ANTIBODIES TO IDIOTYPES OF ISOLOGOUS IMMUNOGLOBULINS*

By NOBUO SAKATO AND HERMAN N. EISEN

(From the Center for Cancer Research and the Department of Biology, Massachusetts Institute of Technology, and the Harvard-Massachusetts Institute of Technology Program for Health Sciences and Technology, Cambridge, Massachusetts 02139)

Antibodies to individually specific antigenic determinants (idiotypes) of immunoglobulins are generally raised in animals that differ genetically from the individual that produces the immunoglobulin (Ig) used as immunogen: i.e. heteroantisera (1) and alloantisera (2), from xenogeneic and allogeneic animals respectively, are the usual sources of anti-idiotypic antibodies (Abs).[1] More recently it has been shown that anti-idiotypes can also be raised in syngeneic animals: BALB/c mice produced Abs to idiotypes of some myeloma proteins of BALB/c origin (3–6), and A/J mice produced anti-idiotypes to antistreptococcal Abs from other A/J mice (7).

Isologous anti-idiotypes (produced in the syngeneic strain) have been particularly interesting because they imply that an individual might be capable of forming Abs to idiotypes of some of his own Igs; and it has, indeed, been recently demonstrated that a rabbit can produce Abs against the idiotypes of other Abs made previously in the same animal (8). However, attempts to raise Abs to idiotypes of isologous Igs seem not to have been uniformly successful (5, 6, 9, 10), and we have therefore attempted in this study to explore further whether most or only exceptional Igs are able to elicit these Abs. BALB/c mice were immunized with five myeloma proteins of BALB/c origin: four were IgA, κ-proteins with antiphosphorylcholine activity (T15, M167, M603, and M511), and one was an IgG2a,κ-protein with no known ligand (LPC-1). Anti-idiotypes were clearly produced to all but one. Tolerance probably accounts for the ineffectiveness of the exceptional protein, T15, whose idiotype has turned out to be remarkably abundant in mice (11, 12). Nevertheless, prolonged immunization with T15 finally induced formation of isoantibodies; most of these Abs had a peculiar specificity (they seemed broadly specific for BALB/c IgA myeloma proteins, especially those with κ-light-chains), but some may have been specific for the T15 idiotype.

Materials and Methods

Tumors. MOPC-167, MOPC-603, MOPC-511, and TEPC-15 were obtained from M. Potter, National Cancer Institute, Bethesda, Md. or from Litton Bionetics, Silver Spring, Md. They were

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[1] *Abbreviations used in this paper:* Abs, antibodies; BSA, bovine serum albumin; PBS, phosphate-buffered saline (0.015 M NaCl-0.01 M K phosphate, pH 7.4).

maintained by serial passage as subcutaneous or intraperitoneal (ascites) tumors in BALB/cAnN or BALB/cJ mice. These tumors arose in purebred BALB/cAnN (13).

Myeloma Proteins. M167, M603, M511, and T15 were isolated from serum or ascites fluid of tumor-bearing mice, as described (14). After precipitation with half-saturated ammonium sulfate, the proteins were subjected to mild reduction (0.01 M dithiothreitol in 0.2 M Tris, pH 8.2) and alkylation (with iodoacetamide), and were then adsorbed on phosphorylcholine-Sepharose 4B, from which they were specifically eluted with 1 mM phosphorylcholine in phosphate-buffered saline, 0.015 M NaCl-0.01 M K phosphate, pH 7.4 (PBS). The eluted proteins were concentrated by precipitation with half-saturated ammonium sulfate, dialyzed against 10 mM K phosphate, pH 7.4, and finally freeze-dried. LPC-1, purified by electrophoresis on agarose, was generously provided by C. Janeway, Jr., National Institute for Allergy and Infectious Diseases, Bethesda, Md.; the tumor producing this protein also arose in a purebred BALB/cAnN mouse (13).

Immunization. As described (3), mice of both sexes were given five (sometimes more) weekly injections of purified myeloma protein in hind foot pads and several (usually four) subcutaneous sites. Protein was incorporated in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) for the first injection, in incomplete Freund's adjuvant (Difco Laboratories) for the second, and in PBS for the remainder. With T15, booster injections of the protein in the complete adjuvant were repeated at approximately monthly intervals after the first course of five weekly injections (see below). Generally, a total of 200 μg protein was given at each injection; but as little as 10 μg can be effective with some of these proteins (unpublished observation).

For some trial immunizations, glutaraldehyde was used to aggregate purified T15 or to coaggregate it with keyhole limpet hemocyanin, or human serum albumin, or M315. The aggregation was carried out with 0.025% glutaraldehyde in PBS, pH 7.0 (15). In special instances, other immunization programs were adopted (see Table IV).

Assay for Anti-Idiotypes. A double Ab radioimmunoassay was used. Typically, 5 μl of antiserum, 10 μl of ^{125}I-labeled myeloma protein (0.1 μg), and 100 μl PBS-bovine serum albumin (BSA) were incubated for 20 min at room temperature, and this was followed by 100 μl of diluted goat antiserum to mouse Igs (corresponding to 25 μl antiserum and diluted by Sepharose absorption, see below). After 1 h at 37°C and about 18 h at 4°C, the immune precipitates were washed twice in the cold with PBS-BSA and counted in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). When dissolved in 1.0 ml of 0.5% sodium dodecyl sulfate, representative precipitates had an absorbancy of 0.2 (at 278 nm). The goat antiserum to mouse Igs was routinely adsorbed with the myeloma proteins used as labeled antigen in the assay (usually by passing 5 ml antiserum over 2 ml Sepharose 4B [packed volume] containing about 6 mg myeloma protein).

