# FREQUENCY OF OCCURRENCE OF IDIOTYPES ASSOCIATED WITH ANTI-*p*-AZOPHENYLARSONATE ANTIBODIES ARISING IN MICE IMMUNOLOGICALLY SUPPRESSED WITH RESPECT TO A CROSS-REACTIVE IDIOTYPE*

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When immunized with a conjugate of keyhole limpet hemocyanin (KLH)[1] with *p*-azophenylarsonate (Ar) groups all A/J mice respond with the production of anti-hapten antibodies, a portion of which share a cross-reactive idiotype (CRI) (1). The percentage of anti-Ar antibody molecules in an individual mouse which bears the CRI generally varies between 20–70%. Since this idiotype has not been found in a large number of other strains immunized with the same antigen, it can serve as a genetic marker for the variable (V) regions of anti-Ar antibodies (2, 3).

If, before immunization, a neonatal or adult A/J mouse is first challenged with rabbit anti-idiotypic (anti-id) antibodies directed to the CRI, the idiotype almost always fails to appear upon subsequent immunization of the recipient mouse (4, 5). Such suppression of idiotype in vivo has been confirmed in other systems (e.g., references 6–8). Despite the suppression of CRI, the immunized mice produce normal amounts of anti-Ar antibodies when challenged with KLH-Ar (5). These antibodies can, in turn, be used to elicit anti-id antibodies in rabbits (9). In contrast to the anti-Ar antibodies with CRI, the idiotypes arising in suppressed, hyperimmunized mice occur at very low frequency, or are not detectable, in other immunized A/J mice, either suppressed or nonsuppressed (9, 10). For convenience, we will refer to the idiotypes of the anti-Ar antibodies of suppressed, immunized mice as "private" idiotypes. Since the radioimmunoassays used for detection of idiotypes are sensitive, minute concentrations of private idiotypes would be detected if present in the anti-Ar antibodies of other mice.

These results have obvious implications with respect to the degree of heterogeneity of the anti-Ar antibodies of the A/J strain. However, a technical limitation becomes apparent in the interpretation of the data. It could be argued that a private idiotype is actually a collection of idiotypes, all recognized by the

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[1] *Abbreviations used in this paper.* anti-id, anti-idiotypic; Ar, *p*-azophenylarsonate; BGG, bovine IgG; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CRI, cross-reactive idiotype; HIS, immunologically suppressed with respect to the cross-reactive idiotype and then hyperimmunized with KLH-Ar; KLH, keyhole limpet hemocyanin; PABT, (*p*-azobenzenearsonic acid)-*N*-acetyl-L-tyrosine; pI, isoelectric pH

autologous rabbit antiserum. Another immunized mouse might produce some, but not all of these idiotypes; only partial inhibition of the binding of the autologous, labeled ligand would then be observed in the radioimmunoassay. To circumvent this possibility, we have extended these experiments by subjecting purified anti-Ar antibodies of three randomly selected, hyperimmune suppressed mice, bearing private idiotypes, to isoelectric focusing on polyacrylamide gels; four individual labeled peaks isolated from the gels were then used as ligands in radioimmunoassays designed to detect the presence of private idiotypes in the anti-Ar antibodies of other A/J mice. The antibodies in the peaks selected refocused with the same isoelectric pH (pI) values; another criterion used for selection was the reactivity of at least 90% of the radiolabeled protein in the peak with its anti-id antibodies.

Data concerning the occurrence of such private idiotypes in other mice are presented here and are discussed in terms of the degree of diversity of anti-Ar antibodies in A/J mice and of possible mechanisms that might account for the diversity.

## Materials and Methods

*Experimental Animals.* Male A/J or BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. A/J mice used for suppression of the cross-reactive idiotype were 6-wk old. Mice used for the preparation of anti-Ar antibodies were 8–12 wk of age at the start of immunization

*Induction of Antibodies in Ascitic Fluids.* This was done by the method of Tung et al. (11). Essentially, it consists of repeated injection of a 9:1 emulsion of complete Freund's adjuvant (CFA) and antigen solution (KLH-Ar, 0.5 mg). The schedule of immunization and the method used for tapping of ascites have been described previously (11).

*Suppression of the Cross-Reactive Idiotype and Induction of Anti-Ar Antibodies in Suppressed Mice* The reagent used for suppression of the CRI, associated with the anti-Ar antibodies of A/J mice, was rabbit anti-id antiserum, prepared and absorbed as described below. Two 0.5-ml injections were given intraperitoneally, 3 days apart The idiotype-binding capacity of the antisera used for suppression was approximately 60 $\mu$g/ml. This quantity of anti-id antibody, which is considerably higher than that used previously, was found to suppress the CRI below the limits of detectability in nearly all subsequently immunized mice. To induce anti-Ar antibodies in ascitic fluids of suppressed mice, essentially the same schedule of immunization was employed as that used for nonsuppressed animals (11); immunization was begun 2 wk after the inoculation of anti-id antiserum.

*Preparation of Anti-id Antibodies.* Anti-id antibodies against the CRI characteristic of the anti-Ar antibodies of A/J mice were prepared by a method that differed from that previously described (1), in that specifically purified anti-Ar antibodies, prepared from a large pool of ascitic fluids, rather than a dissolved precipitate of the antibodies with a rabbit IgG-Ar conjugate, were used as the immunogen. Each inoculation of the rabbit utilized 1–1.5 mg of purified anti-Ar antibody in a 1:1 emulsion of CFA and antigen solution. 3 ml were injected in several subcutaneous and intramuscular sites A total of four injections were given, with 2-wk intervals between injections. Starting 2 wk after the last inoculation, the rabbits were bled weekly for 4 wk. Rabbit antisera were absorbed with a crude globulin fraction obtained from A/J ascitic fluid, induced with CFA alone. The anti-id antiserum obtained by this method was about 10 times stronger, in terms of idiotype-binding capacity per unit volume, than antisera obtained by the method described earlier (1). Tests for anti-id specificity were carried out by radioimmunoassay as described previously (1, 4): they include inhibition by small amounts of idiotype; the failure of large amounts of normal A/J serum, of anti-Ar antibodies from immunologically suppressed A/J mice which have high titers of anti-Ar antibodies, or anti-KLH-Ar antibodies of BALB/c mice, to inhibit the binding of the radioactive ligand (the immunogen) to the absorbed anti-id antibodies (10 ng of $^{125}$I-labeled ligand was used in each test); and the inability of the absorbed anti-id antibody to precipitate $^{125}$I-labeled normal A/J IgG by the indirect (anti-globulin) method.

