ISOLATION FROM INDIVIDUAL A/J MICE OF ANTI-*p*-AZOPHENYLARSONATE ANTIBODIES BEARING A CROSS-REACTIVE IDIOTYPE*

By AMAR S. TUNG and ALFRED NISONOFF

(From the Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois 60612)

Rabbit or human antibodies generally possess unique idiotypic specificities; i.e., they fail to exhibit idiotypic cross-reactivity with antibodies of the same specificity from other individuals (1–4). Such cross-reactions are more frequent among antibodies from rabbits of partially inbred families (5) or from mice of the same inbred strain (6–12).[1] Thus, all A/J mice immunized with KLH-Ar[2] produce anti-Ar antibodies, some of which share idiotypic determinants (8, 13, 14). Since this idiotype does not appear upon immunization of mice of many other strains, it can be used as a genetic marker for V regions of immunoglobulins of the A/J strain (8, 15). Because of the strong intrastrain idiotypic cross-reactions, it is also possible to use anti-idiotypic antiserum, prepared against the anti-Ar antibodies of an individual A/J mouse, to suppress the appearance of the cross-reactive idiotype (CRI), in both primary and secondary responses in neonatal or adult A/J mice (16–18).

Molecules with CRI generally comprise about 20–70% of the anti-Ar population in A/J mice immunized with KLH-Ar. The remainder of the population is probably very heterogeneous; thus, after suppression of the CRI high concentrations of anti-Ar antibodies can still be elicited, but individual suppressed A/J mice produce idiotypically distinct populations (17).

The present investigation is concerned with the isolation and partial characterization of the subpopulation of anti-Ar antibodies bearing CRI. Such antibodies, from each A/J mouse investigated, were found to have a characteristic, narrow range of isoelectric pH. Sufficient quantities have been isolated to permit initiation of structural studies and comparisons of antibodies from individual mice. Since this subpopulation is of restricted heterogeneity, and occurs in every immunized A/J mouse, partial sequence analyses of antibodies from individual

* Supported by grants AI-06281 and AI-10220 from the National Institutes of Health.

[1] Idiotypic cross-reactivity within members of the same strain of mouse is not always observed. For example, intrastrain idiotypic cross-reactions are observed among anti-Ar antibodies raised in A/J, A/He, or A/WySn mice but rarely in the AKR, LP, SM, or C57BR/cd strains (L. L. Pawlak and A. Nisonoff, unpublished observations).

[2] *Abbreviations used in this paper:* Ar, *p*-azophenylarsonate; BGG, bovine IgG; BSA, bovine serum albumin; CRI, cross-reactive idiotype; FCA, Freund's complete adjuvant; IEF, isoelectric focusing; KLH, keyhole limpet hemocyanin; pI, isoelectric point.

mice may provide information concerning the constancy or diversity of structure of antibodies with shared idiotype from different individuals.

Materials and Methods

Anti-p-azophenylarsonate (Anti-Ar) Antibodies. The method of preparation of the KLH-Ar antigen used as the immunogen has been described (8). Male A/J mice, 8–10 wk of age, were inoculated intraperitoneally with 0.5 mg of KLH-Ar in 0.02 ml of 0.15 M NaCl, emulsified with 0.18 ml of Freund's complete adjuvant (FCA). This was repeated after 14, 21, 28, and 35 days. 4 wk after the initial injection about $\frac{3}{4}$ of the mice developed an ascites fluid containing, on the average, 8–10 mg/ml of antihapten antibody precipitable by a bovine IgG-Ar conjugate. Ascites fluid was collected by tapping the abdominal cavity and was immediately frozen. This process was repeated every 3–5 days while the fluid persisted (for about 3 wk). Cheese was included in the diet during this period; this decreased the mortality rate. A maximum of 25 ml, and an average of approximately 6–8 ml, of ascites fluid was obtained from individual mice. Anti-Ar antibodies were specifically purified from ascites fluid as described previously (8), with the exception that lesser amounts of DEAE-cellulose (0.2 cm^3/mg of antibody) were used. Alternatively, purification was carried out by adsorption of the antibodies from ascites fluid onto Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) to which bovine IgG-Ar had been conjugated by the method which utilizes cyanogen bromide (19). EDTA (0.01 M) was added to the fluid before the adsorption to minimize uptake of complement. The adsorbed antibodies were eluted in the cold with a solution containing 1 M propionic acid and 0.15 M NaCl; the eluate was neutralized immediately and dialyzed against neutral buffer.

DEIONIZATION OF UREA. To 1 liter of a freshly prepared 5 M solution of urea (Schwartz-Mann, Orangeburg, N.Y.) was added 15 g of a mixed-bed resin (Bio-Rad Laboratories, Richmond, Calif.). After mixing for 30 min, the solution was filtered through a sintered glass funnel in the cold and was used immediately.

ISOELECTRIC FOCUSING (IEF). Two methods, differing with respect to the supporting medium and the amount of protein applied, were used. In one, IEF was carried out in cylindrical polyacrylamide gels (5 × 115 mm) by a modification of the procedure of Wrigley (20). The gel was prepared with the following reagents: acrylamide, 4.85%; bisacrylamide, 0.15%; riboflavin, 5×10^{-5}%; *N,N,N*^{1},*N*^{1}-tetraethylenediamine, 0.05%; urea, 3.5 M; Ampholine, pH 5–8, final concentration, 2%. Acrylamide, bisacrylamide and the amine (all "electrophoresis grade") were obtained from Bio-Rad Laboratories. The carrier ampholytes (Ampholine) were obtained from LKB Instruments, Inc. (Rockville, Md.). 50–250 μg of protein in 20–30 μl of 2% ampholyte solution, containing 4 mg of sucrose, was applied to the top of the gel. The protein was labeled with ^{125}I (500–1,000 cpm/μg) to permit quantitation. Above this was placed a layer of ampholyte in sucrose solution, both at a lower concentration. IEF was carried out in the cold room starting with a constant current of 1 mA per tube; when the voltage reached 450 V, it was maintained at this level for 6–8 h. Immediately thereafter the gel was fractionated by using an AGDW-8 Auto-Gel Divider (Savant Instruments, Hicksville, N.Y.) at a rate of 1 cm/110 sec.; 0.15 M saline, freshly degassed, was used for elution and five-drop fractions were collected in ice. To every fourth fraction 0.7 ml of degassed saline was added, small particles of gel were allowed to settle, and the pH was determined. Bovine serum albumin (BSA) solution (0.3 ml, 5 mg/ml in borate-saline buffer, pH 8) was then added to each fraction to retard denaturation of the protein in the highly dilute solution. The mixture was centrifuged and the supernate removed; the particles were then washed with 0.3 ml of BSA solution, and the two supernates were combined. The amount of protein was determined by measurement of radioactivity.