For inhibition assays, various inhibitors (unlabeled purified myeloma proteins, or whole mouse serum, or 10 mM phosphorylcholine in PBS) were added to anti-idiotypic serum; then, after 20 min, the ^{125}I-labeled antigen was added and the rest of the assay was as described above.

Mice. BALB/cJ, CE/J, and A/J were obtained from Jackson Laboratories, Bar Harbor, Maine; A/Sn and BALB/cAnN were provided by Dr. Ralph Graff, Washington University, St. Louis, Mo.; BAB/14 and C.AL$_9$ were generously furnished by Dr. Roy Riblet, Salk Institute, La Jolla, Calif.; (BALB/cJ $\times$ A/J)F$_1$ hybrids were raised in our laboratory.

Iodination. Myeloma proteins were labeled with carrier-free ^{125}I according to the method of Sonada and Schlamowitz (16). Sp act were usually about 3×10^6 cpm/μg; for assays whose object was detection of the T15 idiotype in normal sera, the sp act was 3×10^7 cpm/μg.

Other Procedures and Reagents. Proteins were dinitrophenylated and numbers of attached DNP groups per protein molecule were determined as described (17). Ovalbumin, twice recrystallized hen's ovalbumin, and keyhole limpet hemocyanin were obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.

Results

Responses to immunization with the four antiphosphorylcholine Igs are summarized in Table I. Abs to M167, M603, and M511 were detected by 2–3 wk, but Abs to T15 were not detected until a booster injection (in complete adjuvant) was given 1 mo after the final (fifth) injection of the first course. To determine if the Abs were anti-idiotypes, various unlabeled Igs were tested for ability to

TABLE I

Response of BALB/c Mice to Immunization with Phosphorylcholine-Binding Myeloma Proteins (α,κ) of BALB/c Origin

Immunogen	Proportion of mice responding 1 wk after injection number*:							
	2	3	4	5	5‡	5§	6‖	7‖
T15	0/5	0/15	0/15	—	—	—	5/10 (9–34)	15/20 (4–71)
M167	9/9 (20–42)	19/19 (51–89)	18/18 (20–81)	9/9 (59–78)	8/8 (61–70)	9/9 (62–69)	—	—
M603	4/9 (5–7)	14/24 (7–40)	18/24 (6–48)	5/9 (6–37)	5/9 (10–38)	5/9 (8–40)	—	—
M511	1/10 (6)	3/10 (4–9)	3/9 (5–9)	4/9 (10–22)	5/9 (6–25)	4/8 (7–26)	—	—

* Response measured with the ^{125}I-labeled myeloma protein used as immunogen. A response three times above background (control with normal BALB/c serum) was taken as positive. Values in parentheses give the range in percent of ^{125}I-labeled protein precipitated by positive sera from individual mice. All mice injected with T15 and with M511 were BALB/cAnN; of the mice injected with M167, 9 were of the AnN and 10 of the J subline. Of the mice injected with M603, 9 were of the AnN and 15 of the J subline. Background was deducted from values in parentheses.

‡ 2 wk after the fifth injection.

§ 3 wk after the fifth injection.

‖ An approximately 1-mo interval separated injections five and six, and injections six and seven. Injections six and seven were made with T15 in complete Freund's adjuvant.

inhibit the reaction between ^{125}I-labeled immunogen and the corresponding antisera. As is shown in Fig. 1, the reaction in each case was completely blocked by the immunogen. Cross-reacting inhibition by other IgA,κ-myeloma proteins was negligible with anti-M167 and anti-M603, but was pronounced with anti-M511.

Antisera to T15 were different: they were no more effectively inhibited by the immunogen than by the three other α,κ-antiphosphorylcholine proteins or by M460 (also α,κ), which binds DNP, TNP, and vitamin K_3 (18) but not phosphorylcholine. M315, an α,λ2-protein whose ligand-binding specificities are similar to those of M460 (19), inhibited less well; and LPC-1, a γ2a,κ-protein with no known ligands, did not inhibit at all. It appears, therefore, that most of the anti-T15 molecules were specific for IgA proteins in general, especially those with κ-light-chains (Fig. 1). However, the reactions of these sera with T15 were not inhibited by Fab fragments from T15 or the other antiphosphorylcholine proteins, suggesting that the specific determinants are localized in the Fc domain of IgA molecules, but are somehow influenced by the molecule's light chain.

In an alternative approach, specificity was evaluated by comparing the reactivity of the antisera with the labeled immunogen and with other labeled IgA,κ-proteins. This procedure was less satisfactory than the inhibition assay (see above), because various labeled protein preparations were probably denatured to different extents. Nevertheless, the results of the two assays were generally in

