INHERITANCE OF ANTIBODY SPECIFICITY V

Anti-2-Phenyloxazolone in the Mouse*

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A dozen of haptens or simple antigens have been found that induce antibodies of restricted heterogeneity in at least some inbred mouse strains (1–13). A manifestation of this restricted heterogeneity at the level of an individual antiserum is a limited number of isoelectrofocusing (IEF)[1] patterns (spectrotypes). It can also be seen at the strain level, different individual immune sera share some, so-called public, spectrotypes. Other methods can be used for the demonstration of this restricted heterogeneity. The most useful of them has been the antigenic characterization of antibodies (classical idiotypes [14, 15]), but sequencing and fine specificity characterization have also been used.

In some cases, the public spectrotypes account for only a small fraction of the total antibody against a determinant but in other cases, they are the majority population. The presence of such a strain-specific antibody pattern is usually controlled by a Mendelian gene or genes closely linked to, but separable from, the Ig H-chain allotype genes. The simplest explanation is that these V_H genes are structural genes for the variable polypeptides of the H chains, in analogy to the simple postulation that the Mendelian allotype genes are structural genes for the constant polypeptides.

We have screened different anti-hapten antibodies of various mouse strains looking for restricted, strain-specific responses. The hapten 2-phenyloxazolone (phOx) was of particular interest because this compound is known to induce a strong T-cell response (16). Some mouse strains were found to produce restricted anti-phOx antibodies which could be characterized by three methods; isoelectric focusing, use of anti-idiotype antibodies, and determination of fine specificity.

Our screening for 11 compounds have been completed although the number of mouse strains tested was only five in some cases. No strain-specific responses could be demonstrated to seven compounds. The results are unpublished but the compounds were NIP and NNP (5), DIP (6), ABA-NP, ABA-MIP and ABS-HOP (8), and DNP. Strain-specific responses have been demonstrated against four compounds: NP (5), NBrP (6), ABA-HOP (8), and phOx (this report).

Materials and Methods

Mice and Their Immunization. All mice were 3–6-mo-old females. They were bred in our

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; cap, epsilon-aminocaproyl; CSA, chicken serum albumin; IEF, isoelectric focusing; NP, (3-nitro-4-hydroxyphenyl) acetyl; NRS, normal rabbit serum; phOx, 2-phenyloxazolone; its structural analogues are described in Methods; RI, recombinant inbred.

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animal colony, and most breeding nuclei were obtained from The Jackson Laboratory, Bar Harbor, Maine. In addition to normal inbred strains, we studied two congenic strains that carry the immunoglobulin H-chain allotype genes in a BALB/c background (17). One breeding nucleus (CB-20) was obtained from Dr. Michael Potter (National Institutes of Health, Bethesda, Md.), and another (BAB-14) from Dr. Lee Herzenberg (Stanford University, Stanford, Calif.). Five recombinant inbred strains that carry different random combinations of BALB/c and C57BL genes were also studied.

Mice were immunized with a 30-μg i.p. injection of alum-precipitated antigen and 10^9 *Bordetella pertussis* bacteria (day 0). They were bled on day 20, immunized again without *B. pertussis* on day 30, and sometimes on day 60. Bleedings were taken on days 40 and 70.

Haptens

2-PHENYL-4-ETHOXYMETHYLENE-5-OXAZOLONE. 2-Phenyl-4-ethoxymethylene-5-oxazolone was purchased from BDH and recrystallized from ethanol. It is a reactive compound which couples spontaneously to proteins by forming peptide-type bonds with amino groups (18). The ethoxymethylene group is lost, and phOx remains protein bound.

2-PHENYL-5-OXAZOLONE AMINOCAPROYL (phOx-cap). 217 mg of 2-phenyl-4-ethoxymethylene-5-oxazolone (1 mmol) was dissolved in 6 ml of acetone, and 195 mg (1.4 mmol) of epsilonaminocaproic acid in 14 ml of 0.24 M $NaHCO_3$. When the two solutions were miced in a flask immersed in an ice bath, a precipitate formed. The mixture was stirred for 1 h in the ice bath and then overnight at 20°C. The precipitate gradually dissolved indicating formation of phOx-cap. This compound was precipitated by bringing the pH to ≅3.0, the mixture was filtered, and the precipitate was lyophilized. The purity and the nature of the compound was tested with thin-layer chromatography. It has an optical density maximum at 348 nm, and the molar coefficient of extinction is 32,000.

phOx-GLYCINE, phOx-ASPARTIC, AND phOx-$CH_2CH_2NH_2$. 1 mmol of phOx and 1.4 mmol of glycine or aspartic acid were mixed and handled as described above for phOx-cap. For the preparation of phOx-$CH_2CH_2NH_2$, 1 mmol of phOx in 20 ml of chloroform was mixed with 1.5 mmol of 1,2-diaminoethane dissolved into 10 ml of chloroform. After an overnight incubation, 4 ml of 1.1 M NH_4HCO_3 was added, and the flask was gently mixed from time to time. After 24 h, the chloroform and the aqueous layers were separated with centrifugation. The aqueous layer was lyophilized.

2-*o*-IODOPHENYL-4-ETHOXYMETHYLENE-5-OXAZOLONE (IphOx). 5 g of ortoiodohippuric acid (Merck art 820736) was dissolved into a mixture of 8 ml of triethyl ortoformate (Koch-Light, Koch-Light Laboratories, Ltd., Colnbrook, England), 40 ml of ethylacetate, and 16 ml of acetic anhydride. The mixture was refluxed with vigorous boiling (120°C) for 3 h and kept at −20°C overnight. The precipitate (IphOx) was filtered dry and dissolved into 50 ml of boiling ethanol. It was kept at −20°C for overnight, and the resulting crystals were dried. Purity was checked with thin-layer chromatography, and the nature of the compound with nuclear magnetic resonance using phOx as the reference compound.

2-*p*-NITROPHENYL-4-ETHOXYMETHYLENE-5-OXAZOLONE (NO_2phOx). Paranitrophenyl hippuric acid (ICN, 17088, ICN Pharmaceuticals, Plainview, N. Y.) was crystallized twice from ethanol and used for the synthesis exactly as iodohippuric acid was used for the synthesis of IphOx. The product was crystallized twice from hot ethanol and characterized with thin-layer chromatography.

