MONOCLONAL ANTI-IDIOTYPIC ANTIBODIES REACTIVE WITH A HIGHLY CONSERVED DETERMINANT ON A/J SERUM ANTI-p-AZOPHENYLARSONATE ANTIBODIES

By MITCHELL J. NELLES, LORRAINE A. GILL-PAZARIS,§ AND ALFRED NISONOFF

From The Rosenstiel Basic Medical Sciences Research Center, Department of Biology, Brandeis University, Waltham, Massachusetts 02254

The humoral response to the azobenzenearsonate (Ar) hapten in the A/J strain includes at least three definable populations of antibodies. One population comprises the major cross-reactive idiotype (CRI) found in all immunized mice; in individual mice the CRI generally constitutes 20–70% of the anti-Ar population (1). A second population, which has been designated the minor CRI, ordinarily constitutes 5–10% of the anti-Ar antibodies (2–4). These molecules are clearly distinguishable from the major idiotype and appear to be quite heterogeneous. Conventional anti-idiotypic antiserum prepared in a rabbit includes molecules reactive with both the major and minor CRI populations (3, 4). The minor CRI in the A/J strain corresponds to a major CRI in BALB/c mice (5, 6); thus, anti-idiotype directed to the minor CRI of A/J anti-Ar antibodies reacts with 5–10% of an A/J anti-Ar population, but with ~40% of BALB/c anti-Ar antibodies. Approximately the same population of BALB/c antibodies is recognized by anti-CRI prepared against BALB/c anti-Ar. Individual A/J mice also produce anti-Ar antibodies with idiotypes that are unique to individual mice and may be present in undetectable concentrations in other immunized mice of the same strain (7).

Serological and amino acid sequence analyses have been carried out on hybridoma products expressing the major CRI (8–13). These have shown that the major CRI constitutes a large family of related but nonidentical molecules. Variations in amino acid sequence within the family occur both in framework and hypervariable regions. However, the degree of homology, when sequences of heavy or light chains from two members of the family are compared, exceed 90% (8–11) (J. D. Capra; M. Margolies, personal communications).

The presence of a conserved idiotypic determinant in molecules constituting the...
major CRI was indicated by serological studies in which heterologous anti-CRI prepared against one CRI+ hybridoma product (HP) was allowed to interact with a different, labeled HP that also carried the CRI (13). When various unlabeled CRI+ HP were tested as inhibitors of binding, it was found that 12 of 14 HP were quantitatively equivalent in their inhibitory capacities. This suggests the presence of at least one conserved idiotypic determinant in molecules carrying the major CRI but does not provide information concerning the nature of the conserved determinant(s).

We have now taken a direct approach to the question of the presence of conserved idiotypic determinants in A/J serum Anti-Ar antibodies and in CRI+ HP. This was done by preparing monoclonal anti-idiotypic antibodies against A/J serum anti-Ar and studying their interactions with individual CRI+ hybridoma products and with serum anti-Ar. The data indicate that a large proportion of A/J serum anti-Ar antibodies and 8 of 10 CRI+ HP share at least one idiotypic determinant definable by monoclonal anti-CRI antibodies. The shared determinant is associated with the hapten-binding site of the CRI+ HP. In addition, functional studies were carried out to ascertain whether monoclonal anti-idiotypic antibodies are capable of suppressing CRI formation when inoculated into adult A/J mice.