*Preparation of Anti-id Antisera against Anti-Ar Antibodies Arising in Immunologically Suppressed A/J Mice* As reported previously (5), mice that are suppressed with respect to the appearance of the CRI produce normal amounts of the anti-Ar antibodies when immunized by the conventional method with KLH-Ar. Anti-id antibodies can in turn be prepared against these anti-Ar antibodies (9) The immunogen used to prepare such anti-id antibodies was, first, a dissolved precipitate (pH 3.5) of the anti-Ar antibodies with a rabbit IgG-Ar conjugate, emulsified in CFA. After two injections the rabbits were boosted with specifically purified anti-Ar antibodies prepared from the ascitic fluid of the same suppressed mouse The latter inoculations resulted in substantial increases in anti-id titers The rabbit antibodies were absorbed in the usual manner and tested for idiotypic specificity by the radioimmunoassay To maximize sensitivity, only 1.5–2 ng of labeled ligand was used Assays for the presence of the corresponding idiotype in the sera of other immunized or nonimmunized mice were carried out by measurement of the capacity to inhibit the binding of the radiolabeled ligand. In all cases (see Results) the unlabeled autologous antibody was a potent inhibitor of binding.

*Radioimmunoassay for Total Anti-Ar Antibody* This method is based on that described by Klinman and his associates (personal communication). Microtiter plates (model 220-24; Cooke Laboratory Products Div , Dynatech Laboratories Inc , Alexandria, Va.) were used. Individual wells were filled with a solution of bovine serum albumin (BSA)-Ar at a concentration of 1 mg/ml After standing overnight at room temperature, the wells were thoroughly washed, and then filled with a 2% solution of horse serum in borate-buffered saline, pH 8. On the following day, the plates were washed four times with the buffer, and 20 $\mu$l of a 1·10 dilution of normal A/J serum was added. The test for anti-Ar antibodies was then carried out by adding 50 $\mu$l of an appropriate dilution of serum to each well After incubating at room temperature for 3 h, wells were washed four times with buffer. Then, 0.1 ml of specifically purified $^{125}$I-labeled rabbit anti-mouse IgG was added to each well, in a given set of experiments, either 0 25 or 1 $\mu$g of the labeled reagent was used In each experiment a standard curve was obtained with A/J anti-Ar antiserum containing known amounts of anti-Ar antibodies, as determined by precipitin analysis After standing overnight at 4°C, the plates were thoroughly washed with cold tap water, individual wells were cut out, and radioactivity was determined with a gamma scintillation counter Control samples, lacking anti-Ar antibodies, were also run, and the small values obtained were subtracted from experimental data. Specificity was shown in each series of tests by demonstrating the capacity of the hapten, *p*-arsanilate, to inhibit the binding of anti-Ar antibodies to the well. Background values corresponded to the binding of approximately 2–5 ng of anti-Ar antibody The assay in general was useful within a range of 3–40 ng of anti-Ar antibody The average deviation from the mean of duplicate samples was less than 8%.

*Isoelectric Focusing* Isoelectric focusing of specifically purified anti-Ar antibodies was carried out in cylindrical, 5 × 115 mm, 5% polyacrylamide gels; the solvent was 3 5 M deionized urea and the final concentration of Ampholine (LKB Instruments, Inc., Rockville, Md.) was 2% After focusing, the gels were either stained with Coomassie Blue dye or subdivided with a Savant Autogel Divider (Savant Instruments, Inc., Hicksville, N Y.) using degassed 0 15 M NaCl as eluant Each gel yielded approximately 150 fractions of five drops each. Details of the method were reported previously (12). The pH was determined on every fourth fraction by adding 0.5 ml of degassed saline solution and allowing particles to sediment before the measurement. To minimize denaturation, 0 6 ml of a solution containing 1% BSA in borate-buffered saline was added to each of the other fractions. The overall recovery of extracted protein, as estimated from radioactivity measurements, was over 60%. (It was found that the buffer was necessary for efficient extraction ) All samples applied to the gels were labeled with $^{125}$I to permit quantitation of the protein When small amounts of labeled samples were used, normal mouse IgG was added as carrier immediately after the neutralization of chloramine-T in the iodination procedure.

*Identification of the Subclass of Anti-Ar Antibodies.* Radioimmunoassays were used to quantitate the subclass of certain mouse antibody fractions. For these tests $^{125}$I-labeled mouse antibodies were used. Subclass-specific rabbit antisera were obtained from Litton Bionetics, Kensington, Md An excess of rabbit antiserum was added to 1 5 ng of radiolabeled test sample, and an additional 2 $\mu$l of normal rabbit serum was added as carrier This was followed by an excess of goat anti-rabbit Fc that had been adsorbed with a crude globulin fraction of mouse serum. The percentage of radioactivity precipitated was then determined The specificity of these tests was shown by using radiolabeled myeloma proteins of known subclasses, obtained from Litton Bionetics

*Specifically Purified Rabbit Anti-Mouse IgG.* A/J IgG was conjugated to Sepharose-4B using a ratio of 2 mg of protein/ml of packed gel. A portion was mixed with excess rabbit antiserum directed to mouse IgG (1 h at 37°C; overnight at 4°C). The mixture was packed into a column and washed extensively with borate-buffered saline, pH 8. The antibody was eluted with 2 M KSCN and immediately dialyzed and concentrated. Estimated yields were 30–40% and approximately 75% of the protein was precipitable by mouse IgG.