IphOx-cap AND NO_2phOx-cap. They were prepared from IphOx or NO_2phOx as Ox-cap was prepared from phOx. The products were characterized with thin-layer chromatography. The molecular coefficient of extinction was 20,000 at 337 nm maximum for IphOx-cap and 13,000 at 313 nm for NO_2phOx-cap.

Antigens. phOx-chicken serum albumin (phOx-CSA) was prepared by stirring a 75-mg suspension of 2-phenyl-4-ethoxymethylene-oxazolone crystals with 1 g of CSA in 20 ml of 5% $NaHCO_3$ for 24 h at 4°C. After incubation, the mixture was centrifuged for 30 min with the minimum of 30,000 *g* (top of the tube), and then excessively dialyzed against 0.15 M NaCl. The resulting solution had an optical density maximum at 352 nm which coincided with the maximum of phOx-cap. The amount of nondialyzable phOx was estimated from this optical density assuming that phOx coupled to the lysine residues of the protein has the same optical density (OD_{352} = 32,000) as phOx coupled to epsilon-aminocaproic acid. The conjugate

contained 20 molecules of phOx per molecule of CSA ($phOx_{20}CSA$). Two other conjugates, $phOx_{25}$ bovine serum albumin (BSA) and $phOx_{16}BSA$ were prepared in an analogous manner.

Assay of Anti-phOx Antibodies. The method was adopted from Klinman et al. (19) with the modification of Karjalainen and Mäkelä (20). Polystyrene tubes were coated for 90 min at 37°C with 0.5 ml of 0.05% $phOx_{16}BSA$, washed and soaked for 90 min at 37°C with 5% normal rabbit serum (NRS) diluted with saline +0.02% Na-azide. They were often stored with NRS for up to 2 wk at 4°C. Before the actual test, the NRS was removed and the tubes were washed once with saline. Antibody concentrations of sera were determined by adding 0.4 ml from a dilution series into duplicate tubes. A standard serum pool was run in each test. It was standardized with purified anti-phOx antibody.

After an overnight incubation at room temperature, the tubes were emptied and washed twice with 1 ml of saline. 0.3 ml of ^{125}I-labeled specifically purified rabbit anti-mouse IgG was added, it contained ≅20,000 cpm. After an overnight incubation at room temperature, the tubes were washed twice and counted.

Anti-Idiotypic Antibodies. BAB-14, a strain congenic to BALB/c, was used as the donor strain of anti-phOx sera because we had more mice of the former type. A pool of secondary and tertiary response sera (29 ml) was mixed with 1 ml of 50% phOx-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), and incubated with gentle stirring for 4 h. The beads were washed with phosphate-buffered saline until the UV absorption of the washing buffer was <0.002. The packed beads were then suspended in 10 ml of 10^{-2} M phOx-cap and incubated at room temperature for 4 h. The mixture was centrifuged and the supernate exhaustively dialyzed against phosphate-buffered saline. The dialyzed material had only one optical density peak (OD = 1.5) at 280 nm. It was assumed to contain 1.1 mg/ml of anti-phOx antibody.

Two rabbits were subcutaneously immunized with two 0.5 mg injections of the purified anti-phOx antibody in complete Freund's adjuvant (CFA) on days 0 and 31. They were bled on day 38, and the sera were absorbed with mouse Ig-Sepharose until they were unable to bind purified ^{125}I-labeled anti-NP antibody.

Determination of Idiotype Content. Conventional radioimmunoassay was set up for the idiotype quantitation. Samples to be studied (0.1 ml, usually one-half-log dilution series) were incubated with 3 ng (0.1 ml) of the purified antibody described above and 0.3 ml of anti-idiotypic serum for 15–24 h at a room temperature. (This amount of anti-idiotype serum was capable of binding 40% of label). After 24 h at a room temperature, a 0.3-ml excess of sheep anti-rabbit Ig serum was added. To make precipitation more effective, the label solution contained 1% NRS. After 6 h at room temperature, tubes were centrifuged and pellets were counted.

Isoelectric Focusing. The focusing was done as previously described (21, 22) with a few modifications. The final ampholyte (Ampholine, LKB-Produkter AB, Bromma, Sweden) concentration was 4%. Mouse hemoglobin (pH 7.2) was used as marker. For most strains, Ampholine pH 5–8 and 7–9 were mixed in proportions 2/1 but for allotype b sera, the gel contained 2% of Ampholine pH 5–8 and 7–9, each. The focusing took place during 18 h, and the maintenance voltage was 400 V.

Antibody patterns were developed with sheep erythrocytes coupled with $phOx_{25}BSA$. To 2 ml of washed packed erythrocytes, 4 ml of 2.5% $phOx_{25}BSA$ in saline was added. After the addition of 60 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide HCl (Story Chemical Corp., Muskegon, Mich.) in 0.5 ml of saline, the mixture was gently rocked for 2 h at 4°C. The cells were washed and a 20% suspension was prepared into a phosphate-buffered saline, pH 7.0.

195 mg Agarose was dissolved into 25 ml of minimum essential medium eagle, and the pH was brought to ≅8.0 by adding 3 drops of 5.6% $NaHCO_3$. Agar was dissolved by boiling, and 5-ml aliquots were placed into a 42°C water bath. Into each tube 30 μg of anti-mouse Ig, 200 μl of fresh guinea-pig serum, and 0.6 ml of 20% erythrocyte suspension were added in rapid succession. The contents of the tube were quickly poored on a plate of 10 × 16 cm. The plates were incubated at 37°C until the patterns of hemolysis looked good (15–30 min). Then the plates were fixed in phosphate-buffered saline containing 0.25% glutaraldehyde.

phOx-cap-T4 Bacteriophage and Determination of Fine Specificity. 50 mg of phOx-cap, 30 mg of N-hydroxysuccinimide, and 50 mg of dicyclohexylcarbodi-imide were dissolved in 2 ml of methanol. After 90 min at 20°C, 5 ml of 3% $NaHCO_3$ was added. The precipitate that formed (dicyclohexyl urea plus unreacted dicyclohexylcarbodi-imide) was quickly filtered out, and 1