In one set of experiments the polyacrylamide gel was cut into slices 0.5 mm thick. A gel slicer obtained from the Instrument Shop, Physics Dept., Univ. of Michigan, was used for this purpose.

On a larger scale, IEF was carried out on a plexiglass plate by a modification of the method of Radola (21). A thin slab (300 × 150 × 5 mm) of Sephadex G-75 (Superfine) was the supporting medium; 2.5 ml of a 40% Ampholine solution (pH 5–8) was added per 100 cm^3 of gel suspension, and freshly deionized urea was present at a final concentration of 3 M. The gel suspension was degassed under vacuum, poured over the plate, and allowed to settle in the cold room for 6 h. A groove (0.5 × 14 cm) was made, 12 cm from anodal end of the plate, to allow insertion of the protein (50–75 mg). The solution of protein (>20 mg/ml, in 0.1 M Tris-HCl buffer, pH 7.2) was first mixed with dry Sephadex

and Ampholine to give the same consistency as the remainder of the gel. Part of the gel bed (1.5 × 15 cm) was removed from each end and replaced by a "solvent pad" (Gelman Instrument Co., Ann Arbor, Mich.) previously soaked in the appropriate electrolyte solution (0.4 M ethylenediamine or 0.2 M phosphoric acid). Contacts between the paper pads and electrode vessels were made with cellulose acetate strips (Gelman Instrument Company, Ann Arbor, Mich.), shielded by dialysis tubing. IEF was carried out in the cold in a humid chamber. Initially 200 V was applied; this was increased to 400 V after 4 h, and maintained at this level for 34–36 h. Slices 0.5 cm in width were immediately cut from the gel and suspended in 2 ml of saline; after 15 min, the pH of the supernate was read at 25°C. The protein was recovered by placing the gel suspension on a sintered glass funnel and eluting with 2.5 ml of borate-saline buffer, pH 8, ionic strength 0.16, under nitrogen pressure. The concentration of protein was estimated from the optical density at 280 nm, using the extinction coefficient, $E^{1\%}_{1cm} = 15$.

PREPARATION OF AMPHOLYTES WITH A NARROW PH RANGE. Carrier ampholytes covering the pH range 6.3–7.4 were prepared by electrofocusing 10 ml of 40% ampholyte solution (pH 6–8) in an LKB 8101 column (22), and isolating the ampholytes of the desired pH range. The concentration of the resulting carrier ampholyte was approximately 4% (wt/vol). Before using these ampholytes of narrow range, a solution of ampholytes with a pH range of 3–10 was added in a quantity sufficient to constitute 10% of the total ampholytes present.

Rabbit Anti-idiotypic Antibodies. These were prepared against Anti-Ar antibodies, specifically purified from the antiserum of an individual mouse. The methods used for immunization, absorption of rabbit antisera, and proof of anti-idiotypic specificity of the absorbed antisera have been described (8, 14).

ASSAY FOR ANTI-IDIOTYPIC ANTIBODIES. A radioimmunoassay was employed (8), which utilizes 10 ng of ^{125}I- or ^{131}I-labeled anti-Ar antibody of the donor mouse, 0.05 μl of anti-idiotypic antiserum, 3 μl of rabbit antiovalbumin serum (to provide carrier protein), and excess goat antirabbit Fc (50–75 μl). The goat antiserum had previously been absorbed with immunoglobulins of A/J mice. Somewhat less than an optimal amount of anti-idiotypic antibody was used. The percent of radioactive ligand precipitated varied from 54 to 57% (corrected for the small amount of radioactivity (2–6%) precipitated when rabbit antiovalbumin antiserum was substituted for anti-idiotypic antiserum). The presence of idiotypic antibodies in various unlabeled preparations was assayed by their capacity to inhibit the binding of the radioactive ligand (^{125}I-labeled purified anti-Ar antibody). In some instances, ^{125}I-labeled antibodies were tested as inhibitors; in such experiments the ligand was labeled with ^{131}I. Inhibitors were added to the anti-idiotypic antiserum 15 min before the addition of the labeled ligand. All assays were carried out in duplicate or triplicate. The overall average deviation from the mean, expressed as percent ligand bound, was approximately 1.5%.

RADIOLABELING. Specifically purified anti-Ar antibodies from individual mice were trace-labeled with ^{125}I or ^{131}I by using minimal amounts of chloramine-T (23). Labeled proteins were dialyzed until more than 97% of the radioactivity was precipitable by trichloroacetic acid at a final concentration of 5%. The amount of iodine introduced was approximately 0.1 atom per molecule of protein. The presence of the iodine did not significantly alter the IEF spectrum (see Results). Measurements of radioactivity were carried out with a Nuclear-Chicago Autogamma Spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). In some instances ^{131}I was assayed in the presence of ^{125}I; the contribution of ^{125}I to the measured cpm was neglible.

Antisera to Mouse Immunoglobulins. Monospecific anti-IgG1, anti-IgG2a, and anti-IgG2b were prepared in rabbits by using myeloma proteins of the appropriate subclass as immunogens and adsorbing the antisera with myeloma proteins of other classes and subclasses. Goat anti-IgM and anti-IgA were obtained from Meloy Laboratories Inc. (Springfield, Va.) and shown to be unreactive with other classes of immunoglobulin by Ouchterlony analysis.