*Other Methods.* We have previously described procedures for the preparation and purification of anti-Ar antibodies, the preparation and absorption of rabbit anti-id antibodies, radioimmunoassay for the CRI of A/J anti-Ar antibodies (1), preparation of IgG from ascitic fluids (11), and conjugation of proteins to Sepharose-4B (Pharmacia Fine Chemicals, Inc. Piscataway, N. J.) (1, 13). Radioiodination was carried out by the use of chloramine-T (14). The radioimmunoassay was a double-antibody procedure, using goat anti-rabbit Fc to precipitate complexes of labeled idiotype and rabbit anti-id antibodies. The synthesis of (*p*-azobenzenearsonic acid)-*N*-acetyl-L-tyrosine (PABT) has been previously described (15).

## Results

The purpose of these experiments was to study the idiotypic properties of homogeneous subfractions of anti-Ar antibodies arising in A/J mice that had been suppressed with respect to the CRI normally present in the anti-Ar antibodies of the strain. The results presented in Figs. 1–3 show the isoelectric-focusing patterns of specifically purified, $^{125}$I-labeled anti-Ar antibodies of hyperimmune suppressed mice, designated HIS-7, HIS-13, and HIS-5, respectively. The arrows in the figures indicate the peaks that were used in radioimmunoassays to detect the possible presence of private idiotypes in the antisera of other immunized A/J mice; in the case of HIS-7 the ligand was obtained in a separate run, using a more highly labeled protein. The pattern was very similar to that shown in Fig. 1.

That the heterogeneity with respect to pI values was not due to an artifact of the procedure was ascertained by refocusing protein in some of the peaks. The results obtained for two peaks present in the HIS-5 pattern are shown in Fig. 4. The peaks with pI values of 6.3 and 6.9 were mixed and refocused. To further facilitate identification, three times as much of the former material was used in the mixture. The pI values of the refocused peaks agree within 0.0 and 0.1 pH unit with those of the originally extracted materials. The data also show that the protein present in each peak of the heterogeneous mixture refocuses as a single peak (Figs. 3 and 4). Similar results were obtained with the major peak (pI 6.72) of the anti-Ar antibodies of mouse HIS-7. Upon refocusing, a single peak was obtained with a pI value of 6.75.

Another set of control experiments was carried out to ascertain whether iodination causes a shift in the pI value. The number of atoms of iodine incorporated per molecule of protein was less than 0.1 in each case. It seemed possible that the presence of an atom of iodine might cause a shift in pI. If this occurred, then the concentration of anti-Ar antibodies would not correspond precisely with levels of $^{125}$I shown in Figs. 1–3. This question was pursued with each of the preparations represented in those figures. The procedure consisted of adding a 250- to 600-fold excess of unlabeled specifically purified antibody to each labeled preparation. Approximately 100 $\mu$g of the mixture was applied to the polyacrylamide gel and subjected to isoelectric focusing. Two assays were carried out on each of approximately 150 fractions isolated from each run. One was measurement of radioactivity; the other was determination of concentration

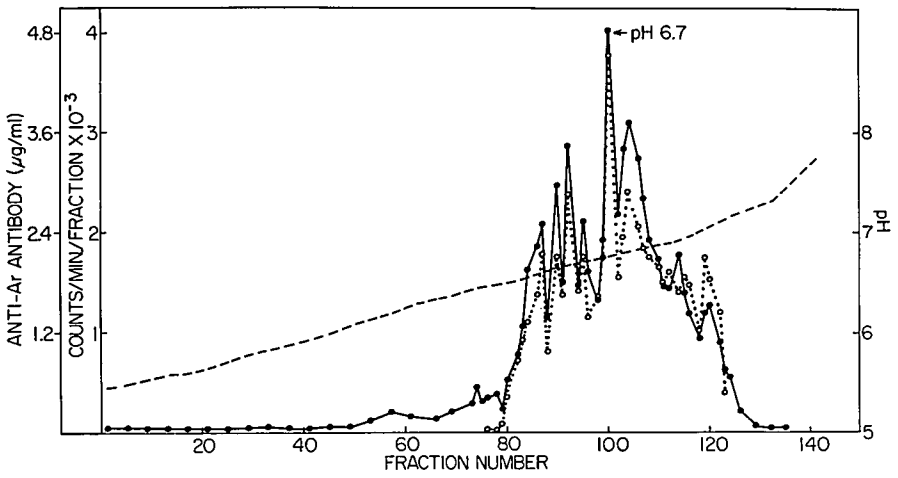


FIG. 1. Isoelectric-focusing pattern of specifically purified anti-Ar antibodies of mouse HIS-7; fractions were obtained with the Autogel Divider. The sample was prepared by mixing the $^{125}$I-labeled antibody with a 600-fold excess of the same, unlabeled preparation. 100 μg was applied to the gel Each fraction was assayed for counts per minute (solid line). A 10 μl aliquot of each fraction was assayed for content of anti-Ar antibody (dotted line) The arrow designates the pI value of the peak used as ligand for inhibition studies. This ligand was obtained in a separate run with highly labeled protein.