TABLE I

Concentrations of Anti-phOx Antibodies in Pooled Sera of Various Mouse Strains

H-2	Allotype	Strain	No. of individuals	Concn. of anti-phOx antibody in the pool
				mg/ml
a	e	A/J	10	0.58
b	b	C57BL/Ka	11	0.39
	a	C57L	20	0.25
	a	CXBG	9	0.16
	a	CXBJ	8	0.60
	b	CXBK	10	0.14
bc	b	LP	9	0.53
d	a	BALB/c	19	0.97
	b	BAB-14	22	1.2
	b	CB-20	8	0.29
	b	CXBD	8	1.0
	b	CXBH	13	0.46
	c	DBA/2	11	0.56
k	d	AKR	7	0.60
	j	CBA	9	0.83
	j	C3H	8	0.20
	c	RF	4	0.14
s	b	SJL	14	0.51
r	g	RIII	11	0.92
v	b	SM	5	0.48
z	d	NZB	7	0.56

Each individual mouse contributed 10 μl to the pool. Mice received one injection of phOx-CSA, and were bled 20 days later.

ml of the filtrate was added into 5 ml of bacteriophage T4 (10^{11} plaque-forming units per milliliter of 3% $NaHCO_3$). The mixture was incubated overnight at 4°C and dialyzed.

Competitive hapten was assayed by inhibition. The indicator reaction was inactivation of phOx-cap-T4 by a standard amount of antibody, and the concentration of the haptens tested varied. For each hapten-antiserum combination the micromolar concentration was determined that reduced the antibody titre to 50% (IC_{50} value). The method has been described in details elsewhere (23). Relative affinity of e.g., IphOx-cap was calculated by dividing the IC_{50} value of phOx-cap by the value of IphOx-cap. To exclude the effect of IgM antibodies, all tests were carried out in 0.1 M 2-mercaptoethanol (final concentration).

Results

Quantity of Anti-phOx Antibodies in Different Mouse Strains. Ando et al. (24, 25) found that the amount of antibodies induced by phOx is partly controlled by H-2-linked genes, partly by allotype-linked genes, and probably by other genes as well. When skin painting was the immunization procedure, strains C57BL and DBA/2 were low responders, whereas BALB/c, AKR, and CBA were strong responders. Also, in the present study C57BL and DBA/2 mice responded more weakly than BALB/c, AKR, or CBA mice (Table I).

Isoelectric Focusing Analysis. The first striking finding in our experiments wa- that anti-phOx antibodies of individual BALB/c mice shared many bands in the isoelectric

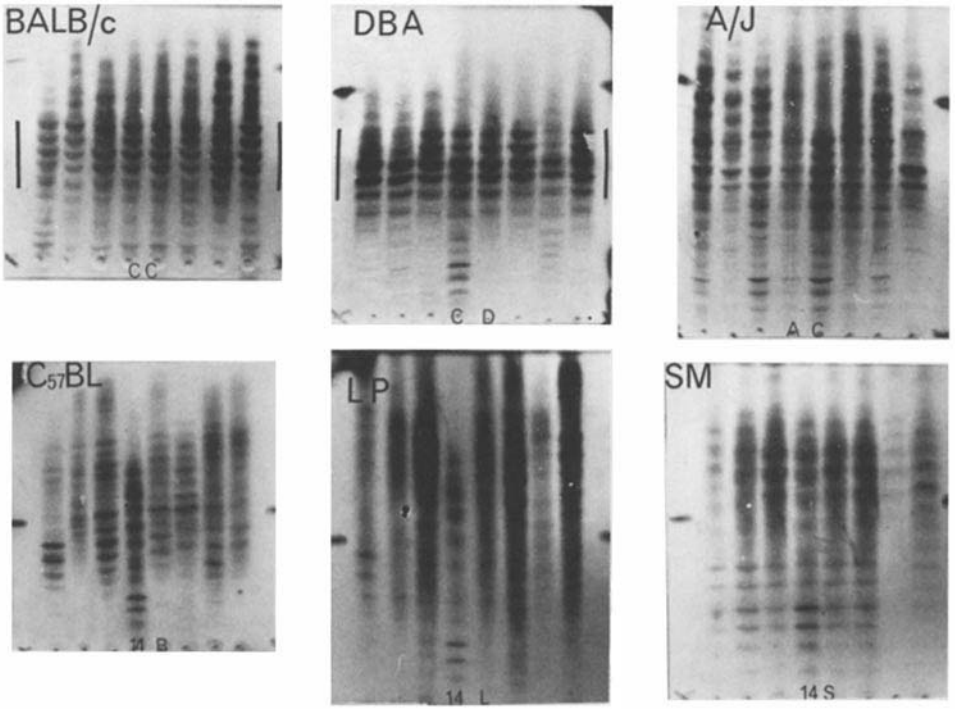

FIG. 1. Isoelectric focusing patterns of anti-phOx antibodies from different mice. Each frame presents patterns of five to six individual sera from a strain. These sera were run in the peripheral tracks. The two central tracks contained pooled sera as indicated by letters. C = BALB/c, D = DBA/2, A = A/J, B = C57BL/Ka, L = LP, S = SM, and 14 = BAB-14. One pH gradient was used for the three strains in the top portions, and another for the three (allotype b) strains in the bottom portion (see Methods). The place of the C57BL hemoglobin marker (pH 7.2) is indicated in many margins by horizontal bars. The place of spectrotype Ox-1 of BALB/c and DBA/2 is indicated by vertical lines in the margins.

focusing pattern. Particularly predominant was a series of three or four bands which we call public spectrotype Ox-1 (Fig. 1).

This spectrotype was also detectable in all tested DBA/2 antisera. A/J mice probably had it, too but the total pattern was different from BALB/c-DBA/2 pattern. On the other hand, C57BL and LP antisera exhibited normal heterogeneous IFF patterns (Fig. 1). Finally, SM antisera had at least two public spectrotypes but they were entirely different from the public spectrotypes of BALB/c, DBA/2, or A/J antisera. One of them was more alkaline than the mouse hemoglobin marker.

Besides the public spectrotypes, many BALB/c, DBA/2, A/J, or SM sera also had individual spectrotypes which were visible only in one mouse. The individual spectrotypes made a large proportion of visible A/J spectrotypes but in the other three strains, public spectrotypes predominated in the IEF pattern.