Results

Data obtained in IEF experiments carried out with polyacrylamide gels are shown in Figs. 1 to 4. The proteins are specifically purified anti-Ar antibodies, labeled with ^{125}I, from individual A/J mice; 250 μg, with a spec act of 500–1,000 cpm/μg, was introduced into each gel. The total yield of protein in the combined eluates,

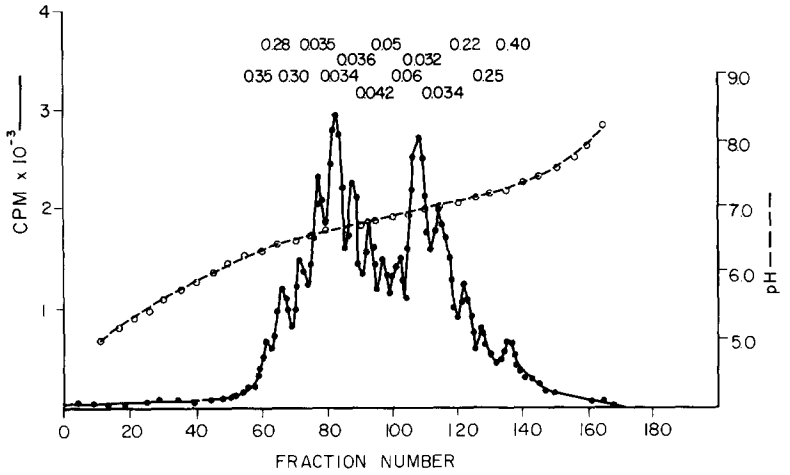

FIG. 1. Results of IEF on polyacrylamide gel (pH range, 5–8) of 250 μg ^{125}I-labeled specifically purified anti-Ar antibody of mouse 79. The protein in each peak was tested for its content of CRI by the standard inhibition assay, using the same antibody, labeled with ^{131}I, as ligand. The anti-idiotypic antibodies employed in the assay were prepared against anti-Ar antibodies of mouse 79. The weight of antibody in each peak, required to cause 50% inhibition of binding, is indicated directly above the corresponding peak in the figure.

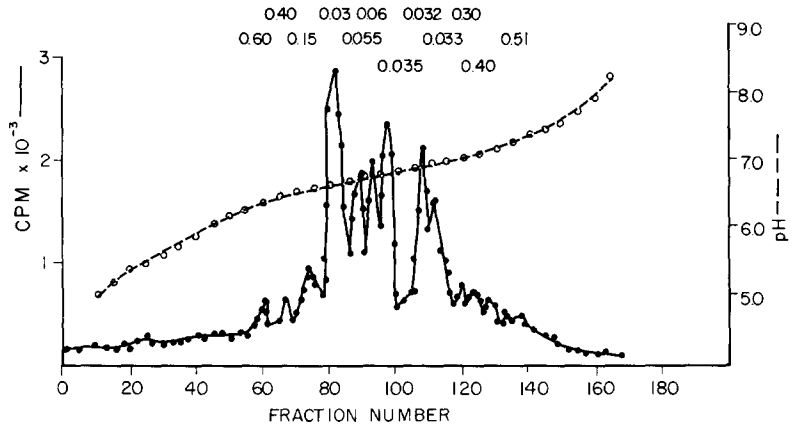

FIG. 2. The procedure is the same as that indicated in the legend of Fig. 1 except that the specifically purified anti-Ar antibody subjected to focusing was from mouse 426. The ligand and anti-idiotypic antibody are the same as in Fig. 1.

as estimated from the radioactivity recovered, varied between 60 and 80%. Each point in Figs. 1 and 2 represents the contents of a five-drop fraction (see Methods). After the addition of BSA, fractions were tested for their capacity to inhibit the binding of 10 ng of ^{131}I-labeled specifically purified anti-Ar antibody from mouse 79 to its anti-idiotypic antibodies. (The same system was used in all tests for idiotype, unless otherwise specified.) The use of the ^{131}I label precluded errors due to the radioactivity of the ^{125}I-labeled inhibitor. The amounts of protein from each fraction that were tested for inhibitory capacity were 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 μg; in a few instances 2.5 and 5 μg quantities were also

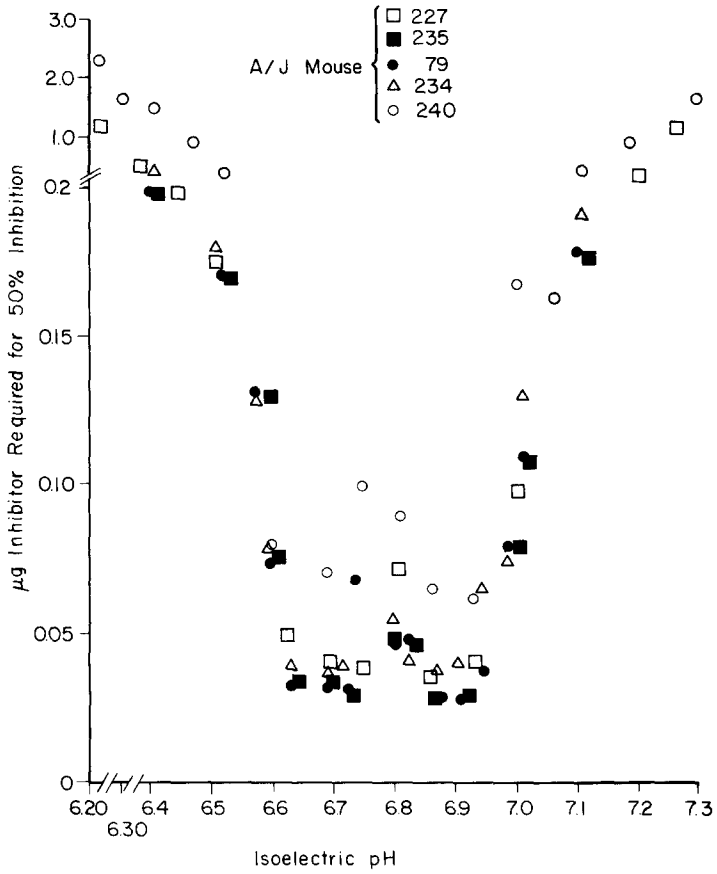

FIG. 3. Composite of data from several mice on the content of CRI in fractions obtained after IEF of specifically purified anti-Ar antibodies on polyacrylamide gels (pH 5–8). The ^{131}I-labeled ligand and the anti-idiotypic antibody are the same as in Fig. 1. Note that the antibodies from four of the five mice appear to be idiotypically almost identical.