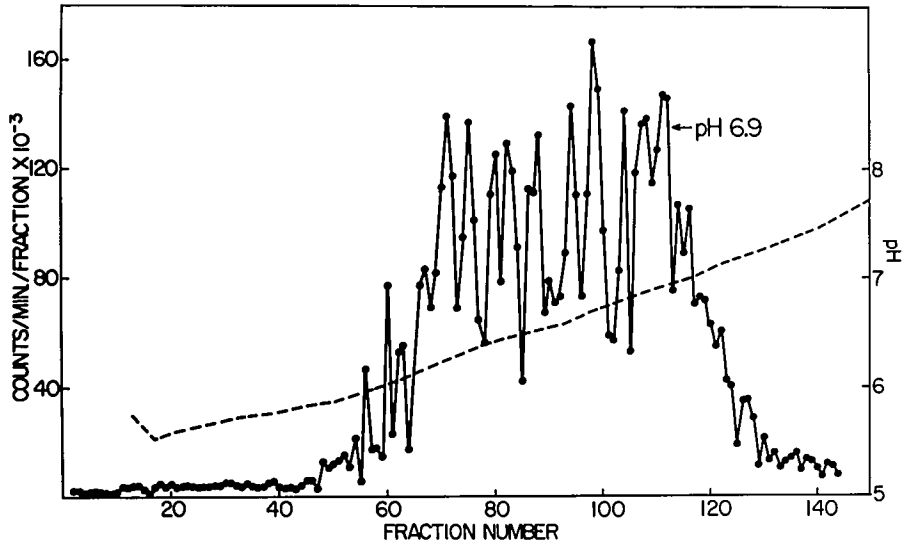


FIG. 2. Isoelectric-focusing pattern of $^{125}$I-labeled specifically purified anti-Ar antibody of mouse HIS-13 The arrow designates the peak used in the radioimmunoassay for idiotype content. Approximately 20 μg of labeled antibody and 80 μg of unlabeled A/J IgG (as carrier) were applied to the gel.

of anti-Ar antibodies by the assay using microtiter plates, described under Materials and Methods. A typical run is shown in Fig. 1. The correspondence between the curves obtained by the two procedures was very close and the peaks of anti-Ar antibody activity corresponded within experimental error with the

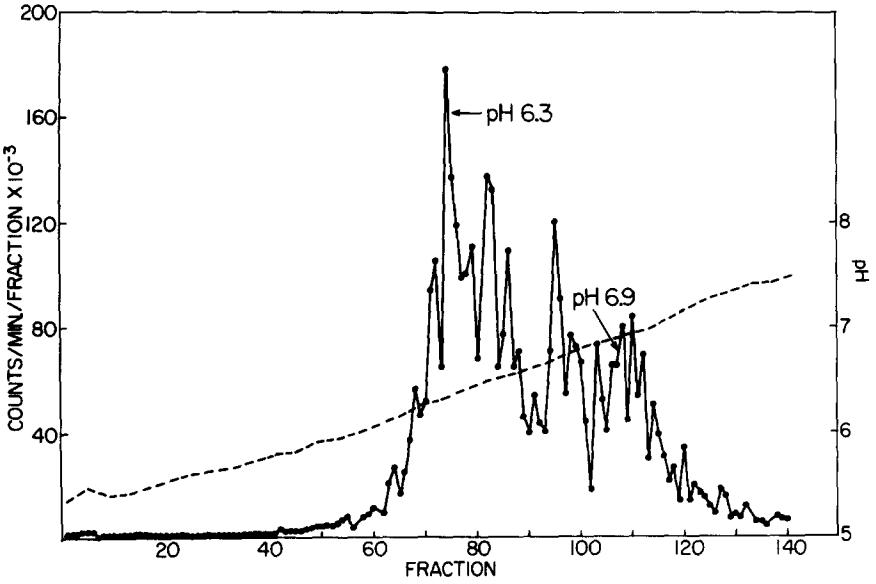


FIG. 3. Isoelectric-focusing pattern of $^{125}$I-labeled specifically purified anti-Ar antibodies of mouse HIS-5 The arrows designate the peaks used in radioimmunoassays for idiotype content. Approximately 20 $\mu$g of labeled antibody and 80 $\mu$g of unlabeled A/J IgG (as carrier) were applied to the gel

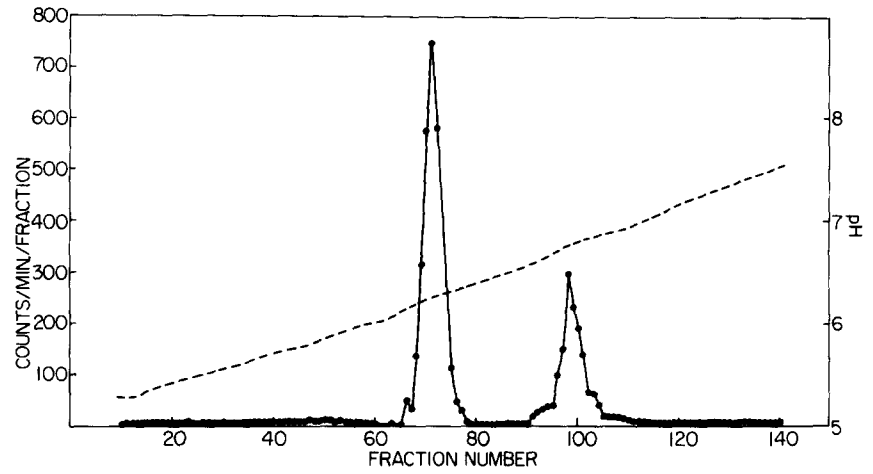


FIG 4 Refocusing of the peaks with pI 6.3 and 6.9 from mouse HIS-5 (see Fig 3). The ratio of amounts of protein applied with pI 6.3 and 6.9 was 3:1.

peaks of radioactivity. Any shift in pI due to iodination is, therefore, not significant. In nearly all of the peaks the ratio of counts per minute to anti-Ar activity was similar. In a few peaks of proteins HIS-5 and HIS-13 the ratio was considerably higher than that observed in nearly all other peaks. This may be due to very low affinity of the anti-Ar antibodies in those peaks, preventing quantitative measurement of antibody content, or to a small degree of contamination by labeled protein lacking antibody activity. In all cases, however, the

peaks of radioactivity and anti-Ar activity coincided. The presence of multiple bands in each preparation was confirmed by using unlabeled antibodies and staining with Coomassie Blue (without extraction).

*Investigation of Subpopulations from Antibodies of Suppressed, Hyperimmune Mice.* The purpose of this work was to study homogeneous subpopulations bearing a single idiotype. Those peaks chosen for further study are indicated by the arrows in Figs. 1–3. In addition to the uniformity with respect to pI, further evidence for homogeneity was the fact that over 90% of the radioactivity in each of the four designated peaks could be precipitated by autologous anti-id antiserum.