Strain-Specific Idiotypes in Murine Anti-phOx Antibodies. Sera of two rabbits that had been immunized (twice) with purified BAB-14 anti-phOx antibodies were absorbed several times with Sepharose 4B that had been coupled with ammonium sulphate-precipitated mouse serum proteins (50% saturation). The effect of the absorption was monitored by the capacity of the absorbed antiserum to bind iodinated anti-phOx

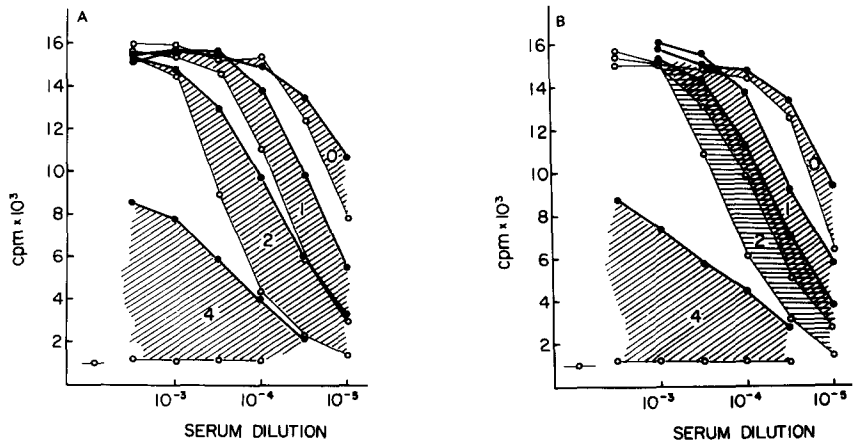

FIG. 2. Specificity of the anti-idiotypic sera 260 and 527 before absorptions and after various stages of the absorption procedure. The capacity of various serum dilutions to bind radioactive anti-phOx antibody (dots) or radioactive anti-NP (open circles) are given. The lines representing the situation after four absorptions are connected by parallel lines, and the shaded area is marked by number 4. The situation after two, one or zero absorptions is similarly illustrated.

TABLE II

Binding of Iodine-Labeled Mouse Immunoglobulin Preparations by an Anti-Idiotype Serum of Rabbit 260

Binding reagent	Ligand, purified and labeled antibody of allotype b mice	
	Anti-NP	Anti-phOx
Anti-id (260)		
1/30	1,800	6,100
1/100	1,900	5,400
1/300	1,400	4,500
1/1 000	1,400	3,900
1/3 000	1,300	3,200
1/10 000	1,400	2,500
1/30 000	1,300	1,800
1/100 000	1,400	1,200
1/∞	1,400	900
Total cpm added	7,900	8,200

Numbers give the counts per minute bound. The anti-NP ligand was obtained from C57BL/6 mice and the anti-phOx ligand from BAB-14 mice.

antibodies (homologous ligand) or iodinated anti-(3-nitro-4-hydroxyphenyl)acetyl (NP) antibodies (heterologous ligand). Both ligands were predominantly IgG_1 antibodies of allotype b mice.

The unabsorbed antiserum bound both ligands with little difference, but after the fourth absorption, neither serum bound the heterologous ligand. Most of the homologous binding had disappeared at the same time but enough remained to make the sera useful anti-idiotypic reagents (Fig. 2). One (260) was used in the studies to be reported. The highest tested concentration (1/30) bound 70% of the radioactive anti-

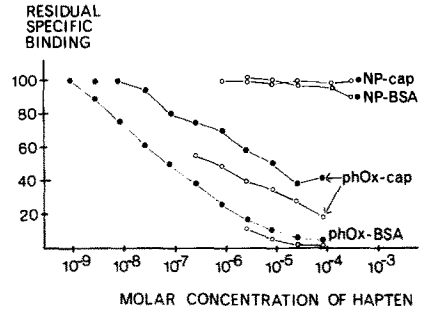

FIG. 3. Binding of the purified ^{125}I-labeled anti-phOx antibody of BAB-14 mice by absorbed serum 260 is inhibited by hapten phOx either as an caproyl conjugate or as a BSA conjugate. The binding is not inhibited by hapten NP similarly conjugated. Two experiments were conducted, one is symbolized by black dots and the other by open circles. Hapten concentrations of the final reaction mixture are given. Concentration of serum 260 was 1/3,000 (see Table II).

phOx antibody (disregarding nonspecifically bound 11%) but only 5% of the purified anti-NP antibody (Table II).

The binding of the radioactive anti-phOx antibodies by the absorbed serum 260 was efficiently inhibited by the hapten coupled to BSA (>95% inhibition by 10^{-4} and >50% inhibition by 10^{-7} M phOx). It was partially inhibited by the hapten phOx-cap (>60% inhibition with 10^{-4} M and 50% inhibition with 10^{-5}–10^{-6} M phOx-cap). This inhibition was specific: 10^{-4} M NP either in the form of NP-cap or NP-BSA inhibited <10% (Fig. 3).

The same binding could also be inhibited by nonradioactive anti-phOx antisera of some mouse strains, notably BALB/c, DBA/2, C57L, A/J, RIII, and SM (Fig. 4). It was weakly (<40%) inhibited by anti-phOx sera of strains C57BL, C3H, AKR, LP, or RF. Sera of strains SJL, CBA, and NZB showed some inhibition, but only at very high concentrations; 500–700 ng required for 50% inhibition whereas 12 ng of BALB/c antibody was sufficient for it. BALB/c antiserum against an unrelated antigen was >1,000-fold less effective in inhibition than anti-phOx serum.

In summary, Table II and Fig. 4 show that the absorbed antiserum 260 reacted with anti-phOx antibodies of some strains but not of others, and it did not react with other immunoglobulins of the BALB/c mice. The binding was specifically inhibited by the hapten phOx (Fig. 3), which suggested that the antibodies in the absorbed serum 260 react with determinants that are close to the combining site of the anti-phOx antibodies. The reactions were characteristic for an anti-idiotype antiserum.

The significance of the weak inhibition by C57BL, LP, AKR, RF, C3H, CBA, NZB, or SJL antibodies is difficult to evaluate. The main alternatives are either that these sera contain a cross-reactive idiotype of the type described in the A5A and S117 systems (9, 26) or they contain small quantities of antibodies that are identical to the BALB/c idiotype. The second alternative has been postulated for the difference between BALB/c and C57BL strains in the anti-phosphorylcholine response (27, 28).