assayed. The total volume of the reaction mixture, not including the goat antirabbit Fc, was held constant at 0.4 ml. The weight of inhibitor needed for 50% inhibition of binding was estimated by plotting the data on a graph and interpolating. The relative error in the data, owing to the interpolation, may be as high as 10–20%; this was acceptable since differences in inhibitory capacity among the proteins in different peaks varied by as much as a factor of 10. The data in Figs. 1, 2, and 3 were obtained with antibodies from individual mice that, with one exception (mouse 240), were selected for their high content of CRI. The results in Fig. 4 are more representative of the random population; the antibodies from only two of the five mice represented in that figure are very rich in CRI. (All immunized A/J mice, however, possess CRI in significant quantity.)

The data in Fig. 1 were obtained by subjecting ^{125}I-labeled anti-Ar antibodies of mouse 79 to IEF in polyacrylamide gel. (The same antibodies had been used to elicit the anti-idiotypic antiserum used in tests of inhibition.) It is evident that the maximal concentration of idiotype, as indicated by the weight of antibody

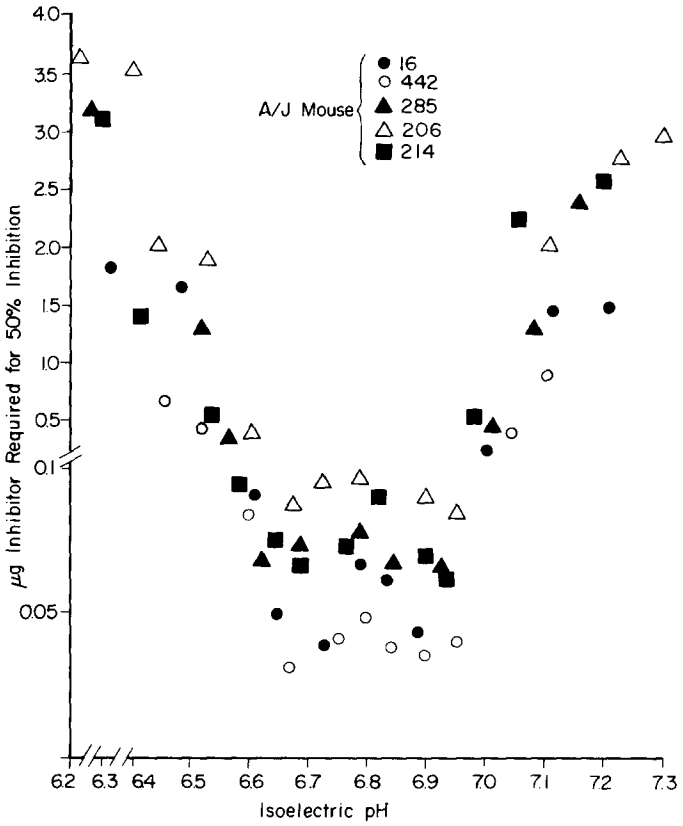

FIG. 4. See legend of Fig. 3. The results obtained with anti-Ar antibodies of five additional mice are shown here.

needed for 50% inhibition, is associated with fractions focusing between pH 6.6 and pH 6.95, and particularly in peaks focusing at pH 6.67 and 6.87. Outside this pH range the inhibitory capacity per unit weight of protein falls off rapidly; e.g., about 10 times as much antibody with a pI of pH 6.4, as compared with that of pI 6.7, is required for 50% inhibition.

Very similar results are evident in Fig. 2. Here, the protein subjected to IEF was ^{125}I-labeled specifically purified anti-Ar antibody of mouse 426. It is evident from Figs. 1 and 2 that maximum inhibitory capacity occurs between pH 6.65 and 6.92 in each system and is approximately the same, per unit weight, for the antibodies of the two mice.

Data obtained upon IEF of anti-Ar antibodies of 10 individual mice, including mouse 79, are summarized in Figs. 3 and 4, in which the weight of protein required to cause 50% inhibition is plotted against pI. The most effective inhibitors in each experiment have an isoelectric point between pH 6.65 and 6.95; the inhibitory capacity falls off very markedly outside this range and, to a much lesser extent, around pH 6.8.

Four of the five mice represented in Fig. 3 produced anti-Ar antibodies which gave patterns of pI vs. inhibitory capacity that are very similar to one another.

The four include mouse 79, whose anti-Ar antibodies were used to elicit the anti-idiotypic antibodies used for quantitative tests. As noted above, the antibodies of the fifth mouse (no. 240; Fig. 3) were somewhat less inhibitory in the test system; however, the material with maximum inhibitory capacity from mouse 240 has approximately the same range of pI as that in the other four preparations. Three of the antibody preparations represented in Fig. 4 have less CRI per unit weight of antibody than the four preparations discussed above; however, maximum inhibitory capacity is found, in each case, in the same range of isoelectric pH.

Refocusing of Proteins in the Major Peaks. Since the anti-Ar antibodies with pI 6.7 and 6.9 (± 0.05) had similar contents of CRI per unit weight, the question arose as to whether the appearance of two peaks in each preparation might be an artifact of the IEF procedure. The proteins in each peak were therefore isolated and refocused. Specifically purified anti-Ar antibody of mouse 80 was first divided into two portions; one was labeled with ^{131}I and the other with ^{125}I. The two preparations were then separately subjected to IEF. The ^{125}I-labeled protein in the peak with pI 6.64 (Fig. 5) was mixed with ^{131}I-labeled protein of pI 6.85 and the mixture was again subjected to IEF. The results are indicated by filled circles in Fig. 5. It is apparent that each of the two proteins refocused at a pI value nearly identical to that obtained in the first run. The double peak must therefore be related to microheterogeneity of the protein and not to the IEF procedure. This experiment also indicates that the protein in each peak is homogeneous with respect to pI.