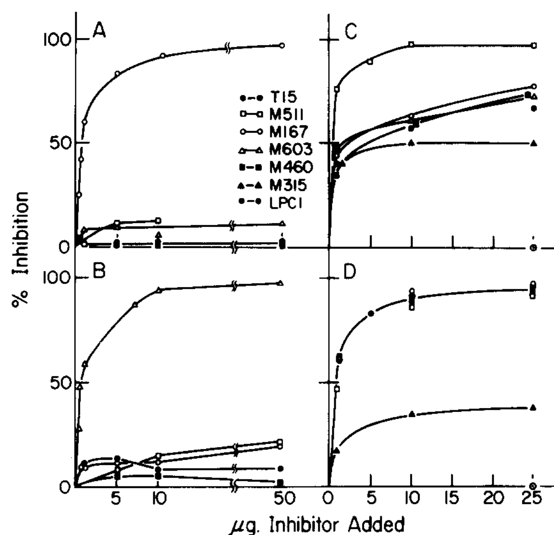

FIG. 1. Specificity of BALB/c antisera to four BALB/c myeloma proteins. Each quadrant shows the inhibitory activity of various myeloma proteins (listed with their symbols in A) for radioimmunoassay reactions between antisera and the corresponding ^{125}I-labeled immunogen. Sera in A, C, and D were from BALB/cAnN; serum in B was from BALB/cJ. (A) Approximately 0.1 μg of [^{125}I]M167 plus 5 μg of pooled antisera to M167 (obtained 1 wk after the second immunizing injection with M167). In the uninhibited control, 44% of the added [^{125}I]M167 was precipitated, and 80% of this amount was inhibitable by 3 mM phosphorylcholine (final concentration). (B) Approximately 0.1 μg [^{125}I]M603 plus 5 μl of pooled, positive antisera to M603 (obtained 1 wk after the third injection of M603). In the uninhibited control 20% of the added [^{125}I]M603 was precipitated, and 65% of this amount was inhibitable by 3 mM phosphorylcholine. (C) Approximately 0.1 μg [^{125}I]M511 plus 5 μl pooled, positive antisera to M511 (obtained 1 wk after the fifth injection of M511). In the uninhibited control 16% of [^{125}I]M511 was precipitated, of which 26% was inhibitable with 3 mM phosphorylcholine. (D) Approximately 0.1 μg [^{125}I]T15 plus 5 μl pooled, positive antisera to T15 (obtained 10 days after the second booster injection of T15; see Table I and Fig. 2). In the uninhibited control 35% of the T15 was precipitated, of which <10% was inhibitable by 3 mM phosphorylcholine.

agreement. For instance, as is shown by the representative results of Table II, anti-M167 and anti-M603 discriminated sharply between the respective immunogens (M167 and M603) and the other myeloma proteins, while anti-M511 was less discriminating, and anti-T15 discriminated poorly, if at all (e.g., it reacted as well with [^{125}I]M511 as with [^{125}I]T15).

Phosphorylcholine, which is bound specifically by M167, M603, M511, and T15 (20), extensively inhibited the reactions of M167, M603, and M511 with their respective antisera, but it inhibited only slightly one of the two anti-T15 sera (Table III). It should be noted that inhibition by phosphorylcholine diminished progressively in successive bleedings of mice immunized with M167. Whether the decrease is due to an increase in serum concentration of anti-idiotypes, or to an increase in their affinity for the idiotype, or to a change in their specificity remains to be determined.

We conclude from these results (Fig. 1 and Tables II and III) that the BALB/c Abs to M167 and to M603 were almost exclusively anti-idiotypes, and those to M511 were largely anti-idiotypes. However, the BALB/c Abs to T15, which

TABLE II
*Specificity of Isologous (BALB/c) Anti-Idiotypes**

Immunogen	Bleeding after injection no:	^{125}I-labeled proteins					
		M167	M603	M511	T15	M315	M460
T15	N‡	33	39	71	**62**	21	—
M167	2	**54**	12	10	0	4	4
	3	**65**	13	14	2	6	8
M603	3	0	**30**	0	0	0	0
M511	5	2	8	**14**	2	—	3
	7	4	13	**19**	5	—	7

* Values are the percent of added ^{125}I-labeled protein (ca. 0.1 μg) that precipitated with 5 μl of immune serum. Values in bold face were obtained with labeled immunogen. Generally 1–3% of ^{125}I-labeled protein was precipitated with normal mouse serum, used as a control of each assay. Values less than 10% (three times control) are of dubious significance. The mice injected with T15 and M511 were BALB/c AnN; those given M167 and M603 were BALB/cJ. Background (normal mouse serum controls) was deducted from the values shown.

‡ N, 10 days after booster injection (no. 7, Table I).

formed only after exceptionally intensive immunization, were specific for IgA proteins (especially with κ-chains) rather than the idiotype of T15; a small proportion of the anti-T15 molecules may, however, have been specific for the idiotype of T15 (see inhibition by phosphorylcholine, line 16, Table III).

To induce a distinct anti-idiotypic response to T15 a variety of additional immunization procedures were explored. Glutaraldehyde was used to aggregate T15, with and without various "carrier" proteins. As is shown in Fig. 2, aggregated T15 was no more immunogenic in BALB/c mice than monodisperse T15, but coaggregates with BSA, M315, and especially with hemocyanin, induced earlier formation of anti-T15 Abs. However, these Abs resembled in specificity those formed after prolonged immunization with monodisperse T15: they discriminated poorly, if at all, between T15 and other antiphosphorylcholine α,κ-proteins (Fig. 3). That some Abs may have been formed to the idiotype of T15 was, however, indicated by marginal inhibition with 3 mM phosphorylcholine: with 10 serum samples from BALB/cJ mice bled 1 wk after the third to fifth injections of T15-hemocyanin, the inhibition was 8.4 $\pm$ 5.7 (mean $\pm$ standard deviation). Fralker et al. (6) have recently also observed that attachment of other carrier proteins (various Igs) augments the immunogenicity of another BALB/c myeloma protein (LPC-1, see below) for BALB/c mice.