Relationship Between the Strain-Specific Spectrotypes and the Antigenically Defined Idiotype. Binding data of Table II indicate that >70% of the purified anti-phOx antibodies of BAB-14 mice are positive for the idiotype. On the other hand, it seems likely from Fig. 7 that the public spectrotypes make up a major fraction of (IgG) anti-phOx antibodies. This means that the idiotypically defined antibody population must

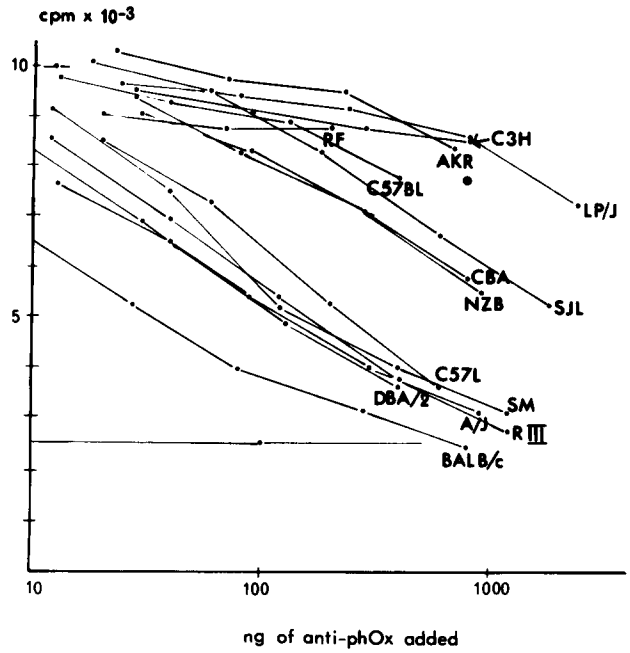

FIG. 4. Inhibition of idiotype binding by pooled crude anti-phOx sera of different mouse strains. Anti-idiotype serum 260 was used in dilution 1/3,000 (see Table II), and the pools of Table I were the inhibitors. The horizontal line in the upper left corner indicates the binding without inhibitor and the lower horizontal line background binding (normal rabbit serum instead of serum 260). Highest serum concentrations tested were 1/100 dilutions. The large black dot indicates inhibition by BALB/c serum that had been immunized with an unrelated antigen.

TABLE III

Structural Analogues of phOx-cap Tested in a Competitive Inhibition Assay

Compound	IC_{50} value
phOx-cap	0.42
phOx-gly	1.2
phOx-asp	7.2
phOx-$(CH_2)_2NH_2$	5.5
IphOx-cap	1.1–6.6*
NO_2phOx-cap	8.4

* BALB/c mice had the mean value of 1.1 μM and C57BL mice the value of 6.6 μM.

The residue was replaced by glycine, aspartic, or $—CH_2CH_2NH_2$ in three analogues. In two of them, an additional structure (I or NO_2) was in the benzene ring. The second column gives the micromolar concentration of the compound (geometric mean, at least 15 sera were tested) that reduced antibody titre to 50% (IC_{50} value).

overlap with the public spectrotypes: the fact that all the five strains that have large quantities of the idiotype also have easily detectable public spectrotypes, whereas none of the remaining nine strains do, further support this idea.

Fine Specificity of Anti-phOx Antibodies. Initially, six structurally related compounds were tested for their capacity to inhibit anti-phOx antibodies. Table III shows the mean concentrations of those required to reduce the antibody titre to 50% (IC_{50}

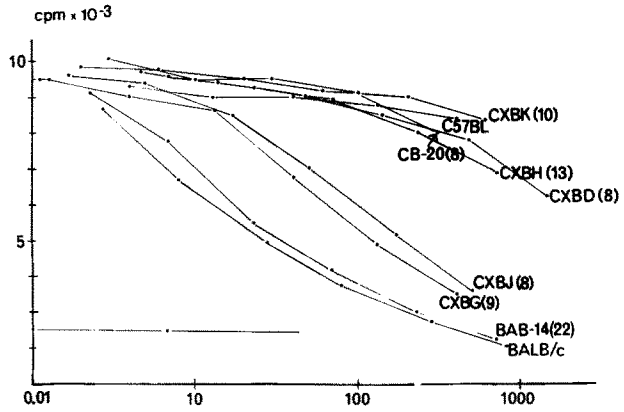

FIG. 5. Inhibition of idiotype binding by pooled crude anti-phOx sera of congenic and recombinant inbred strains of mice. Sera were bled 20 days after a single injection of phOx-CSA. For further explanations see Fig. 4.

concentration). phOx-cap is the reference compound because it closely resembles the antigenic determinant, phOx coupled to lysine. The other compounds did inhibit to a lesser degree, but most of them were equally effective regardless of the strain (BALB/c, CBA, C57BL, DBA/2, AKR, NZB, A/J, and RIII were tested). Only IphOx-cap discriminated between strains, the mean IC_{50} was 1.1 μM in C57BL but 6.6 μM in BALB/c mice.

Inheritance of the Strain-Specific Characteristics of BALB/c Anti-phOx Antibodies. Anti-phOx antibodies of BALB/c mice have three strain-associated characteristics: the IEF pattern, the idiotype, and the low affinity (high IC_{50} value) for IphOx-cap. We studied the inheritance of these characteristics by using inbred strains that carry different mixtures of BALB/c and C57BL genes in a homozygous state. Two (CB-20 and BAB-14) were allotype congenic strains, developed by Potter (17), which both have the heavy chain allotype genes (C_H genes) of the C57BL in a BALB/c background. Five were recombinant inbred strains developed by Bailey (29), which contain random mixtures of BALB/c and C57BL chromosomes (and perhaps a few recombinant chromosomes), each in a homozygous state.

Of these strains, five (C57BL, CXBD, CXBH, CXBK, and CB-20) have totally or predominantly C57BL V_H genes in their genome (all have also allotype b). Three (CXBG, CXBJ, and BAB-14) have totally or predominantly BALB/c V_H genes (17). Of these, CXBG and CXBJ also have allotype a, but BAB-14 apparently has a recombinant chromosome, in which the C_H genes (allotype) are of C57BL origin but most V_H genes of BALB/c origin.