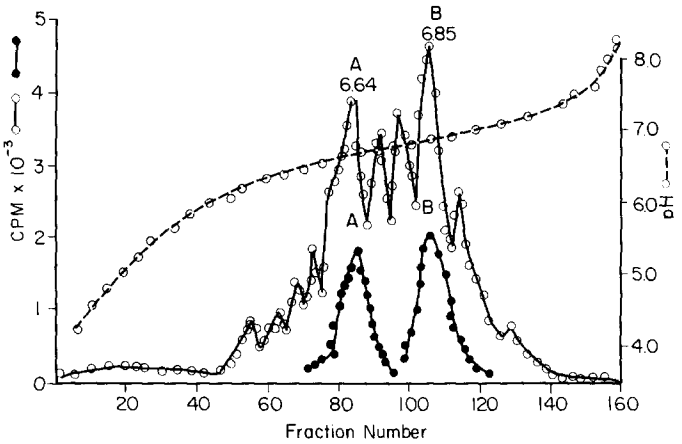

FIG. 5. Results obtained after refocusing proteins from the two major peaks on polyacrylamide gels (pH 5–8); the specifically purified anti-Ar antibodies of mouse 80 were used. A total of three runs was carried out. The first utilized ^{125}I-labeled antibody, the second, ^{131}I-labeled antibody. The results of these two runs were virtually identical after correction for the difference in cpm per unit weight of protein. Data obtained with the ^{125}I-labeled protein are shown as the upper curve (open circles) (The second run is not shown). Next, 23,000 cpm of ^{125}I-labeled peak A (first run), was mixed with 26,000 cpm of ^{131}I-labeled peak B (second run) and the mixture was refocused. The results are indicated by the filled circles. In peak A, the cpm are those of ^{125}I; in peak B, ^{131}I; the pI values of the two refocused peaks are 6.66 and 6.84, respectively.

Possible Presence of Residual Hapten in the Specifically Purified Anti-Ar Antibody. Since haptens (*p*-arsanilate and phenylarsonate) are used for the specific purification of anti-Ar antibody, the possibility was considered that, despite exhaustive dialysis, one or two molecules of hapten might be associated with some of the purified antibodies. Since the hapten carries a negative charge this could introduce heterogeneity with respect to pI. For this reason, an alternative procedure, involving specific adsorption and elution of antibody at low pH without hapten, was employed for the purification (see Methods). The isoelectric spectra of anti-Ar antibodies purified by the two procedures from the same pool of ascites fluid (from mouse 238) were compared. The results, shown in Fig. 6, indicate that the two preparations are identical with respect to their

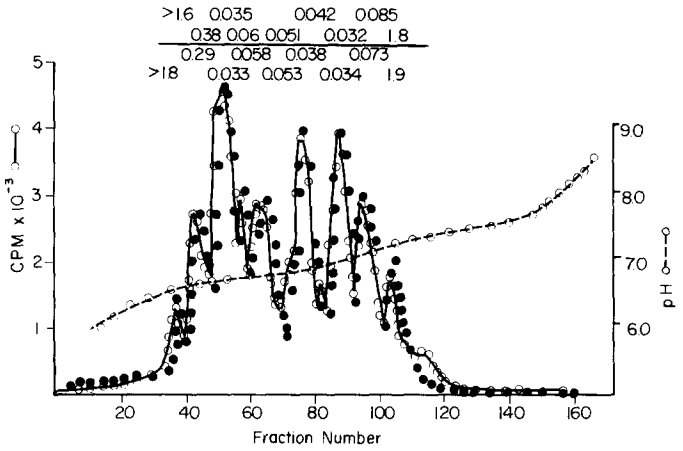

FIG. 6. Comparison of IEF patterns (polyacrylamide gel, pH 6.3–7.4) of ^{125}I-labeled anti-Ar antibodies of mouse 238, specifically purified by the precipitation method, which utilizes hapten (open circles) or by adsorption followed by elution at pH 2.3 without hapten (filled circles). (See Materials and Methods for purification procedures.) The numbers above the graph give the number of μg of protein, in the peak below the number, required to cause 50% inhibition of binding in the standard assay system described in the legend of Fig. 1. The upper two rows of numbers correspond to the peaks in the curve with filled circles; the lower two rows to the open circles.

patterns of isoelectric pH. Further support for this conclusion was obtained by determining the inhibitory capacities, in the standard assay system for idiotype, of material in several corresponding peaks. No significant differences were detected (Fig. 6).

Equivalence of Idiotype in Proteins with Isoelectric pH 6.7 or 6.9. The regular occurrence of two peaks, with approximate pI values of 6.7 and 6.9, led us to investigate the question whether the antibodies focusing in each peak are idiotypically the same. This was approached by subjecting anti-Ar antibodies of mouse 79, labeled with ^{131}I or with ^{125}I, to IEF on polyacrylamide gel. The ^{131}I-labeled proteins isolated after IEF focusing were used as ligands and the ^{125}I-labeled proteins as inhibitors in the standard assay for idiotype. The results are shown in Table I, where the data are expressed in terms of the weight of

TABLE I

*Equivalence of Idiotype in Proteins with pI 6.67 and 6.87**

^{131}I-labeled ligand	pI of inhibitor	μg required for 50% inhibition
Peak with pI 6.67 (10 ng)	Unfractionated	0.056
	6.37	>0.3
	6.45	>0.3
	6.51	>0.2
	6.62	0.032
	6.67	0.030
	6.72	0.034
	6.79	0.053
	6.82	0.051
	6.87	0.030
	6.92	0.038
	6.99	>0.1
	7.08	>0.2
	7.2	>0.2
Peak with pI 6.87 (10 ng)	Unfractionated	0.055
	6.37	>0.3
	6.45	>0.2
	6.51	>0.2
	6.62	0.035
	6.67	0.031
	6.72	0.035
	6.79	0.051
	6.82	0.050
	6.87	0.029
	6.92	0.037
	6.99	>0.1
	7.08	>0.1
	7.2	>0.2

* The proteins subjected to IEF were ^{125}I- and ^{131}I-labeled specifically purified anti-Ar antibodies from mouse 79; the latter were used as ligands in the inhibition assays. In the absence of inhibitor 52% (pI 6.67) and 54% (pI 6.87) of the ^{131}I-labeled ligand was bound by anti-idiotypic antibody. (A less than optimal amount of anti-idiotypic antibody was used in the tests). The radioimmunoassay for inhibition of binding is described under Materials and Methods. The anti-idiotypic antibody was prepared against the anti-Ar antibodies of mouse 79.