**Materials and Methods**

*Preparation of Hybridomas Secreting Anti-CRI Antibodies.* Adult BALB/c mice (The Jackson Laboratory, Bar Harbor, Maine) were immunized with specifically purified A/J anti-Ar antibodies that had been polymerized with glutaraldehyde by the method of Daugharty et al. (14). Mice were immunized a total of three times intraperitoneally with 250 μg of anti-Ar antibody emulsified in complete Freund's adjuvant. 3 d before killing, animals were immunized via both the intraperitoneal and intravenous routes, with the latter immunization given in saline. Spleen cells depleted of erythrocytes from a single mouse were used to prepare hybridomas by the method of Köhler and Milstein (15), as modified by Gefter et al. (16). The BALB/c tumor line used, Sp2/O-Ag14, was developed by Shulman et al. (17); it does not secrete heavy or light chains before or after fusion. 1 × 10⁸ splenic leukocytes and 2 × 10⁷ tumor cells were fused with 30% polyethylene glycol (PEG-1000, J. T. Baker Chemical Co., Phillipsburg, N. J.). Immediately after fusion, cells were transferred to 96-well tissue culture plates (Costar Co., Cambridge, Mass.) at a concentration of 3.3 × 10⁴ tumor cell equivalents per well, in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, and antibiotics (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.). 24 h later, hybrid cells were selected in medium that contained hypoxanthine, thymidine, and aminopterin, as described by Littlefield (18). Of the 504 wells originally plated, 103 showed macroscopic growth 2½ wk after fusion. Culture supernatants were assayed for anti-CRI antibody by solid-phase radioimmune assay (19) using polyvinyl chloride microtiter trays containing adsorbed purified A/J anti-Ar antibodies. All assays were carried out in the presence of normal A/J serum to avoid detection of antibodies with specificity for A/J allotypic determinants. Bound anti-CRI antibodies were detected by measuring the subsequent uptake of ¹²⁵I-labeled purified A/J anti-Ar antibody to the tray. Individual cell culture supernatants obtained from growth-positive wells were assayed in this manner and three were found to contain anti-CRI antibodies. Each hybrid was derived from a separate culture well, and, because cells were plated out immediately after fusion, each represents the product of an independent fusion event. The cells from anti-CRI-positive wells were transferred into larger (24-well) Costar plates and subsequently cloned in soft agarose, as described by Köhler and Milstein (15). Clones were visible in 14–21 d. Individual clones were transferred with a Pasteur pipet to microtiter plates and grown into mass culture. Large amounts of monoclonal antibody were produced in mice that had been primed with pristane (Aldrich Chemical Co., Milwaukee, Wis.) (20). A globulin fraction of each monoclonal anti-CRI was first prepared by precipitation with ammonium sulfate (40% saturation). The dissolved globulin was fractionated on DEAE-cellulose by stepwise elution with increasing concentrations of phosphate buffer, pH 8. Each of the three monoclonal
MONOCLONAL ANTI-IDIOTypIC ANTIBODIES

anti-CRI antibodies (7B7.10, 2F6.4, IF2.1) migrated as a single sharp band upon electrophoresis in agarose; the three proteins differed in electrophoretic mobility. Ouchterlony analysis showed that each protein is IgG1K.

Reagents. All of the anti-Ar hybridoma proteins used in these studies, with the exception of 123E6 and 124E1, were the generous gift of Dr. Edmundo Lamoyi, Brandeis University, and were specifically purified as previously described (13). Anti-Ar HP 123E6 and 124E1 were kindly supplied by Dr. J. D. Capra, University of Texas Health Science Center, Dallas, Texas (13). Serum designated HIS (hyperimmune, suppressed) was obtained from A/J mice that had been idiotypically suppressed by inoculation of heterologous anti-CRI serum (21) and then hyperimmunized with keyhole limpet hemocyanin-Ar (KLH-Ar). Hyperimmunized animals that are immunologically suppressed for the A/J CRI (HIS) serum contain a high concentration of anti-Ar antibodies that are not inhibitory in the standard radioimmune assay for CRI.

Proteins were conjugated to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) by using cyanogen bromide (22). Approximately 200 μg of anti-CRI antibody was conjugated per cm² of beads. Rabbit serum containing anti-CRI antibody specific for the major CRI of the Ar-binding hybridoma protein R16.7 [anti-CRI (R16.7)] was the generous gift of Dr. Thomas Kresina, Brandeis University. Sepharose columns containing bound anti-CRI antibodies were assayed for their binding capacities by determining the weight of ¹²⁵I-labeled purified A/J serum anti-Ar antibody bound by a given volume of beads at saturation. Proteins were labeled with ¹²⁵I by the chloramine-T method of Hunter (23).