Results of the idiotype assay are given in Fig. 5. The strains fall into two distinct classes. The sera of all the three strains whose V_H genes originate predominantly from the BALB/c parent were clearly positive for the idiotype. BAB-14 antibodies were as efficient inhibitors as BABL/c antibodies of idiotype binding. CXBG and CXBJ antibodies were less efficient inhibitors but the slopes were the same as the slopes of BALB/c or BAB-14 sera, and all four types of sera caused almost complete inhibition of idiotype binding. The simplest explanation is that only one-fifth of CXBG and CXBJ anti-phOx antibodies bear the idiotypic determinants. Anti-phOx antibodies of the five strains that bear C57BL V_H genes were as weak inhibitors as C57BL

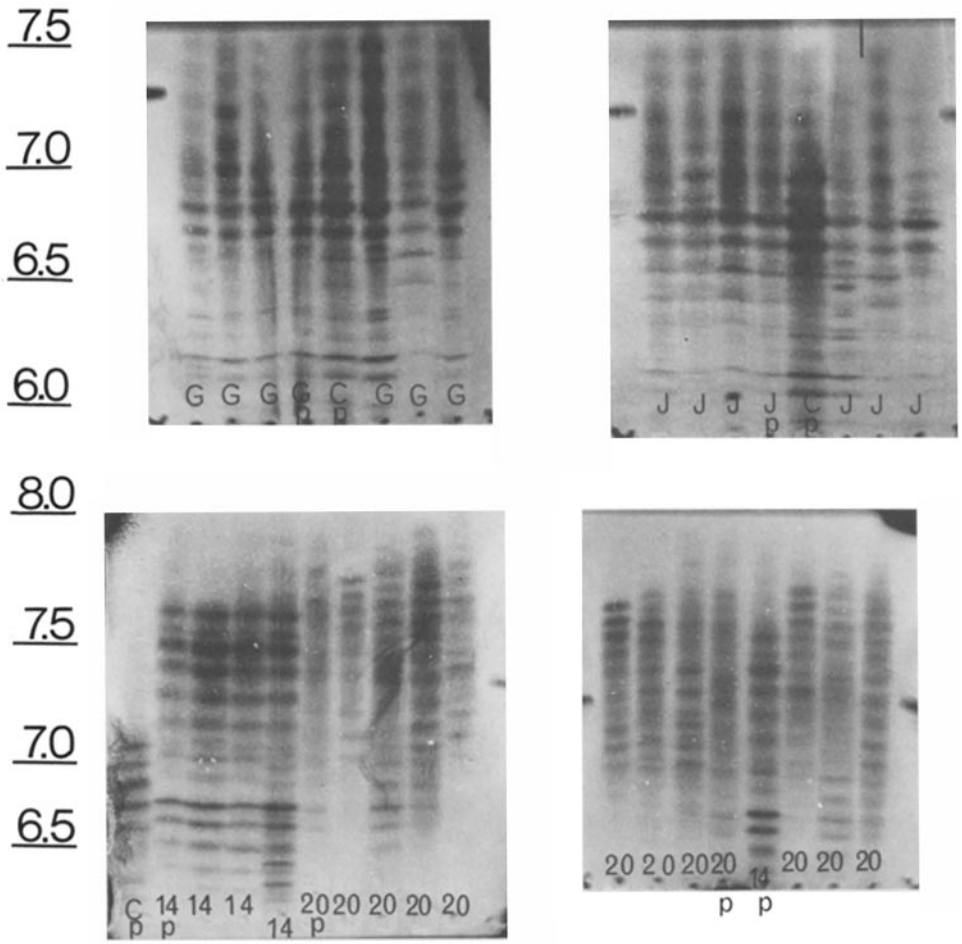

FIG. 6. Isoelectric focusing patterns of anti-phOx antibodies of BALB/c (C), CXBG (G), CXBJ (J), BAB-14 (14), or CB-20 (20) mice. Individual sera were analyzed in most cases, pooled sera are indicated by letter p. For the top frames, Ampholines pH 5–8 and pH 7–9 were used in proportions 2/1 and for the lower frames, in proportions 1/1. For further explanations see Fig. 1. Independent pH measurements were conducted from the gel, the results are given on the left margin.

antibodies (Fig. 5). The pool of CB-20 sera that was tested in Fig. 5 may not give a true picture, however. We have found individual CB-20 sera that inhibit more strongly than this pool. The possibility cannot be excluded that CB-20 mice produce small amounts of the idiotype that makes up the majority population in BAB-14 and BALB/c antibodies.

Anti-phOx sera of the same strains were submitted to the IEF analysis. All the four strains with C57BL V_H genes resembled this strain; no public spectrotypes could be detected. The pattern of CB-20 is given in Fig. 6. All the three strains that have inherited mainly BALB/c V_H genes had public spectrotypes. Strains and CXBJ had patterns hardly distinguishable from the BALB/c pattern (Fig. 6). The public spectrotypes of BAB-14 mice were different from those of BALB/c mice. On the other hand, the idiotype analysis and the fine specificity analysis suggest similarity of the

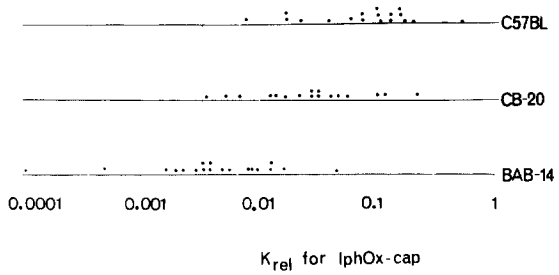

FIG. 7. Fine specificity of anti-phOx antibodies of C57BL/Ka and of mice that have C57BL H-chain genes in BALB/c background. Each dot represents an individual mouse, and its horizontal localization indicates relative affinity of its antibodies for a structural analogue IphOx-cap. Sera bled 20 days after one injection of antigen were tested. All the three strains have statistically significant differences in their distributions. The p values in different comparisons were as follows: C57BL vs. BAB-14, and CB-20 vs. BAB-14 ($p < 0.001$); C57BL vs. CB-20 ($P < 0.01$).