^{125}I-labeled protein required to cause 50% displacement of the ligand. It is apparent from the quantitative cross-inhibition data that the proteins from these peaks are identical with respect to their content of CRI.

Furthermore, it was found that an excess of protein from either peak was capable of completely displacing the ^{131}I-labeled protein from the other peak, and no significant differences were noted in the amounts of protein required for complete inhibition (data not shown).

Assay for CRI by Direct Binding. The quantitative data on CRI presented so

far were obtained by measurements of inhibitory capacity in the standard assay system. Table II presents a composite of results obtained by labeling various anti-Ar antibody preparations with ^{125}I and measuring the percentage of labeled protein bound by rabbit anti-idiotypic antibody directed against the anti-Ar antibody of mouse 79. The indirect precipitation method was employed. The values shown are those obtained with 10 ng of labeled ligand and an excess of anti-idiotypic antibody (0.5–2 μl). It is evident, first, that the highest concentration of idiotype is present in the same pI range (6.7 to 6.9 [± 0.05]) as that identified by the inhibition assay. Second, as much as 94% of the protein in those peaks rich in CRI was bound by the anti-idiotypic antibody. Some uncertainty is introduced by the control values (up to 6%) obtained by using ^{125}I-labeled

TABLE II

Binding by Anti-Idiotypic Antibody of Fractions of ^{125}I-Labeled Specifically Purified Anti-Ar Antibodies Subjected to IEF

Anti-Ar antibody from A/J mouse no.‡	% ^{125}I-labeled antibody bound by anti-idiotypic antibody* Isoelectric pH (±0.02):										
	6.40	6.51	6.55	6.67	6.72	6.77	6.81	6.87	6.95	7.1	7.2
79	22	34	48	91	90	84	82	91	89	37	28
206	8	11	28	58	61	54	54	58	65	12	7
214	9	ND	29	67	69	61	52	63	72	28	14
227	13	ND	27	89	87	80	63	90	92	38	19
234	16	34	51	89	93	81	83	91	93	39	21
235	18	28	42	94	92	88	84	95	92	34	17
240	11	ND	21	56	59	41	38	55	46	16	9
285	9	12	33	69	67	54	57	71	68	20	13
16	10	14	ND	77	79	64	62	76	72	28	17

* The anti-idiotypic antibody was prepared against the anti-Ar antibody of mouse 79. The data indicate the plateau values obtained using excess anti-idiotypic antibody and 10 ng of ^{125}I-labeled ligand.

‡ 50 μg of each ^{125}I-labeled antibody preparation was subjected to IEF on polyacrylamide gel. The spec act was between 2×10^6 and 4×10^5 cpm/μg.

nonspecific mouse IgG as ligand and an excess of anti-idiotypic antibody. While this number should probably be subtracted from the lower values in the table, it is uncertain whether to subtract it from the higher values. (If 100% of the protein carried the idiotype, the observed value would be 100%, not 106%).

IEF on a Preparative Scale, using Sephadex Gel. The results of IEF on a preparative scale, using Sephadex G-75 Superfine, as the supporting medium are shown in Fig. 7, together with data obtained by focusing the same protein on polyacrylamide gel. The weights of protein introduced into the Sephadex or polyacrylamide gel were 50 mg and 250 μg, respectively. The protein subjected to IEF on polyacrylamide gel was first labeled with ^{125}I to permit quantitation. Concentrations of the protein fractionated on Sephadex were estimated by measurements of absorbancy at 280 nm after elution from the gel. The weights of

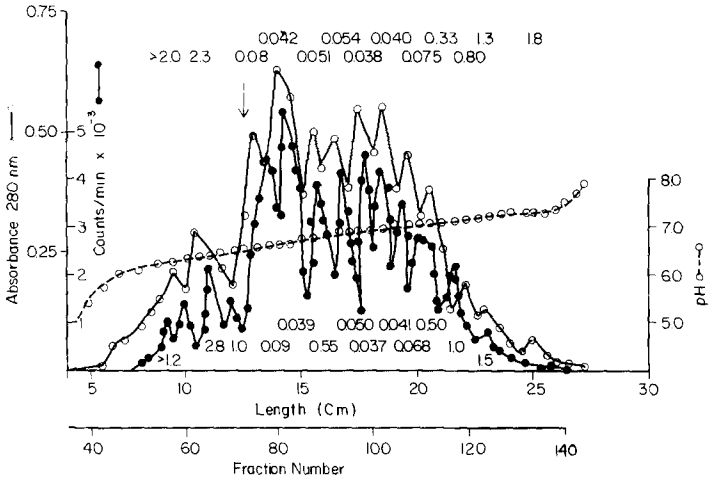

FIG. 7. Comparison of IEF profiles obtained with polyacrylamide gel (250 μg of protein, open circles) or with Sephadex G-75 Superfine (55 mg of protein, filled circles) as supporting medium. The protein is specifically purified anti-Ar antibody from mouse 23. The numbers shown in the graph indicate the weight of protein in the corresponding peak required to cause 50% inhibition of binding in the standard assay system (same system as in Fig. 1). The upper set of numbers refers to the upper curve (open circles); the lower set to the lower curve (filled circles). The length plotted on the abscissa refers to the distance measured along the length of the Sephadex slab; the fraction numbers refer to the polyacrylamide gel. The pH range in each case was 5–8. The arrow indicates the position of application of the sample in the Sephadex gel.

protein, in μg, required to cause 50% inhibition of binding in the standard assay system for idiotype, with Sephadex or polyacrylamide gel as supporting medium, are indicated by numbers at the top and bottom, respectively, of the graph. It is evident that the two methods give very similar results although the resolution on polyacrylamide gel appears somewhat greater.