Quantitative tests for IgG subclasses were carried out on the labeled individual peaks by the radioimmunoassay described under Materials and Methods; 83–90% of the four proteins were IgG1; the remainder of the four proteins radioactivity was precipitable by antibodies to IgG2a and IgG2b. The totals for the three precipitations ranged from 96 to 102%. Radioimmunoassays for IgA or IgM were not carried out but the unfocused, purified antibodies all failed to form precipitin lines with anti-IgA or anti-IgM in the Ouchterlony test. This was as expected since the purification procedure includes passage through DEAE-cellulose in 0.04 M phosphate buffer, pH 8.

*Effects of Haptens on the Binding of Anti-id Antibodies to Ligands Isolated by Isoelectric Focusing.* These results are shown in Table I. In all instances, the hapten, PABT, was a potent inhibitor of binding. This compound, that resembles a substituted tyrosyl side chain in the immunogen, has an average binding affinity for anti-Ar antibodies of nonsuppressed A/J mice of approximately $5 \times 10^5$ to $1 \times 10^6$ $M^{-1}$ (15); *p*-arsanilate, which is smaller and would also be expected to have a lower binding affinity since it resembles the immunogen less closely, was somewhat less potent as an inhibitor. *p*-Aminobenzoate had no significant inhibitory effect on binding.

*Individuality of Private Idiotypes in Antibodies Isolated by Isoelectric Focusing.* Tables II–V present data relating to the presence of "private" idiotypes in anti-Ar antisera of a large number of mice. The four radioactive ligands used are the antibodies in the individual peaks indicated by the arrows in Figs. 1–3. Two of the ligands are from protein HIS-5 (peaks with pI 6.3 and 6.9); the other two are individual peaks obtained by isoelectric focusing of proteins HIS-7 and HIS-13. The idiotypes under investigation were shown by cross-inhibition studies to be unrelated, using specifically purified, unfocused anti-Ar antibodies as inhibitors. The weights of proteins HIS-5, HIS-7, and HIS-13 tested as inhibitors were 125, 150, and 80 $\mu$g, respectively.

Inhibitors tested in the various systems (Tables II–V) were ascitic fluids from A/J mice, or sera of BALB/c mice, immunized with KLH-Ar. The total anti-Ar antibody content was determined by the radioimmunoassay (using Microtiter plates) described under Materials and Methods.

In the radioimmunoassay for private idiotypes, 25 $\mu$l of each sample was tested in duplicate as the inhibitor. There was wide variation in the anti-Ar antibody concentration of the samples tested (0.4–8 mg/ml). As indicated in the tables, some of the samples were from hyperimmunized A/J mice that had been immunologically suppressed with respect to the CRI before immunization. The

TABLE I
*Effect of Haptens on the Reactions of Anti-id Antibodies*

| Source of labeled ligand* | Hapten | | | | | |
|---|---|---|---|---|---|---|
| | PABT | | *p*-Arsanilate | | *p*-Aminobenzoate | |
| | 1.6‡ | 20 | 1.6 | 20 | 1 6 | 20 |
| | *$^{125}$I-label bound, % of controls*§ | | | | | |
| HIS-5, pI 6.3 peak | 45 | 8 | 107 | 54 | 113 | 130 |
| HIS-5, pI 6.9 peak | 25 | 19 | 55 | 60 | 89 | 119 |
| HIS-13, pI 6.9 peak | 26 | 26 | 55 | 57 | 101 | 97 |
| HIS-7, pI 6 7 peak | 9 | 2 | 12 | 12 | 112 | 84 |
| CRI‖ | 57 | 52 | 85 | 78 | 90 | 109 |

* The $^{125}$I-labeled ligands (1.5–2 ng) were proteins in the peaks of specifically purified antibodies designated by arrows in Figs. 1–3. The proteins were present in a buffered mixture containing 1% BSA. The amount of anti-id antibody used was somewhat less than optimal The pH of each mixture was 8.0.

‡ Concentration in millimoles per liter

§ Expressed as percentage of the amount of ligand bound in the absence of hapten.

‖ Specifically purified anti-Ar antibody from a nonsuppressed mouse, comprising approximately 60% of the CRI.

TABLE II
*Displacement from Anti-Idiotypic Antibody of the Major Peak (pI 6.7) of the Anti-Ar Antibody of the Suppressed A/J Mouse HIS-7*

| Inhibitor (source of anti-Ar antibody) | No of Mice | Nanograms Anti-Ar antibody required for 50% inhibition* |
|---|---|---|
| Autologous (HIS-7, specifically purified) | | 8 |
| Nonsuppressed A/J mice | 5 | >10,000 |
| | 71 | >25,000 |
| | 68 | >75,000 |
| A/J Mice suppressed for CRI | 14 | >10,000 |
| | 13 | >25,000 |
| | 10 | >75,000 |
| Total no of mice tested | 181 | |

* 1.5 ng of labeled ligand was used in the assay

criterion for the presence of a private idiotype was the capacity to cause more than 50% inhibition of binding in the radioimmunoassay. Nearly all noninhibitory sera or ascitic fluids caused less than 20% inhibition. The same set of samples was tested as inhibitors in each system, with the exception that BALB/c sera were used only in the experiments of Table V. (The reason for testing the BALB/c sera in this system will be indicated below.) Thus, the experiments were designed to identify the presence of any of the four private idiotypes in the samples tested as inhibitors.

Two of the private idiotypes studied (from mice HIS-7 and HIS-13) could not be detected in any of the mice investigated (Tables II and III). In the case of HIS-7,

TABLE III

*Displacement of Labeled Anti-Ar Antibodies of A/J Mouse HIS-13 (pI 6.9 fraction) from their Anti-id Antibodies*

| Inhibitor (source of anti-Ar antibody) | No. of Mice | Nanograms Anti-Ar antibody required for 50% inhibition* |
|---|---|---|
| Autologous (HIS-13, specifically purified) | | |
| Unfocused | | 85 |
| Focused (pI 6 9) | | 15 |
| Nonsuppressed A/J Mice | 5 | >10,000‡ |
| | 71 | >25,000 |
| | 68 | >75,000 |
| A/J mice suppressed for CRI | 14 | >10,000 |
| | 13 | >25,000 |
| | 10 | >75,000 |
| Total no of mice tested | 181 | |

* 2 ng of labeled ligand was used in the assay.