The haptens, p-arsanilic acid (Eastman Kodak Co., Rochester, N. Y.), o-arsanilic acid (Aldrich Chemical Co., Milwaukee, Wis.), and p-aminobenzoic acid (Fisher Scientific Co., Fairlawn, N. J.) were recrystallized twice from hot water. The 2-ring hapten m-azobenzene-p'-arsonate-3(p-hydroxphenyl)-propionic acid (Ar-HPP) was the gift of Dr. Edmundo Lamoyi, and m-arsanilic acid was the gift of Dr. Oliver Roholt, Roswell Park Institute, Buffalo, N. Y.

Assays for Anti-Ar Antibodies and CRI. Anti-Ar antibodies in serum were quantified by the polyvinyl chloride tray method of Klinman et al. (19), using BSA-Ar-coated trays with ¹²⁵I-labeled specifically purified rabbit anti-mouse Fab as the developing reagent. The content of CRI in serum samples was determined by radioimmune assay, as previously described (1, 12). The assay utilized 10 ng of ¹²⁵I-labeled purified A/J serum anti-Ar antibody together with heterologous anti-CRI in a quantity sufficient to bind slightly less than a maximum amount of ligand. Complexes were precipitated with excess goat anti-rabbit Fc antiserum. Unlabeled sera or antibodies were tested as inhibitors of binding.

Characterization of Anti-CRI Hybridoma Proteins. Solid-phase radioimmune assays using polyvinyl chloride microtiter trays were used to study anti-CRI hybridoma proteins (anti-CRI HP) in a number of different assays. In one assay system, wells were coated with various anti-CRI HP for determination of their fine specificity. Inhibitors were added, followed by 10 ng ¹²⁵I-labeled purified serum anti-Ar antibody or ¹²⁵I-labeled CRI' HP R16.7. Data are expressed as the weight of unlabeled inhibitory protein causing a 50% reduction in binding of radiolabeled anti-Ar antibody or R16.7 to the immobilized anti-CRI HP. To determine whether anti-CRI HP interact with CRI' antibody molecules through a determinant close to the antigen-binding site, wells were coated with HP R16.7; hapten diluted in saline-borate buffer containing 2% horse serum was added, followed by ¹²⁵I-labeled anti-CRI HP. Data are expressed as the concentration of hapten required to cause a 50% reduction in the binding to R16.7 of the various radiolabeled anti-CRI HP. To compare the avidities of the various anti-CRI HP, wells were coated with purified serum anti-Ar antibody or HP R16.7 and incubated with unlabeled anti-CRI HP. 10 ng of a particular ¹²⁵I-labeled anti-CRI HP was then added. Data are expressed as the weight of unlabeled anti-CRI HP required to cause a 50% reduction in the binding of individual radiolabeled anti-CRI HP to immobilized CRI' proteins.

Results

Table I shows the maximum percentages of labeled purified A/J serum anti-Ar antibodies or of the CRI' HP R16.7 that were bound by several anti-CRI preparations, including the three anti-CRI HP. Preliminary experiments were done to ensure
TABLE I
Maximum Percentages of Labeled, Purified A/J Anti-Ar Antibody or of Labeled CRI* HP R16.7 Bound by Various Anti-CRI Preparations

| 125I-labeled protein | Adsorbent (anti-CRI conjugated to Sepharose) | Percent labeled protein bound |
|----------------------|---------------------------------------------|------------------------------|
|                      | 7B7.10*                                     | 2F6.4*                       |
| Purified anti-Ar¶    | 34                                          | 41                           |
| R16.7**              | 84                                          | 77                           |
| nMlG                 | 2                                           | 2                            |

* Anti-CRI HP.
† Mixture of equal weights of 7B7.10, 2F6.4, and 1F2.1.
§ Rabbit anti-CRI serum specific for HP R16.7 (CRI*).
¶ Rabbit anti-CRI serum specific for A/J serum anti-Ar antibody.
** R16.7 is a CRI* HP.