Effect of Trace-Labeling with Iodine on pI. This question was explored by labeling specifically purified anti-Ar antibodies of mice 79 and 235 with ^{125}I, introducing approximately 0.1 atom of I per molecule of protein. Each labeled antibody preparation was then mixed with a large excess (400–500 μg) of the corresponding unlabeled antibody. After IEF of each mixture in polyacrylamide gel the protein was fixed, then stained with Coomassie brilliant blue dye. Thin slices were removed from the gel and their radioactivity determined. Peaks of radioactivity corresponded precisely with maximum intensities of the dye, indicating that shifts in pI due to trace-labeling with I were not significant. (A radioactive disintegration necessarily indicates the presence of an atom of I on the molecule).

Class of Immunoglobulin-Carrying CRI. Ouchterlony tests with monospecific rabbit antisera to mouse IgG1, IgG2a, IgG2b, IgA, or IgM were carried out with proteins with pI 6.7–6.9, isolated from specifically purified anti-Ar antibody preparations that were rich in CRI. Antibodies of three mice were tested. A precipitin band was obtained in each case only with anti-IgG1.

Discussion

The experiments reported here represent initial efforts to isolate and characterize those anti-Ar antibodies which carry the CRI specificity present in all immunized A/J mice. The technique used for isolation is IEF of specifically purified anti-Ar antibody; fractions are tested for the presence of CRI by radioimmunoassay. Separations were carried out on a semipreparative or preparative scale, using polyacrylamide or Sephadex gel, respectively, as supporting medium. The two methods gave very similar results, although the former appears to provide slightly higher resolution (Fig. 7). In the two procedures, the amounts of protein fractionated per run were of the order of 50–250 μg and 50–75 mg, respectively.

By either technique, CRI was found to be present principally on molecules with pI 6.65 to 6.95. Two peaks with pI 6.7 (±0.05) and 6.9 (±0.05) were generally observed.[3] The antibody in either peak refocuses with the same pI value (Fig. 5) but cross-inhibition studies indicated that the antibodies in the two peaks are idiotypically identical (Table I). Therefore, this is not an example of cross-idiotypic specificity (24). The basis for this microheterogeneity is not yet known. Variation in carbohydrate and/or amide content, owing to postsynthetic changes (e.g., 25), as well as amino acid composition may be contributing factors. The microheterogeneity was shown not to be attributable to the presence of hapten in some antibody-combining sites (Fig. 6) nor to the presence of the iodine label. All or nearly all of the antibody-bearing CRI is IgG1.

The fact that protein with pI 6.7 or 6.9 refocuses with the same pI value indicates that this observed range of pI is not an artifact of IEF; second, it shows that the protein in each major peak is homogeneous with respect to pI (Fig. 5). Further, and more conclusive evidence for restricted heterogeneity of anti-Ar antibody from the major peaks has come from N-terminal amino acid sequence analyses of H chains from antibody of an individual mouse or from a pool (26). The sequences include the first hypervariable region.

Although considerable variation is seen among individual A/J mice with respect to content of CRI per unit weight of antibody, the antibody-containing CRI is always localized at its highest concentration in the same range of pI (Figs. 1–4). A restriction of CRI to pI 6.65–6.95 was confirmed by measurements of direct binding of anti-Ar antibodies to anti-idiotypic antibodies after focusing (Table II). Small amounts of CRI are present in antibodies with pI values below and above the 6.65–6.95 range. This suggests considerable heterogeneity of charge among molecules bearing CRI. The possibility has not been ruled out that the idiotype in such molecules is cross-reactive but not identical to that in the major peaks. Antibody populations with relatively low concentrations of CRI generally have smaller peaks of protein with pI 6.7–6.9, and their IEF patterns appear more heterogeneous.

Two factors are relevant to the isolation of substantial amounts of anti-Ar antibodies from individual A/J mice. One is the production of an ascites fluid (2–25 ml per mouse, with an average of about 7 ml), which contains a high

[3] The overall range of pI values of A/J anti-Ar antibodies is about 6.3–7.2.

concentration of anti-Ar antibody (frequently 8–10 mg/ml), after four or five intraperitoneal injections of KLH-Ar in FCA. (There is considerable literature on the production of ascites fluids in mice by a variety of techniques.) Not all strains respond in this way; BALB/c, DBA, C57BR, and C57BL mice produce large amounts of ascites fluid which, however, contains very little antibody. RF and SWR mice form ascites fluid containing substantial amounts of anti-Ar antibodies, but these do not exhibit strong intrastrain idiotypic cross-reactions.[4] Among strain A mice, (A/J, A/WySn A·Sw/Sn,[5] A·By/Sn[6]) all produce anti-Ar antibodies with CRI but the highest concentrations of anti-Ar antibody are found in the sera or ascites fluids of the A/J strain. For use in sequence studies, antibodies can be obtained from those A/J mice which produce large amounts of antibody, and whose antibodies possess a high proportion of CRI; about one mouse out of 10 fulfills both criteria.

Our principal aim is to make structural comparisons among antibodies of restricted heterogeneity, and with shared idiotype, from individual inbred mice; such studies have not heretofore been feasible. Data presented elsewhere indicate that the H chains are sufficiently homogeneous to permit N-terminal amino acid sequence analysis through the first hypervariable region (26, footnote 7).

Summary

Immunization of A/J mice with a KLH-*p*-azophenylarsonate conjugate induces the formation of antihapten antibodies, some of which share idiotypic specificity common to all recipients. The subpopulation carrying the idiotype generally comprises 20–70% of the total antibody content. Large quantities of antihapten antibody (occasionally over 100 mg) were obtained from individual mice through the induction of an ascites fluid. This facilitated isolation of antibodies with the cross-reactive idiotype by isoelectric focusing. Most of this subpopulation has pI values between 6.65 and 6.95 and essentially all is of the IgG1 subclass. Two peaks, near pI 6.7 and 6.9, were frequently observed. Upon refocusing, the protein in each peak reappeared with the same pI value; thus, the range of pI is not an artifact of the procedure, but indicates microheterogeneity. The antibodies in the two peaks were found to be idiotypically identical by measurements of cross-inhibition. Preliminary studies have indicated that it is feasible to initiate investigations of primary structure with antibodies from individual inbred mice.