We also applied to T15 the procedure used by Janeway and Paul (5) to raise BALB/c Abs to the idiotype of LPC-1, a γ2a,κ-myeloma protein of BALB/c origin. In this procedure, which is based on the earlier work of Iverson (10), alum-precipitated, lightly dinitrophenylated myeloma protein (about 2 DNP groups/150,000 daltons) was injected into mice that had been previously immunized with DNP-hemocyanin. As is shown in Table IV, the effectiveness of this procedure for LPC-1 was confirmed, but it failed to elicit Abs to T15.

TABLE III
Hapten Inhibition of the Reaction between Myeloma Proteins and the Respective Isologous Anti-Idiotypes

Immunogen	Strain	Immunization schedule*	Bleeding after injection no.:	Inhibition by phosphorylcholine‡	No. of Mice
				%	
M167	BALB/cAnN	I	2	76.0 ± 4.1	9
		I	3	52.9 ± 21.6	9
		I	4	41.2 ± 17.1	8
M167	BALB/cJ	I	3	68.7 ± 20.1	10
		I	4	45.8 ± 10.2	8
		II	2	88.6 ± 4.0	5
		II	3	72.6 ± 23.8	5
		II	4	40.4 ± 25.6	5
		III	1§	86.1 ± 10.2	6
		III	2‖	68.3 ± 11.5	6
M603	BALB/cJ	I	3¶	27.7 ± 23.2	8
		I	4	29.4 ± 29.9	11
M511	BALB/cAnN	I	5	27.3**	4
		I	6	30.2**	5
		I	7	28.8**	4
T15	BALB/cAnN	I	N-1‡‡	17.7 ± 4.7	3
		I	N-2‡‡	3.8 ± 5.0	7

* I, Weekly injections of immunogen: first in complete Freunds adjuvant; second in incomplete adjuvant; third, fourth, and fifth in PBS (see Materials and Methods). II, Same as I, but first injection in incomplete adjuvant. III, first injection, immunogen adsorbed on precipitated alum and injected intraperitoneally (i.p.) 4 h after an i.p. injection of 2×10^9 *B. pertussis* organisms. Second injection, immunogen injected i.p. in PBS.

‡ Final ligand concentration, 3 mM. Values are mean ± standard deviation for the number of individual animals listed in extreme right-hand column.

§ 3 wk after first immunization (with alum ppt.)

‖ 4 wk after first immunization (1 wk after second injection, in PBS, i.p.).

¶ 5 wk after first immunization (2 wk after second injection).

** Pooled antiserum.

‡‡ N-1, 10 days after first booster injection; N-2, 10 days after second booster injection. (For intervals, see Table I and Fig. 1.)

To explore the basis for the poor immunogenicity of the T15 idiotype in BALB/c mice, the responses to T15 were examined in some other strains and in some hybrids. The results are summarized in Table V, and specificities of the resulting antisera are illustrated in Fig. 4. The responses of hybrid (BALB/cJ $\times$ A/J)F_1 mice were only slightly better (if at all) than those of purebred BALB/c, but all the other strains responded well. The responses of A/J, A/Sn (not shown), and CE/J mice were similar: some Abs in their early bleedings were probably specific for allotypes of BALB/c α-chains (see inhibition by M315 and M460 in Figs. 4 A and C). Other Abs made in these strains were specific for T15: though

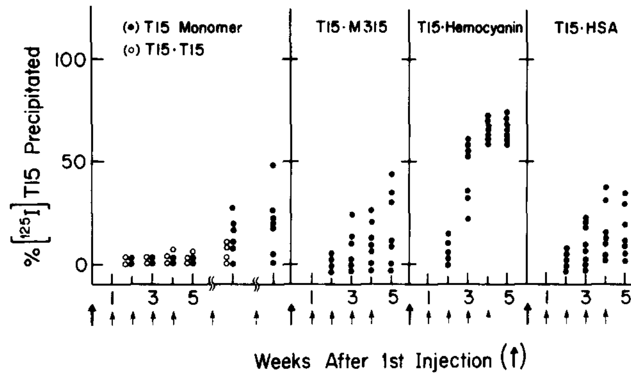

FIG. 2. Increased immunogenicity of aggregated T15. The injected protein was either unaggregated (T15 monomer, ●) or aggregated via glutaraldehyde with itself (T15-T15 polymer, O) or ith M315, or hemocyanin, or human serum albumin (HSA). See Table I for time intervals beyond 5 wk (with T15 and T15-T15 aggregate). Each symbol represents the bleeding of an individual BALB/cAnN mouse.

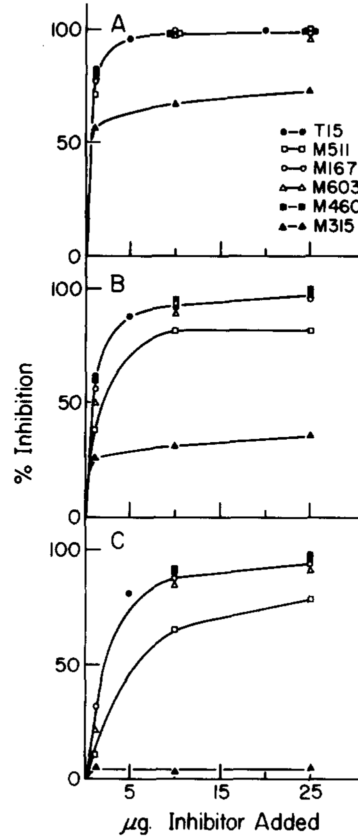

FIG. 3. Specificity of the BALB/c antisera produced by immunization with aggregated T15 (see Fig. 2). The myeloma proteins used as inhibitors are listed with symbols in A. Each test used approximately 0.1 μg [^{125}I]T15 and 5 μl antiserum (pooled, positive sera). The proportion of added [^{125}I]T15 that precipitated in uninhibited controls was 19% in A, 63% in B, and 71% in C. There was no significant inhibition by 3 mM phosphorylcholine. (A) Sera obtained 1 wk after third injection of T15-HSA. (B) Sera obtained 1 wk after third injection of T15-hemocyanin. (C) Sera obtained 1 wk after fifth injection of T15-hemocyanin.

