TSA hapten **4** at 1 μM; bar 4, after incubation of antibody F9.1 with ester **1**, the rabbit anti-product antibodies were not added; bar 5, the anti-product antibodies were added in the presence of free product acid **2** at 0.1 mM; and bar 6, an anti-**4** noncatalytic antibody with ester **1**. These results are typical of those obtained with all the selected catalytic clones.

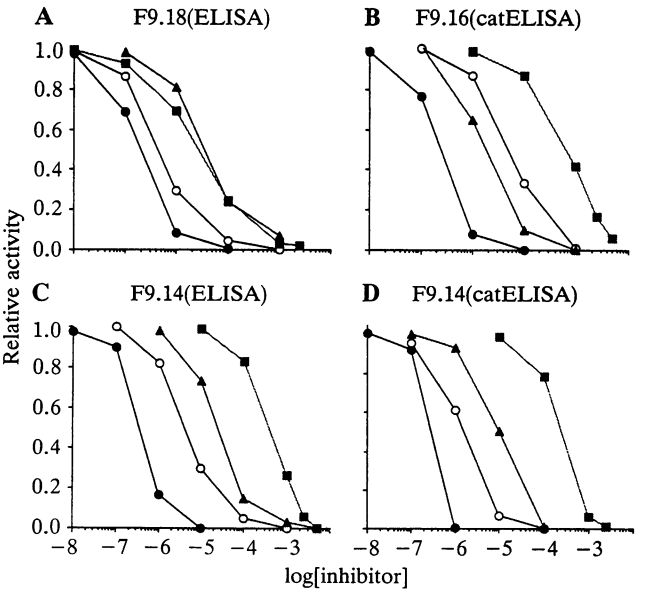

FIG. 3. Affinity patterns of catalytic clones isolated from MRL/*lpr* mice (F9) were obtained by competitive inhibition assays of binding and of catalytic activity. Supernatants of various clones preincubated with increasing concentrations of TSA hapten **4**, as the benzylamide (●); a "short" TSA (mono-*p*-nitrobenzyl methylphosphonate) (○); amide substrate **5**, as the 5-carboxyamylamide (■); or *p*-nitrobenzyl alcohol (**3**) (▲) were assayed by ELISA (*A* and *C*) or by catELISA (*B* and *D*). Data were originally collected in absorbances at 690 nm and are presented here in relative units.

immunization (F6 and F9, respectively) were estimated by catELISA to be in the lower range (i.e., k_{cat}/k_{uncat} = 50–1000 and k_{cat}/K_m = 0.1–10 $M^{-1}s^{-1}$). Antibodies obtained from these mice after longer immunization periods (i.e., F8 and F14) exhibited significantly higher rate accelerations (10^3–10^5). The catalytic power of these antibodies is comparable to that of esterolytic antibodies which were elicited in BALB/c mice after prolonged immunization as well (F1a; see ref. 6).

In general, murine and human autoimmune disorders are characterized by the presence of high titers of antibodies to a number of self-antigens such as DNA. None of the catalytically active monoclonal antibodies isolated from MRL/*lpr* or SJL mice exhibited significant ELISA cross-reactivity toward single- or double-stranded DNA. Likewise, DNA (double-stranded, 20 μg/ml), or cardiolipin, another known self-antigen (at concentrations up to 0.5 mg/ml), failed to inhibit binding or catalytic activity of these clones.

DISCUSSION

The results presented in this study clearly indicate that the number and the occurrence of esterolytic antibodies in autoimmunity-prone MRL/*lpr* mice and in SJL mice immunized with a TSA, is remarkably higher than in normal mouse strains (e.g., BALB/c, C57BL, or MRL/++). Attempts to generate catalytic antibodies in autoimmunity-prone mice were not, to the best of our knowledge, previously reported. In fact, experiments such as those presented here—i.e., screening of the *complete* repertoire of hybridomas obtained from a number of fusions—became feasible only with the development of an appropriate screening methodology—i.e., catELISA (6).

The generation of catalytic antibodies in these mice was found to be antigen-selected. The catalytic antibodies manifest specificity for the antigen (for examples, see Fig. 3) and do not display cross-reactivity with major self-antigens—e.g., DNA and cardiolipin—that share common structural epitopes with the antigen (i.e., the negatively charged phosphonate of hapten

4 and the phosphodiester in DNA or cardiolipin; see ref. 9). Antibodies from the same fusion displayed different affinity patterns and binding parameters such as K_S/K_{TSA} or K_P/K_S (see Fig. 3), different catalytic efficiencies, and different isotypes. These features indicate a clonal variability. It still has to be determined if these differences reflect a diverse usage of germ-line V-region genes, a restricted repertoire which has been diversified by somatic hypermutations, or both. It should be emphasized that the generation of *autoantibodies* in the MRL/*lpr* mice (by a self-antigen such as DNA) is also antigen-selected and not a result of a nonspecific polyclonal activation; moreover, a highly variable germ-line V-gene usage, hypermutations, and affinity maturation were all observed in these mice (9, 10).