‡ These numbers are the same as those in Table II because the same 181 sera were used in the tests

TABLE IV

*Displacement of Labeled Anti-Ar Antibodies of A/J Mouse HIS-5 (pI 6.9 fraction) from their Anti-id Antibodies*

| Inhibitor (source of anti-Ar antibody) | No of Mice | Nanograms Anti-Ar antibody required for 50% inhibition* |
|---|---|---|
| Autologous (HIS-5, specifically purified) | | |
| Unfocused | | 50 |
| Focused (pI 6 9) | | 15 |
| Focused (pI 6 3) | | >135 |
| Nonsuppressed A/J mice | 1 | 4,400 |
| | 2 | 40,000, 100,000 |
| | 4 | >10,000 |
| | 70 | >25,000 |
| | 67 | >75,000 |
| A/J mice suppressed for CRI | 14 | >10,000 |
| | 13 | >25,000 |
| | 10 | >75,000 |
| Total no. of mice tested | 181 | |

* 1.5 ng of labeled ligand was used in the assay

8 ng of unlabeled autologous antibody caused 50% inhibition of binding. This degree of inhibition could not be achieved with 25 $\mu$l of any of the other samples, containing between 10,000 and 200,000 ng of anti-Ar antibody. A total of 181 A/J mice were tested, of which 37 were suppressed with respect to the common, cross-reactive idiotype. Thus, less than one molecule in 1,200 to less than one

TABLE V

*Displacement of Labeled Anti-Ar Antibodies of A/J Mouse HIS-5 (pI 6.3 fraction) from their Anti-id Antibodies*

| Inhibitor (source of anti-Ar antibody) | No. of Mice | Nanograms Anti-Ar antibody required for 50% inhibition* |
|---|---|---|
| Autologous (HIS-5, specifically purified) | | |
| Unfocused | | 37 |
| Focused (pI 6.3) | | 8 |
| Focused (pI 6.9) | | >70 |
| Nonsuppressed A/J mice | 3 | 800, 1,400, 2,000 |
| | 18 | 3,000–10,000 |
| | 25 | 11,000–120,000 |
| | 5 | >10,000 |
| | 49 | >25,000 |
| | 44 | >75,000 |
| A/J mice suppressed for CRI | 2 | 3,000–10,000 |
| | 3 | 11,000–150,000 |
| | 13 | >10,000 |
| | 10 | >25,000 |
| | 9 | >75,000 |
| BALB/c mice | 12 | >10,000 |
| | 9 | >25,000 |
| | 5 | >75,000 |
| Total no. of A/J mice tested | 181 | |

* 1.5 ng of labeled ligand was used in the assay

molecule in 25,000 contained the HIS-7 idiotype; the actual concentrations may be much lower since 50% inhibition was never achieved.

The same group of sera and ascitic fluids were tested as inhibitors in the HIS-13 system, again with consistently negative results. In this case, 85 ng of unfocused, autologous protein (specifically purified HIS-13 antibody) caused 50% inhibition of binding, whereas 15 ng of unlabeled protein with pI 6.9 (the same as that of the labeled ligand) caused 50% inhibition. Unlabeled antibody of the same mouse, HIS-13, but with pI 6.3, was noninhibitory; only 150 ng was tested because of the necessity of isolating the material after focusing.

The possibility was considered that the private idiotype might actually be present in some noninhibitory samples, but masked in some manner through interaction with another component. Therefore, 10 ng of unlabeled HIS-7 anti-Ar antibody was added to 25 $\mu$l of each of 20 randomly selected, noninhibitory ascitic fluids represented in Table II. The mixtures were then tested as inhibitors in the radioimmunoassay for the HIS-7 idiotype (pI 6.7). In all cases more than 50% inhibition was observed (maximum, 74%). A similar experiment was done with 20 noninhibitory ascitic fluids with labeled HIS-5 (pI 6.9) as ligand. Addition of 50 ng of unlabeled, unfocused HIS-5 purified antibody caused more than 50% inhibition in each case.

Table IV shows the results obtained with one of the two idiotypes isolated

from the anti-Ar antibodies of mouse HIS-5 (pI 6.9). In this case 3 of the 181 mice tested contained inhibitory protein. However, the concentration in these three ascites was far lower than that in protein HIS-5, since 4,400–100,000 ng of antibody were required for 50% inhibition. In this system, 50 ng of unlabeled, unfocused protein HIS-5 and 15 ng of focused protein having the same pI value as the labeled ligand were required for 50% inhibition of binding. Unlabeled antibody of the autologous mouse (HIS-5), but with a different pI value (6.3), was noninhibitory at the highest concentration tested (135 ng caused 8% inhibition of binding).

The idiotype of the anti-Ar antibodies of the same mouse, HIS-5, but with pI 6.3, differed markedly from the other three idiotypes tested, in that a substantial number of the samples tested were inhibitory (Table V); however, the concentration of the idiotype was always much lower than that in mouse HIS-5. Of 181 A/J mice tested, 51 contained enough of the idiotype to cause 50% inhibition. Owing to the large amounts of protein required, the data do not establish whether the idiotypes are identical or cross-reactive (16), or how many idiotypes comprise the inhibitory protein.

Supporting the possibility that the observed inhibition reflects an identical or cross-reactive idiotype was the absence of inhibitory capacity in 26 individual BALB/c sera containing 0.32–4.4 mg/ml anti-Ar antibodies; 25 $\mu$l was tested. In addition, 21 individual A/J sera containing high titers of antibodies to a conjugate of KLH with *p*-azophenyltrimethylammonium groups were noninhibitory. The presence of high concentrations of antibodies to both the protein and the hapten in each sample was shown by Ouchterlony analysis.