TABLE IV
Induction of Isologous Anti-Idiotypic Antibodies to LPC-1 but not to T15 by Injecting DNP-Proteins into DNP-Primed Mice

Immunization*		Antigen bound‡	
Primary	Secondary	1st bleeding§	2nd bleeding¶
		%	%
OVA	T15	0 (5)	0 (5)
OVA	DNP_3-T15	0 (5)	0 (5)
DNA_3-OVA	T15	0 (5)	0 (5)
DNP_3-OVA	DNP_3-T15	0 (5)	0 (5)
KLH	DNP_3-T15	0 (5)	0 (5)
DNP_4-KLH	DNP_3-T15	0 (5)	0 (5)
DNP_3-OVA	DNP_5-LPC-1	29 (3)	40 (3)
DNP_4-KLH	DNP_5-LPC-1	25 (2)	52 (2)

* 100 μg of protein in complete Freund's adjuvant was injected (primary) into hind foot pads. 10 days later the mice were injected (secondary) with 100 μg of protein in alum-precipitated form 4 h after 2×10^9 *B. pertussis* organisms were injected i.p. 21 days afterwards the mice were injected i.p. with the same protein as in the secondary injection, but in PBS.

‡ Antibody to T15 was measured by radioimmunoassay (see Materials and Methods), using [^{125}I]T15. Antibody to LPC-1 was measured by Farr assay using 0.3 μg of ^{125}I-labeled Fab fragment of LPC-1 and 10 μl antiserum (5); values are means (percent added antigen bound) for individual bleedings from the number of mice shown in parentheses. 0 means $<5\%$. Normal mouse serum (background) values were deducted from the tabulated entries.

§ 21 days after secondary injection.

¶ 12 days after third injection.

they were just as readily inhibited by M511 as by T15, they clearly distinguished these from other α,κ-antiphosphorylcholine proteins (M167 and M603), and the discrimination was especially pronounced in late bleedings (Figs. 4 B and D).

In the BAB/14 congenic strain[2] the genome is BALB/c except for genes of the Ig heavy chain linkage group, which are derived from C57BL/6 (21). About half the Abs made against T15 in BAB/14 were readily inhibited by M315 and M460 and appear, therefore, to be specific for allotypes of BALB/c α-chains (Figs. 4 E and F). Other anti-T15 Abs made in BAB/14 resembled in specificity the anti-T15 molecules formed in BALB/c, the isologous strain: they did not distinguish T15 from the other antiphosphorylcholine proteins (Figs. 4 E and F).

The congenic C.AL_9 strain[2] also has the BALB/c genome, but its genes for the Ig heavy chain linkage group are derived from the AL strain (21). The anti-T15 Abs made in one C.AL_9 mouse cross-reacted extensively with other α,κ-proteins (Fig. 4 G); but in a second mouse of this strain some of the anti-T15 molecules

[2] BAB/14 mice were developed by Dr. Leonard Herzenberg (Stanford University, Stanford, Calif.) from backcross mice provided by Dr. Michael Potter, National Cancer Institute: after a BALB/cAnN × C57BL/Ka cross and 14 successive backcrosses to BALB/cAnN, homozygosity was established for the C57BL/Ka IgC_H allotype (21) and maintained by brother-sister matings. A similar procedure established the C.AL_9 line: after a cross of BALB/cAnN × AL and nine successive back-crosses to BALB/cAnN, homozygosity was established for the AL IgC_H allotype (21) and maintained by brother-sister matings.

TABLE V
Production of Antibodies to T15 in Mice of Various Strains

Strain	Proportion of mice responding after injection number*:			
	2	3	4	5
A/J	9/9 (40.1 ± 23.7)	8/8 (77.6 ± 6.6)	8/8 (88.2 ± 2.1)	8/8 (73.6 ± 5.2)
A/Sn	10/10 (50.1 ± 17.2)	10/10 (84.1 ± 1.3)	10/10 (86.7 ± 2.4)	10/10 (66.4 ± 4.0)
CE/J	10/10 (58.9 ± 9.0)	10/10 (85.1 ± 5.9)	9/9 (76.7 ± 0.7)	10/10 (78.5 ± 2.4)
(A × BALB/c)F_1	0/7	1/7 (8.6)	5/7 (10.2 ± 4.7)	5/7 (11.3 ± 5.0)
BAB/14	3/4 (11.3 ± 7.9)	4/4 (27.4 ± 17.8)	4/4 (55.1 ± 16.8)	4/4 (59.7 ± 12.9)
C.AL_9	5/5 (10.2 ± 10.0)	5/5 (35.9 ± 14.2)	5/5 (49.3 ± 15.2)	5/5 (54.8 ± 18.8)

* Weekly injections as in Materials and Methods (i.e., the same as schedule I of Table III). Responses were measured by radioimmunoassay with [^{125}I]T15. A response three times above background (1-3% of the [^{125}I]T15 precipitated with normal BALB/c serum) was taken as positive. Values in parentheses are mean ± standard deviation for percent [^{125}I]T15 precipitated by positive sera from individual mice. Background was deducted from values in parentheses.

resembled the anti-idiotypes made in A and CE strains: i.e., they distinguished T15 from M167 but not from M511 (Fig. 4 H).