TABLE IV

Primary, Secondary, and Tertiary Antibody Responses of BAB-14 and BALB/c Mice to $phOx_{20}$ CSA Conjugate

	Concentration of anti-phOx	ng of Anti-phOx needed for 50% inhibition of idiotype binding
	mg/ml	
BAB-14 prim. pool of 22 sera	0.7	17
sec. pool of 22 sera	1.0	15
tert. pool of 22 sera	0.6	16
BALB prim. pool of 19 sera	0.8	13
sec. pool of 19 sera	2.0	34
tert. pool of 19 sera	0.7	15
BALB/c individual	1.0	11
sera of	1.4	19
the primary	0.8	22
response	1.2	27
	0.7	11
BALB/c individual	1.2	29
sera of the	1.4	32
secondary	1.6	28
response	1.1	19

V_H regions of the BALB and BAB-14 antibodies. The discrepancy between the IEF data and other data is best explained by allotype-associated charge differences in he C_H regions. Anti-phOx antibodies of both strains seem to be mainly IgG_1 because they are unreactive with protein A (30). In IgG_1 there is a charge difference between allotypes a and b; allotype a chains are more alkaline than allotype b chains (36). The public spectrotypes of BALB/c antibodies are more alkaline than those of BAB-14 antibodies. To this picture fits the fact that the public spectrotypes of SM antisera (another allotype b strain) were indistinguishable from BAB-14 spectrotypes (Fig. 1).

The fine specificity characteristics were less useful in this case than in the study of some other V_H genes. Although strains C57BL and BALB/c antibodies had different

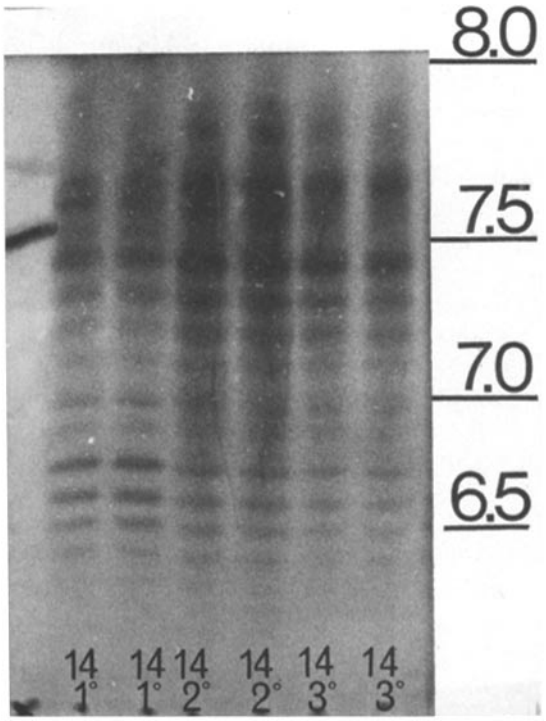

FIG. 8. Isoelectric focusing patterns of anti-phOx antibodies in BAB-14 mice at various stages of an immunization course (primary, secondary, and tertiary response). Pooled sera of the same 22 mice were analyzed. The same pool was run in two adjacent tracks. pH values in the gel after the run are given on the right margin.

affinities for compound IphOx-cap, the difference was not sufficient to permit family studies. For this reason we did adequate population studies with only three strains C57BL, CB-20, and BAB-14. The V_H gene effect was best studied by comparing strains CB-20 and BAB-14 who share the background genes and also allotype genes, and only have different V_H genes. The effect of background genes could be studied by comparing strains CB-20 and C57BL because CB-20 has the V_H genes of C57BL in a different background.

The data of Fig. 7 show that the background genes had a modest effect on the fine specificity; CB-20 mice had a lower relative affinity for IphOx-cap (mean = 0.027) than C57BL mice (mean = 0.076). The effect of the V_H genes was greater, however, the mean K_{rel} of BAB-14 mice was six times lower than the value of its congenic partner strain CB-20.

Persistence of the Inherited Characteristics of Anti-phOx Antibodies during an Immunization Course. A group of 22 BAB-14 mice and another of 19 BALB/c mice were immunized on days 0, 30, and 60 and bled on days 20, 40, and 70. Some individual sera and some pooled sera were tested for antibody and idiotype concentration and also submitted to an IEF analysis. The data suggested that the idiotypic population remained as the major anti-phOx antibody population in both BAB-14 and BALB/c mice throughout the 70-day period (Table IV). It was more surprising to us that the public spectrotypes also remained virtually unchanged. The IEF analysis of the pooled BAB-14 sera is presented in Fig. 8.

Discussion

Antibodies of different mouse strains to the hapten phOx were studied with three types of assay, IEF, determination of fine specificity, and use of anti-idiotypic antibody. Of these methods, isoelectric focusing has the greatest resolving power (31) whereas the other two have the advantage of being useful not only for the characterization of conventional antibodies but of T-cell receptors, too (32–35). The data obtained in this study suggest that a major idiotype is detectable in anti-phOx antibodies of many mouse strains, and it can be demonstrated by all the three assays.

Our first observation to this effect was the presence of an identical IEF pattern (spectrotype) in anti-phOx sera of all BALB/c and DBA/2 mice. It is called spectrotype Ox-1. These two strains belong to different allogroups, but these two allogroups have no detectable differences in the C_H regions of IgG_1 (36). Anti-phOx antibodies of at least BALB/c mice seem to be mostly IgG_1, and identity of the public spectrotypes is thus possible. All A/J antisera shared at least a part of the public spectrotype pattern of the BALB/c and DBA/2. SM antisera had an entirely different public spectrotype pattern whereas C57BL and LP antisera had only private spectrotypes without apparent sharing between individuals.

Anti-phOx antibodies of C57BL mice did not have idiotypic determinants, or the idiotypic antibodies were only present in 100 times lower concentrations in sera of C57BL mice, compared to sera of BALB/c mice after immunization of both strains with Ox_{20}CSA. Anti-phOx antibodies of almost all the individual BALB/c mice had idiotypic determinants. The third difference between anti-phOx antibodies of the two strains was detected in fine specificities: BALB/c antibodies had a lower relative affinity for a chemical analogue of phOx (IphOx) than antibodies of C57BL mice. mice.

Inheritance of all the three characteristics was studied in congenic strains and recombinant inbred (RI) strains which bear different combinations of BALB/c and C57BL genes in a homozygous state. The general conclusion was that all three characteristics were controlled by an allotype-linked gene(s). The congenic strain BAB-14 and the RI strains CXBJ and CXBG were similar to BALB/c, whereas the congenic strain CB-20 and RI strains CXBD, CXBH, and CXBK were similar to C57BL. All three characteristics together defined a new V_H marker, V_{HphOx}.