The authors are grateful to Miss Cynthia Subach, Miss Georgia Andrews, and Mr. Ralph Cress for excellent technical assistance.

Received for publication 9 September 1974.

References

1. Kunkel, H. G., M. Mannik, and R. C. Williams. 1963. Individual antigenic specificities of isolated antibodies. *Science* (*Wash. D.C.*). **140**:1218.

[4] Pawlak, L. L., and A. Nisonoff. Unpublished observations.

[5] Congenic mice with the *H-2* type of the Sw strain on a strain A/WySn background.

[6] Congenic mice with the *H-2* type of the By strain on a strain A background.

[7] Capra, J. D., A. S. Tung, and A. Nisonoff. Manuscript submitted for publication.

2. Oudin, J., and M. Michel. 1963. Une nouvelle forme d'allotypie des globulines y du sérum de lapin apparemment liée à la jonction et à la specificité anticorps. *C. R. Seances Acad. Agric. Fr.* **257**:805.
3. Gell, P. G. H., and A. S. Kelus. 1964. Anti-antibody or clone-product. *Nature (Lond.).* **201**:687.
4. Hopper, J. E., and A. Nisonoff. 1971. Individual antigenic specificity of immunoglobulins. *Adv. Immunol.* **13**:57.
5. Eichmann, K., and T. J. Kindt. 1971. The inheritance of individual antigenic specificities of rabbit antibodies to streptococcal carbohydrates. *J. Exp. Med.* **134**: 532.
6. Cohn, M., G. Notani, and S. Rice. 1969. Characterization of the antibody to the C-carbohydrate produced by a transplantable mouse plasmacytoma. *Immunochemistry.* **6**:111.
7. Potter, M., and R. Lieberman. 1970. Common individual antigenic determinants in five of eight BALB/c IgA myeloma proteins that bind phosphorylcholine. *J. Exp. Med.* **132**:737.
8. Kuettner, M. G., A. L. Wang, and A. Nisonoff. 1972. Quantitative investigations of idiotypic antibodies. VI. Idiotypic specificity as a potential genetic marker for the variable regions of mouse immunoglobulin polypeptide chains. *J. Exp. Med.* **135**:579.
9. Eichmann, K. 1972. Idiotypic identity of antibodies to streptococcal carbohydrate in inbred mice. *Eur. J. Immunol.* **2**:301.
10. Sher, A., and M. Cohn. 1972. Inheritance of an idiotype associated with the immune response of inbred mice to phosphorylcholine. *Eur. J. Immunol.* **2**:319.
11. Carson, D., and M. Weigert. 1973. Immunochemical analysis of the cross-reacting idiotypes of mouse myleoma proteins with anti-dextran activity and normal anti-dextran antibody. *Proc. Natl. Acad. Sci. U.S.A.* **70**:235.
12. Lieberman, R., M. Potter, E. B. Mushinski, W. Humphrey, Jr., and S. Rudikoff. 1974. Genetics of a new IgV_H (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *J. Exp. Med.* **139**:983.
13. Nisonoff, A. 1972. Genetic Control of Immune Responsiveness. Proceedings of Brook Lodge Symposium, May 1972. M. Landy and H. O. McDevitt, editors. Academic Press, Inc., New York.
14. Pawlak, L. L., and A. Nisonoff. 1973. Distribution of a cross-reactive idiotypic specificity in inbred strains of mice. *J. Exp. Med.* **137**:855.
15. Pawlak, L. L., E. B. Mushinski, A. Nisonoff, and M. Potter. 1973. Evidence for the linkage of the IgC_H locus to a gene controlling the idiotypic specificity of anti-*p*-azophenylarsonate antibodies in strain A mice. *J. Exp. Med.* **137**:22.
16. Hart, D. A., A. L. Wang, L. L. Pawlak, and A. Nisonoff. 1972. Suppression of idiotypic specificities in adult mice by administration of antiidiotypic antibody. *J. Exp. Med.* **135**:1293.
17. Hart, D. A., L. L. Pawlak, and A. Nisonoff. 1973. Nature of antihapten antibodies arising after immune suppression of a set of cross-reactive idiotypic specificities. *Eur. J. Immunol.* **3**:44.
18. Pawlak, L. L., D. A. Hart, and A. Nisonoff. 1974. Suppression of immunological memory for a cross-reactive idiotype in adult mice. *Eur. J. Immunol.* **4**:10.
19. Axen, R., J. Porath, and S. Ernback. 1969. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature (Lond.).* **214**:1302.
20. Wrigley, C. W. 1968. Analytical fractionation of plant and animal proteins by gel electrofocusing. *J. Chromatog.* **36**:362.
21. Radola, B. J. 1973. Isoelectric focusing in layers of granulated gels. I. Thin-layer isoelectric focusing of proteins. *Biochim Biophys. Acta.* **295**:412.
22. Freedman, M. H. 1972. The use of preparative isoelectric focusing for the further

purification of rabbit antihapten antibodies. *J. Immunol. Methods.* **1**:177.
23. Hunter, R. 1970. Standardization of the chloramine-T method of protein iodination. *Proc. Soc. Exp. Biol. Med.* **133**:989.
24. Kunkel, H. G. 1970. Individual antigenic specificity, cross specificity, and diversity of human antibodies. *Fed. Proc.* **29**:55.
25. Awdeh, Z. L., A. R. Williamson, and B. A. Askonas. 1970. One cell-one immunoglobulin. Origin of limited heterogeneity of myeloma proteins. *Biochem. J.* **116**:241.
26. Nisonoff, A., A. S. Tung, and J. D. Capra. 1974. Factors determining Immune Responsiveness at the level of B-cells. *Prog. Immunol.* **2**:in press.