These results suggest but do not establish that the inhibition by certain ascitic fluids in the HIS-5 (pI 6.3) system is due to the presence of anti-Ar antibodies. To test this possibility, ascitic fluids from 10 mice with inhibitory capacity were adsorbed with Sepharose-4B conjugated to bovine IgG (BGG)-Ar. As a control, similar adsorptions were carried out with A/J IgG in place of BGG-Ar; the amounts used are specified in Table VI. Mixtures were allowed to stand with frequent agitation at room temperature for 1 h, then overnight at 4°C. The inhibitory capacity was decreased in all of the supernates of fluids adsorbed with the BGG-Ar conjugate; in 7 of the 10 mice the removal was essentially complete (Table VI). After adsorption with the BGG-Ar conjugate, all samples lost the ability to form a precipitin band with BGG-Ar in the Ouchterlony test but retained their precipitating activity against KLH.

## Discussion

The experiments were designed to determine the frequency of occurrence of "private" idiotypes of anti-Ar antibodies that arise on immunization with KLH-Ar of mice that are immunologically suppressed with respect to the intrastrain CRI associated with these antibodies in normal A/J mice. For use as ligands in the radioimmunoassay anti-Ar antibodies were obtained from four single peaks after isoelectric focusing of the specifically purified antibodies from three randomly selected mice. Over 90% of each ligand was precipitable by autologous anti-id antibodies, using the double-antibody procedure. These proteins refocused as single peaks with little or no change in pI, and it was shown that the

TABLE VI

*Association Between the Capacity to Inhibit Antibodies to the HIS-5 (pI 6.3) Idiotype and the Presence of Anti-Ar Antibodies**

| Source of inhibitor, A/J mouse no. | Protein Conjugated to Sepharose-4B | |
|---|---|---|
| | A/J IgG | BGG-Ar |
| | *$^{125}$I-ligand bound, % of Control‡* | |
| 19 | 53 (1) | 105 (10) |
| 10 | 40 (2) | 104 (6) |
| 16 | 19 (1) | 99 (7) |
| 48 | 19 (1) | 96 (15) |
| 39 | 32 (4) | 96 (4) |
| 44 | 22 (0) | 92 (12) |
| 24 | 59 (10) | 88 (6) |
| 49 | 53 (1) | 70 (1) |
| 37 | 35 (6) | 56 (4) |
| 22 | 30 (3) | 51 (1) |

* 200 μl of ascitic fluid from each of 10 randomly chosen mice expressing the HIS-5 (pI 6.3) idiotype were adsorbed with 300 μl of packed Sepharose-4B conjugated with bovine IgG-Ar or normal A/J IgG (2 mg/ml of packed beads). 25 μl of the supernate was tested as inhibitor in the radioimmunoassay for the idiotype.

‡ Mean values of duplicate tests, with average deviations in parentheses.

iodination procedure did not significantly affect the pI value. The usefulness of isoelectric focusing, to enhance detectability of the idiotype in other mice, was supported by the identification of two unrelated idiotypes (pI 6.3 and 6.9),[2] in the anti-Ar antibodies of mouse HIS-5, and by the heterogeneity of all of the isoelectric-focusing patterns.

Small amounts of labeled ligand (1–2 ng) were used in the radioimmunoassays to maximize the sensitivity of detection of the idiotype in the unlabeled preparations tested as inhibitors. A total of four labeled ligands were used; two from proteins HIS-7 and HIS-13 and the two unrelated idiotypes from protein HIS-5. All of the anti-id antisera were inhibited, to varying degrees, by free haptens (Table I).[3]

Using the criterion of 50% inhibition of binding of the labeled ligand for the presence of the corresponding idiotype, two of the four private idiotypes studied (HIS-7 and HIS-13) could not be detected in any immunized mouse other than the donor. The amounts of anti-Ar antibodies tested varied from 10 to 200 μg. In

[2] These 2 idiotypes arise independently, as shown by the fact that the idiotype associated with anti-Ar antibody of pI 6.3 was detected quite frequently in other immunized A/J mice, whereas the other idiotype was not.

[3] A term sometimes used in discussing those idiotypes that are not directly affected by the presence of a small hapten, i e , are not ligand modifiable, is "framework idiotype " The use of this term would seem to require further justification since the hypervariable region of an antibody is much larger than a typical hapten. Even those anti-id antibodies which are not inhibited by the presence of hapten might be directed entirely or in part to hypervariable segments. Also, haptens may fail to inhibit binding at concentrations high enough to saturate their binding sites, because of low affinity in comparison to the anti-id antibodies with which they are competing (17)

contrast, 8 and 15 ng of the two unlabeled ligands caused 50% inhibition of binding, respectively. The private idiotypes thus represented less than 1 part in 1,250 to less than 1 part in 25,000 of the anti-Ar antibodies of the other 181 mice tested. This group included 37 mice that had been suppressed with respect to the CRI before immunization. That the failure to detect the idiotype was not due to some factor that masked the idiotype was shown by adding very small amounts of autologous idiotypes to noninhibitory ascitic fluids; in all cases strong inhibition was observed.

There is obviously a striking contrast between the CRI which appears in the anti-Ar antibodies of every normal A/J mouse, and the private idiotypes which occur at very low frequency or are not detectable in other mice. It seems possible that the CRI represents the product of a germ line gene or genes, or arises from germ line genes by a very small number of mutations that take place in every mouse (1). The infrequency of occurrence of the two private idiotypes appears consistent with the hypothesis that they are generated by a substantial number of somatic mutations, so that their probability of occurrence in any individual mouse is exceedingly small. The very low frequency seems to us to suggest a random, rather than a programmed (18–20) somatic process for the generation of diversity. The results do not formally prove this, since one might postulate that an extremely large repertoire of anti-Ar antibodies is produced in a sequential manner, with only a few idiotypes capable of expression at any given moment. Our sample of inhibitors, although large, might have been inadequate. It should be stressed, however, that each of the hyperimmunized mice tested undoubtedly produced many clones of anti-Ar antibodies (judging from those that were studied by isoelectric focusing) and that only a few nanograms per milliliter would have been detected, as evidenced by experiments in which small amounts of the unlabeled idiotype were added to noninhibitory antibody-containing fluids. Also, the data on which the hypothesis of programmed mutation are based do not appear conclusive.[4]