To account for the variety of responses to T15, the level of this idiotype in serum from normal mice of various strains was estimated with a radioimmune inhibition assay, whose specificity and sensitivity are shown in Fig. 5. The antisera for this assay were produced in A/J and CE/J mice and absorbed with M460 (α,κ): though specific for T15 the adsorbed sera did not distinguish it from M511. Despite the resulting ambiguity, the natural prevalence of the T15 (M511) idiotype seems pretty clearly to be inversely correlated with ability to produce Abs against this idiotype: these Abs were not produced by mice that normally contain the T15 idiotype (BALB/c, A/J × BALB/cJ hybrids, and BAB/14), whereas they were readily formed by strains in which the idiotype is just at or below the level of detection (A/J and CE/J). However, in one of two C.AL_9 mice the Abs appeared to be relatively specific for T15 (compare Fig. 4 H with Figs. 4 A and C), and yet normal serum from C.AL_9 mice contains this (or the M511) idiotype. The apparent inconsistency could be due to inability of the key antiserum to distinguish between T15 and M511 (Fig. 5). Similar lack of absolute specificity might also account for disagreements in the literature regarding the presence (22) or absence (11) of the T15 idiotype in serum of C57BL/6 mice.

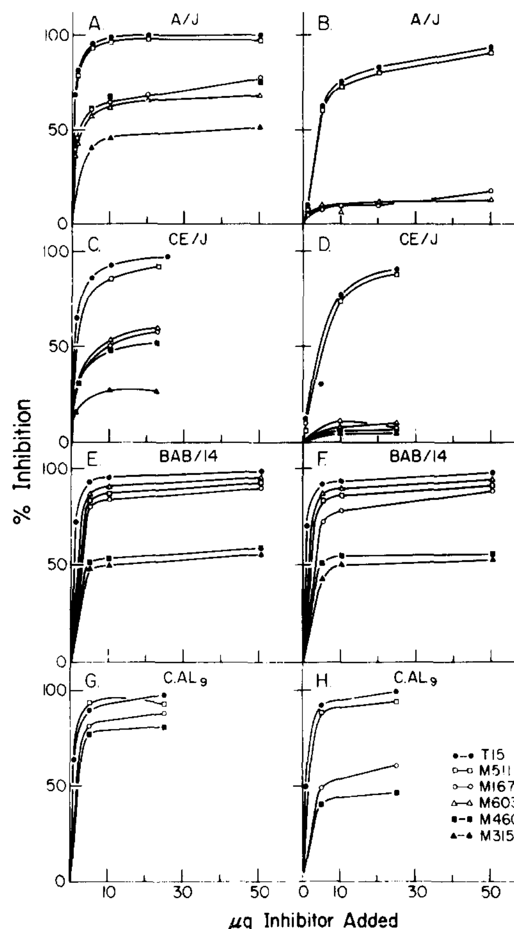

FIG. 4. Specificity of antisera raised in various mouse strains to T15. Approximately 0.1 μg T15 and 5 μl antiserum was used in each assay (for inhibition assay see Assay for Anti-Idiotypes, Materials and Methods). (A and B) Pooled sera from A/J mice bled 1 wk after the second (A) or the third (B) injection. (C and D) Pooled sera from CE/J mice bled 1 wk after the second (C) or fifth (D) injection. (E and F) Pooled sera from BAB/14 mice bled 1 wk after the third (E) or fourth (F) injection. (G and H) Individual sera from two $C.AL_9$ mice bled 1 wk after the fourth injection.

Discussion

The four myeloma proteins on which this report is focussed—T15, M167, M603 and M511—are similar in structure, function, and genetic background (13). They are all α,κ-proteins of BALB/c origin; they bind phosphorylcholine and precipitate specifically with a heteropolymeric pneumococcal polysaccharide; from partial amino acid sequences it appears that three of them (T15, M603, and M511) have the identical sequence for their heavy (α) chains from the N terminus through the first hypervariable region (positions 1-35), and that the heavy chain of the fourth (M167) differs from these only by valine in place of leucine at

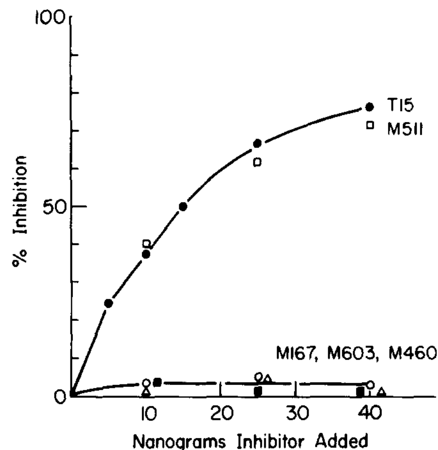

FIG. 5. Specificity and sensitivity of the radioimmunoassay used to measure serum levels of proteins with the T15 (M511) idiotype. The complete reaction (0.125 ml) contained 0.01 μg [^{125}I]T15, 0.025 μl antiserum to T15, obtained from A/Sn mice (1 wk after the third injection of T15 and absorbed with M460), 1 to 10 μl of test material (normal serum from various strains or purified myeloma proteins), and sufficient normal A/J serum to bring the total mouse serum to 10 μl (to provide mouse Ig for the goat antiserum). After incubation, T15-absorbed goat antiserum to mouse Ig was added.

position four (reference 23 and footnote 3). Their light (κ) chains, however, differ considerably in sequence (reference 23 and footnote 3) and the proteins also differ in idiotype and in immunogenicity for isologous (BALB/c) mice.