This general conclusion requires two qualifications. Firstly, although BALB/c and BAB-14 antibodies were indistinguishable in the idiotype assay and similar in the fine specificity assay, their spectrotypes were entirely different. For reasons discussed in Results, this difference is probably caused by charge differences in the C_H regions of the (IgG_1) antibodies. The IEF pattern of the BAB-14 strain was indistinguishable from the pattern of the SM strain, another idiotype-positive strain that shares the C_H region with BAB-14. The data are compatible with the concept that all four strains, BALB/c, DBA/2, BAB-14, and SM have indistinguishable V regions in their idiotype-positive anti-phOx antibodies. The second qualification is that allotype-unlinked genes have an effect on the quantity of the idiotype. The best evidence for this is the lower proportion of the idiotype in CXBG and CXBJ than in BALB/c anti-phOx antibodies.

We cannot exclude the possibility that the idiotype-negative strains such as C3H have small amounts of the idiotype in their response. We can, however, estimate an upper limit, which in this case is such that the proportion of the idiotype in the C3H

anti-phOx antibodies is <2% of the proportion in BALB/c antibodies. This limit value has been pushed lower down in three earlier cases. A/J anti-Ar antibody has >100 times as much CRI idiotype as BALB/c antibody (3), A/J anti-Strep A CHO antibody has >1,000 times as much A5A idiotype as BALB/c antibody (4), and C57BL anti-NP antibody has >10,000 times as much NP-b idiotype as BALB/c anti-NP antibody (20). To our knowledge, the proven difference strains positive for an idiotype and those negative for it has been 50-fold, at most, in all other idiotypic systems.

The murine anti-phOx response is unique because of a combination of two characteristics. One is the constant dominance of spectrotype Ox-1 in all studied BALB/c and DBA/2 individuals throughout a 10-wk immunization course, and the other is the occurrence of one idiotype in many different mouse strains. Especially striking is the finding of the same spectrotype Ox-1 in two strains that belong to different allogroups a and c. An identical spectrotype has been previously detected in anti-NP antibodies of different mouse strains (20) but only in allotypically identical strains. The present finding demonstrates considerable evolutionary conservatism in a specific antibody response, an exception to the rule that one idiotype or one spectrotype is unique both in the total immunoglobulins of an individual (37) and in specific antibodies (38).

Great conservatism has previously been found in phosphorylcholine-binding mouse immunoglobulins (39), but hapten-binding immunoglobulins exhibit, as a rule, light (L)-chain heterogeneity (40). Spectrotype Ox-1 may not be the only widespread homogeneous anti-hapten antibody, however. Another example is suggested by the presence of spectrotype N-1 (22) in three strains of laboratory mice and in a small-sized subspecies *Mus-musculus molossinus* (M. Potter, M. Karjalainen, and O. Mäkelä, Unpublished observations).

Mouse strains that bear the allogroup b in their C_H locus have a remarkably similar composition of V_H markers according to published studies. All strains studied are positive for markers NP^b, $ABA\text{-}HOP^b$, ESE-D, Nase-2, U10-173, and BGL but negative for all other markers (31, 41, 12). The published data have included only up to five strains of this allogroup, and they have omitted strain SM. SM is probably an odd strain in this group. Unlike other members of the group, it is clearly positive for the $V_H$phOx marker and negative for the V_HNP^b marker (20).

The position of the $V_H$phOx marker in relation to other markers of the BALB/c chromosome is unknown, but preliminary information has been obtained from a congenic mouse strain that has been developed in Professor Weiler's laboratory. It has a cross-over chromosome (it combines the allotype gene from C57BL with the V_HDEX marker from the BALB/c strain). This mouse strain is positive for idiotype NP-b, but positive for the phOx idiotype.[2] Thus, the cross-over event appears to have cut the BALB/c chromosome between the V_HDEX and $V_H$phOx markers on one hand, and V_HNP and allotype genes on the other.

Whereas the characteristics of anti-phOx antibodies are controlled by allotype-linked genes, their amount in the primary response seems to be controlled by H-2 linked genes. This was suggested in an earlier report; the weak antibody responses

[2] Cornelia Kolb, J. Seppälä, K. Eichmann, M. Kaartinen, J. Pelkonen, K. Karjalainen, and O. Mäkelä. Manuscript in preparation.

caused by phenyloxazolone painting and demonstrable by using a haptenated phage, were low in most H-2^b strains but high in most H-2^a, H-2^d, and H-2^k strains (25). In this study, we measured antibody concentrations rather than antibody titers, and the immunizing hapten was coupled to a good carrier protein, CSA. Also in this study, H-2^a, H-2^d, and H-2^k strains had higher concentrations of anti-phOx antibodies (geometric mean = 0.62 mg/ml) than H-2^b strains (geometric mean = 0.49 mg/ml). H-2 allele r seems to be associated with still higher concentrations of anti-phOx antibodies (Table I) but because this allele is represented by only two strains, their high responses can be due to non-H-2 genes. Some of these concentrations are high for a primary anti-hapten response but we have seen as high concentrations in another V_H-gene controlled response (20). The H-2-linked control of the anti-phOx responses obviously requires further studies.

Summary

Antibodies to hapten 2-phenyloxazolone (phOx) of all BALB/c and DBA/2 mice have the same idiotype and the same major (public) isoelectric focusing pattern whose main spectrotype is called Ox-1. Neither of these characteristics could be readily demonstrated in anti-phOx antibodies of C57BL, C3H or LP mice; these antibodies were heterogeneous, and lacked public spectrotypes. Also, a fine specificity difference could be demonstrated between anti-phOx antibodies of BALB/c and C57BL mice; the latter have a higher relative affinity than the former for a structural analogue of phOx (2-*o*-iodophenyloxazolone). The three BALB/c characteristics were inherited in congenic and recombinant inbred strains as an allotype-linked block, defining a new V_H marker, V_{HphOx}.

Murine anti-phOx antibodies were found to exhibit three types of conservatism: (*a*) Every individual mouse of strains BALB/c, DBA/2 or BAB-14 had an almost indistinguishable IEF pattern. (*b*) These patterns (and the cross-reactive idiotype) remained virtually unchanged during an immunization course of 70 days. (*c*) An identical idiotype (and in some cases IEF pattern) was present in mouse strains of five different allogroups.

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