So far we have discussed the two private idiotypes that were not detectable in other mice. A third private idiotype, associated with protein HIS-5 (pI 6.9) was present in 3 of 181 immunized mice, but at very low concentrations (Table IV). The fourth idiotype (protein HIS-5, pI 6.3) was detected by our assay in 28% of A/J mice immunized with KLH-Ar, in all cases at a low concentration in comparison to that in mouse HIS-5. When present, it comprised from 1 part in 100 to 1 part in 25,000 of the anti-Ar population. We do not know whether the inhibitory material is an identical idiotype or a cross-reactive idiotype (16).[5] However, its concentration was reduced by adsorption with a BGG-Ar-Sepharose conjugate, and it was completely removed in 7 of the 10 ascitic fluids tested. Also, the anti-Ar antibodies of 26 BALB/c mice, and antibodies to an unrelated hapten

[4] For example, the appearance of the TEPC 15 idiotype on day 6 might reflect the activation of a germ line gene at this time rather than a systematic timed series of mutations (21). In other relevant investigations (19, 20), idiotypes or spectrotypes were not defined, although evidence supporting a programmed appearance of antibody specificities was presented.

[5] In this publication it was also shown that idiotypes characteristic of certain human Bence Jones proteins or myeloma proteins are present in a concentration below 1 part in $10^6$, or 1 part in $10^7$, respectively, in pooled normal human γ-globulin.

on the same carrier from 21 A/J mice were not inhibitory. The data thus suggest that the inhibitor, when present, is associated with an identical or cross-reactive idiotype. The relatively frequent appearance of this idiotype would be accommodated within our hypothesis by the assumption that it arises through a smaller number of mutations than the other three idiotypes investigated, with a consequently higher probability of appearance. Even this idiotype, however, never represented a major proportion of the anti-Ar response of a mouse, and thus contrasts markedly with the CRI characteristic of nonsuppressed A/J mice.

The fact that maps of $V_H$ genes can be constructed (22) does not disprove the possibility of somatic mutations, but rather suggests that those genes used in the construction of the maps are germ line genes or closely related to germ line genes through a small number of mutations which occur in every mouse. Only those genes whose products are found in most or all mice of a strain can be used for mapping, and our data on the anti-Ar antibodies in the A/J strain would suggest that most immunoglobulin gene products could not be applied to map construction, owing to their restricted occurrence within the members of a strain.

Experiments have also been done in which mice of seven strains other than A/J were immunized with KLH-Ar (23). A total of 16 anti-id antisera were prepared against anti-Ar antibodies of individual mice and tested against the anti-Ar antibodies of other mice of the same strain. Strong intrastrain idiotypic cross-reactions among the anti-Ar antibodies were not observed in any of these strains, although a weak set of intrastrain cross-reactions was noted in BALB/c mice with one of three anti-id antisera tested. The other strains used were AKR/J, C57Br/CdJ, LP/J, RF/J, SM/J, and SWR/J.

The present data support those of other investigators (24–26), in indicating that the repertoire of anti-hapten or anti-protein antibodies in an individual strain is very large. In our experiments a few major spectrotypes and a number of minor spectrotypes of anti-Ar antibodies typically appeared upon immunization of an individual mouse.

A finding that is somewhat difficult to reconcile with the data obtained with inbred mice is that of DuPasquier and Wabl (27), who worked with genetically identical (isogenetic) frogs. Such animals produce antibodies to the dinitrophenyl hapten group or to sheep erythrocytes that exhibit virtually identical isoelectric-focusing patterns. Possible explanations include the following: (*a*) Inbred mice do not all have identical sets of germ line *V* genes, although regions of identity must exist to account for intrastrain CRI; (*b*) somatic mutational processes might be of much greater significance in mammals than in amphibia; (*c*) somatic mutations are programmed by genes that are identical in isogenetic frogs but which may exhibit variability in inbred mice; (*d*) all immunoglobulins are the products of germ line genes, with mice having the greater repertoire. Recent data on mRNA-DNA hybridization, suggesting the existence of a limited number of germ-line *V*-region genes in mice (e.g., 28–30) argue against the latter possibility. Our data do not bear directly on the validity of the "gene-insertion" model (31, 32) to account for the presence of hypervariable regions, except to suggest that, if it is correct, the number of such genes must be very large.

## Summary

Inoculation of rabbit anti-idiotypic (anti-id) antibodies suppresses the subsequent appearance of a cross-reactive idiotype (CRI) associated with the anti-*p*-azophenylarsonate (anti-Ar) antibodies of A/J mice. Such suppressed mice produce normal concentrations of anti-Ar antibodies which lack the CRI, but against which anti-id antisera can be prepared. The anti-Ar antibodies of an individual, suppressed mouse do not in general share idiotype with anti-Ar antibodies of other A/J mice, either suppressed or nonsuppressed. The present experiments were undertaken to quantitate several "private idiotypes" in a large number of hyperimmunized A/J mice. Anti-Ar antibodies of three mice, suppressed for the CRI, were labeled with $^{125}I$ and subjected to isoelectric focusing. Four single peaks, that were over 90% reactive with autologous anti-id, were randomly selected for use as ligands in a radioimmunoassay, and ascitic fluids containing anti-Ar antibodies from 181 A/J mice were tested as inhibitors. Two of the four idiotypes could not be detected in any mouse other than the donor. The concentration of the idiotype was less than 1 part in 1,250 to less than 1 part in 25,000 of the anti-Ar antibody population; these are minimum values. A third idiotype was detected in 3 of the 181 mice, but at very low concentrations. The fourth idiotype was present in 28% of the mice, again at a low concentration. The data support the existence of a very large repertoire of anti-Ar antibodies in the A/J strain and are consistent with a process of random somatic mutation for generating diversity in hypervariable regions. It is proposed that the cross-reactive idiotype may be controlled by a germ line gene or a gene related to a germ line gene through a small number of somatic mutations; and that the idiotypes that were not detectable in other mice were the products of genes that had undergone extensive mutations, with a low probability of recurrence in other mice.

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