In ability to induce the formation of isologous antiidiotypic Abs, the order is M167 > M603 > M511 > T15 (Table I). The same order applies to the specificity of the resulting isoantibodies. The inhibition studies of Fig. 1 (all with unabsorbed sera) show that the BALB/c Abs raised against M167 reacted only with this protein, and that the Abs produced against M603 were almost as selective in their reaction with M603. Antisera raised against M511 reacted best with this protein, but they also cross-reacted extensively with other α,κ-proteins.

In contrast to the other antiphosphorylcholine proteins, Abs were produced to T15 only after unusually prolonged immunization of BALB/c mice, and they seemed largely to be specific for α-chains in general, particularly when associated with κ-light-chains (Fig. 1 D). These anti-T15 molecules probably represent an autoimmune response evoked by exceptionally intense immunization. It would be of interest to determine whether there are pathologic changes in mice that make this response (e.g. renal lesions from immune complexes; reference 24).

It must be emphasized that all the isoantisera used here (except in the assay of Fig. 5 and Table VI) were not absorbed. Hence they probably represent an unbiased reflection of the specificities of B cells in normal BALB/c mice. These mice evidently possess B cells that can recognize and respond to the idiotypes of many BALB/c Igs. This has been demonstrated here for three myeloma proteins (M167, M603, and M511), and in previous studies with three others (M315 and M460, reference 3; LPC-1, references 5 and 6; and confirmed in Table IV). B cells with specificity for the idiotypes of isologous Igs are not confined to BALB/c mice

[3] The partial amino acid sequences of heavy and light chains of T15, M603, and M167 are in reference 23; the corresponding sequences of M511 are from E. Appella (personal communication).

TABLE VI
*Concentration of the T15 (M511) Idiotype in Normal Serum from Mice of Various Strains**

Strain	Amount of serum tested	Inhibition	T15/milliliter serum
	μl	%	*μg-equivalents*
BALB/cJ	5	52	4
A/J	10	<5	<0.1
(BALB/c × A)F_1	10	72	3
AKR/J	10	<5	<0.1
CE/J	10	16	0.4
C57BL/6J	10	64	3
BAB/14	1	50	13
C.AL_9	10	65	2
BALB/cJ‡	1	60	24

* The assay system: 0.025 μl A/J anti-T15 serum, 10 ng [^{125}I]T15 (about 20,000 cpm), 1–10 μl serum from normal mice of the indicated strain and a sufficient volume of normal A/J serum to bring total vol of normal mouse serum to 10 μl, sufficient excess of goat-antimouse Ig (absorbed with T15) to precipitate all the Ig in 10 μl of normal mouse serum. To test for the T15 idiotype in normal A/J serum, normal and anti-T15 serum of the CE/J strain was used.

‡ Serum from BALB/cJ mice bled 2 wk after immunization with pneumococcal vaccine (0.1 ml, 2 × 10^9 organisms injected i.p.).

and their myeloma proteins: A/J mice can produce anti-idiotypes to Abs raised by immunizing other A/J mice with a streptococcal vaccine (7).

Why isologous anti-idiotypes to BALB/c myeloma proteins have not been elicitable in some studies (e.g., 9) is not clear. The idiotypes of the Igs tested might have been almost as prevalent as that of T15 (see below); another possibility is that the relatively insensitive precipitin assay was used to detect idiotype to anti-idiotype reactions (9). Whatever the reason, it now seems clear that the Igs capable of eliciting formation of isologous anti-idiotypes are not exceptional; it is possible that almost any Ig can be made to stimulate the production of these Abs.

T15 appears so far to be unusual. Of the seven myeloma proteins tested in this laboratory, it alone failed to elicit distinct anti-idiotypes in isologous (BALB/c) mice when administered by procedures or with adjuvants that are effective with the other proteins (i.e., the use of complete Freund's adjuvant, or injection of the protein as a coaggregate with various "carrier" proteins, or as a lightly dinitrophenylated molecule adsorbed on alum and given to mice that were previously injected with *Bordetella pertussis* and primed to make anti-DNP antibodies). The unresponsiveness to the idiotype could be due to tolerance, resulting from the prevalence of this idiotype in normal BALB/c mice. It seems that virtually all the antiphosphorylcholine Abs made in this strain share the T15 idiotype: thus, injection of a specific anti-T15 serum (produced in A/He mice) into newborn BALB/c suppresses their ability, later in life, to make antiphosphorylcholine Abs (in response, for instance, to immunization with a pneumococcal vaccine [25]). Moreover, this idiotype is extraordinarily abundant in serum of BALB/c mice, even without deliberate immunization (reference 11 and Table

VI): with a total serum Ig level in unimmunized BALB/c of, say, 5 mg Ig/ml serum (probably much less) the finding of 5 μg Ig/ml with this idiotype (Table VI; or higher level, reference 11) means that over 1/1,000 Ig molecules of normal BALB/c have this idiotype.