A striking difference has been observed in the occurrence of catalytic antibodies derived after a short and a long immunization of SJL and MRL/*lpr* mice. In fact, following multiple immunizations, the number of catalytic antibodies in SJL and MRL/*lpr* mice was as low as observed in the normal mouse strains. In view of the current models of maturation of the antibody response (11), we believe that such a behavior results from repeated hypermutation and selection processes occurring during repeated immunization cycles. Apparently, the gene repertoire dominating the early response, and encoding antibodies with catalytic activity, is of limited diversity. Part of the clones can be still of germ-line origin and were induced to proliferate after the primary immunization. The catalytic power of the clones displaying hydrolytic activity is derived primarily from their differential affinity towards the TSA over the substrate. After multiple immunizations and hypermutations, B-cell clones with relatively high binding affinity will be selected, propagate, and dominate the response. Mutations which result in higher affinity towards the nitrophenyl or acyl moieties of the TSA hapten render the antibody noncatalytic. Similarly, V domains which are covalently acylated by the substrate (i.e., a stoichiometric reaction with no further turnover) will not score in the catELISA. Another possible mechanism is that some of the catalytic antibodies can cross-react with self-antigens and are therefore forbidden; B cells expressing such antibodies are eventually anergized or tolerized. As such, they do not differentiate into memory cells, which are triggered to proliferate upon antigenic rechallenge during hyperimmunization. Comparison of the V-domain sequences between early and late, catalytic and binding antibodies obtained from different mouse strains will help to resolve some of these issues.

Traditionally, catalytic antibodies are elicited against a TSA. It is the complementarity of the active site to the TS, rather than to the substrate, that provides the decrease in the activation energy of the reaction and thereby to the acceleration of its rate (1, 2). An interesting question is whether a substance (i.e., an analog of the ground state substrate rather than of the transition state) can induce antibodies that catalyze its own cleavage (3, 6, 12). After immunization with amide **5**, a stable analog of ester substrate **1** (which is too labile to be used for immunization), we could not detect any antibodies that catalyze the hydrolysis of ester **1** or of amide **5** itself. The same results were obtained in BALB/c (see ref. 6) and in MRL/*lpr* autoimmunity-prone mice (Table 1, entry F19). Thus, even under conditions in which catalytic antibodies readily appear, selection of the proper antibody-forming B cells by TSA **4** seems to be necessary.

The differences observed between SJL mice, which respond similarly to MRL/*lpr*, and NZB×NZW, in which a normal occurrence of catalytic antibodies was observed, are hard to account for. SJL mice are vulnerable to develop particular autoimmune disorders *upon induction*—e.g., with mercury (13) or thyroglobulin (14); these disorders were not induced under the same conditions in normal strains, e.g., BALB/c or C57BL/6. NZB×NZW mice *spontaneously* develop a lupus-like disease (15). In both SJL and NZB×NZW strains, large deletions of the T-cell receptor (TCR) V_β genes were identified (15, 16); the mechanisms leading to autoimmunity in these strains are complex (17). On the other hand, for the MRL/*lpr* strain, a detailed explanation is available. The *lpr* mutation in mice was found by Nagata and coworkers (18) to correlate with defects in Fas antigen, which mediates programmed cell death, or apoptosis, during T- and B-cell development. Expression of the mutated *lpr* causes a massive proliferation of T cells, phenotypically resulting in an autoimmune state (10, 15). Indeed, our experiments show a relatively large number of hapten-binding clones after the immunization of these mice (720 vs. 104 in MRL/++). Still, it is not only the absolute number of catalytic clones that is dramatically elevated in the *lpr* mutant mice over its wild type (600 vs. 4 in MRL/++) but more significantly, their fraction in the overall repertoire of hapten-binding clones (82% vs. 4% in MRL/++).

One aspect of the results described in the present study may apply to the future generation of catalytic antibodies. Comparative studies have indicated that catalytic antibodies derived from a particular fusion share significant structural and biochemical similarity (5, 19). Hence, the use of a variety of mouse strains including autoimmunity-prone strains may allow us to largely expand the repertoire of catalytic clones elicited against a certain TSA hapten. Thus, new antibody catalysts that are too rare to be easily found or that have features different from those found in normal mouse strains might be isolated from autoimmune mice. For example, some of the esterolytic antibodies obtained in SJL mice, in particular after prolonged immunizations, exhibit relatively high catalytic efficiencies, yet with completely different substrate specificities than those antibodies elicited in BALB/c mice.

Another important aspect is the possible linkage between autoimmunity and the appearance of catalytic antibodies. The high frequency of autoantibodies found in the *lpr* phenotype is the direct consequence of an interference in the normal pattern of B- and T-cell selection and maturation (15, 18). Our results may indicate that, in the absence of such selection, a marked and preferential increase in the appearance of catalytic antibodies is observed. The above, together with the indications for the presence of catalytic antibodies in autoimmune patients (3, 4), evokes some intriguing speculations: is the frequent appearance of catalytic clones related to an autoimmune disorder?; is there normally a selection *against* antibodies with catalytic activity?; and do catalytic antibodies play a role in the pathogenesis of some autoimmune diseases (20)?.

The present study has dealt with a model antigen and a reaction of no apparent physiological significance. Important questions still remain unresolved—e.g., how do clones that exhibit catalytic activity differ from ordinary binding clones selected for the same antigen?; and what is the mechanism which leads to the decline in the occurrence of catalytic antibodies after prolonged immunizations? More conclusions are expected from the V-gene analysis and amino acid sequences of the current series of catalytic antibodies. Studies of more reactions and haptens in autoimmune mice and the identification of additional naturally occurring catalytic antibodies under autoimmune conditions may answer some of the questions posed above.

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