An alternative explanation for BALB/c unresponsiveness to the T15 idiotype is that these animals lack a required immune response gene (26). Responsiveness should then be dominant, and (BALB/c $\times$ A)F_1 hybrids should resemble purebred A/J, i.e., they should form anti-idiotypes to T15. Instead, like purebred BALB/c, these hybrids are not responsive to this idiotype, which is also readily detected in their normal serum.

It thus seems clear that the inability of BALB/c mice to form anti-idiotypes to T15 is due to tolerance, resulting from the natural abundance of this idiotype in this strain. Though the ensuing depletion (or inactivity) of anti-T15 B cells is likely to be extensive, a few active cells probably persist in some animals. This would account for the finding that phosphorylcholine inhibits slightly (but probably significantly) the reaction between T15 and a few of the BALB/c antisera to T15 (obtained after prolonged immunization with monomer T15 or after briefer immunization with T15-hemocyanin coaggregates).

It is generally believed that each myeloma protein represents one of the many Igs that make up the immensely heterogenous pool of normal Igs. However, with a sensitive hemagglutination-inhibition assay Kunkel has shown that in pooled human Ig the respective idiotypes of several myeloma proteins are present at less than 1 part in 1–30 million, if at all (27). A similarly low frequency of BALB/c myeloma proteins in normal BALB/c mice would probably account for their lack of tolerance and the regularity with which they can make anti-idiotype responses to many of these proteins. What concentrations of idiotypes, or of other indigenous proteins, are required to establish and maintain natural self-tolerance is still not clear. These critical levels can, perhaps, be eventually determined by correlating in individual mice the serum level of certain idiotypes, such as T15, with the capacity to form the corresponding anti-idiotype.

It seems not to have been generally expected that BALB/c mice would be able to form Abs to idiotypes of BALB/c myeloma proteins, perhaps because this seems at first glance to contradict the role of "associative recognition" in the induction of Ab formation. According to this concept (28), an immunogenically effective myeloma protein should contain, in addition to its idiotype, other determinants to which the responding animal is not tolerant. It has been suggested that allotypes can serve as the other determinants, and that the only mice able to form anti -idiotypic Abs to mouse Igs would be those that lack both idiotype and allotype of the immunogen (9). In support of this idea, attachment of IgG from C57BL/6 mice (whose heavy chain allotypes differ from those of BALB/c) to LPC-1, a BALB/c IgG2a myeloma protein, was particularly effective in augmenting BALB/c Ab response to the idiotype of LPC-1 (6). Allotypic disparity probably also contibuted to the immunogenicity of T15 in BAB/14 and C.AL_9 mice (Fig. 4): these congenic BALB/c animals lack the heavy chain allotypes of purebred BALB/c (instead, they have the heavy chain allotypes of the C57BL/6 and AL strains, respectively), and they could thus respond effectively to the BALB/c heavy chain allotype on T15. Nonetheless, the present

results reinforce earlier evidence (3–5) that allotypic discordance is not essential for stimulating the production of Abs to isologous idiotypes.

Since members of an inbred strain can form Abs to idiotypes of Igs from other members of the same strain, it is not surprising that a given individual can produce anti-idiotypes to some of his own Abs (8). The consequences for the Ig repertoire are significant: an individual can form Abs not only to a vast number of different antigens, but also to the combining sites of some (or perhaps many) of these Abs. Anti-idiotypes, moreover, must have their own idiotypes, with which other isologous or autochthonous Igs (anti-anti-idiotypes) might react, etc. This could mean that the number of Igs an individual can make is virtually limitless, or that individual Ig molecules can bind many different antigens (29,30). A number of recent findings provide some evidence for multispecificity of Abs (31–36).

Jerne has proposed that many Igs are "natural" anti-idiotypes, capable of reacting specifically with the combining sites of other Igs, and that through reaction with appropriate B cells these anti-idiotypes might regulate the production of Igs with the corresponding idiotypes (37). The possibility that anti-idiotypes might serve as physiological regulators of Ig production by B cells is supported by the consistency with which mice are able to form Abs to idiotypes of Igs from syngeneic animals, and by the additional finding, to be reported elsewhere, that thymus-derived lymphocytes can also recognize idiotypes of isologous Igs.[4] Though these capabilities have so far been prominently expressed only after intensive immunization, it has recently been reported that autologous anti-idiotypes can be formed spontaneously against Abs that arise in the course of a conventional immune response (38). It is also possible, however, that instead of stimulating the production of autologous anti-idiotypes, a rising level of Ab might serve as a tolerogen, inducing specific unresponsiveness to its idiotype (39).

Summary

To determine if the immunoglobulins (Igs) capable of eliciting the formation of isologous anti-idiotypic antibodies are rare exceptions, BALB/c mice were immunized with five myeloma proteins of BALB/c origin. Anti-idiotypes were produced against all but one. The idiotype of the exception (T15) is remarkably abundant in BALB/c mice, whose unresponsiveness is probably due to tolerance. Nevertheless, prolonged immunization with T15 finally induced the formation of isologous antibodies that seemed largely to be specific for IgA proteins, especially those with κ-light-chains; the reactions of a few of these isologous antisera with T15 were slightly inhibited by phosphorylcholine, suggesting that some anti-idiotypes were probably formed even to this unusually prevalent idiotype. It is likely that under appropriate conditions almost any myeloma protein can elicit isologous anti-idiotypes.

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