...that the column capacity was in large excess with respect to the weight of labeled ligand used.

There is considerable variation in the percentage of labeled purified serum anti-Ar bound by the five anti-CRI preparations. Rabbit anti-CRI specific for R16.7 bound 66% of this ligand, whereas conventional rabbit anti-CRI bound 80%. The difference is attributable to the presence of a “minor” cross-reactive idiotypic population within A/J sera (1-4). The conventional anti-CRI reacts with the minor population, whereas anti-CRI directed against R16.7, a representative of the major idiotypic population, does not. The minor idiotype is serologically distinct from the major cross-reactive idiotype. Of the three anti-CRI HP 2F6.4 bound the highest percentage (41%) of the purified A/J serum anti-Ar antibodies. The fact that anti-CRI HP bound a smaller percentage of the CRI* antibodies than did rabbit anti-CRI directed to R16.7 is consistent with microheterogeneity in the CRI* population (3, 12). All three anti-CRI HP bound labeled R16.7, which is a CRI* HP (74-84% bound); that 100% binding was not attained is probably attributable to partial denaturation of the R16.7 HP during iodination.

The most significant aspect of these results is that the monoclonal HP 2F6.4 was able to bind 41% of the purified anti-Ar serum antibody or about 60% of the antibodies that are recognized by rabbit anti-CRI directed to R16.7. This result indicates that at least 60% of the CRI* population contains a highly conserved idiotypic determinant, recognizable by a monoclonal antibody. The fact that a mixture of the three anti-CRI HP bound the same percentage of purified anti-Ar that was bound by HP 2F6.4 (Table I) indicates that the three monoclonal anti-CRI antibodies react with overlapping subpopulations of the anti-Ar antibodies.

To determine whether anti-CRI HP 2F6.4 bound a subpopulation of antibodies that were bound by rabbit anti-CRI(R16.7), labeled serum anti-Ar that remained unbound by an anti-CRI(R16.7) column was applied to a Sepharose column containing immobilized anti-CRI HP, 2F6.4. Only 1% of the label was bound by the second column, indicating that 2F6.4 binds a subfraction of the anti-Ar antibodies that are bound by anti-CRI(R16.7).

Inhibition of the Interaction of Anti-CRI HP with 125I-labeled Anti-Ar Antibody or with 125I-
labeled CRI\textsuperscript{+} HP R16.7. To study the fine specificity of the anti-CRI HP, competition experiments were carried out in which various unlabeled anti-Ar preparations were allowed to compete with labeled purified anti-Ar antibodies for binding to anti-CRI. The results are shown in Tables II and III. In Table II the labeled ligand is purified A/J serum anti-Ar antibody; in Table III the labeled ligand is the CRI\textsuperscript{+} HP, R16.7.

As controls for the experiments of Table II, normal mouse IgG (nMIgG) and serum containing anti-Ar antibodies from HIS mice were tested as inhibitors. These preparations failed to inhibit significantly the binding of labeled anti-Ar to any of the anti-CRI preparations tested. Similarly, the CRI\textsuperscript{-} anti-Ar HP R18.11, R19.9, and R21.10 were noninhibitory.

Among the CRI\textsuperscript{+} HP, considerable variation was observed in their capacity to displace labeled purified anti-Ar antibodies from rabbit anti-CRI. The weights required for 50\% inhibition of binding varied from 15 to 2,000 ng. The pattern of inhibition seen for these particular HP is very similar to that observed in previous experiments (13) in which a double antibody precipitation assay was used.

In contrast, 8 of 10 of the CRI\textsuperscript{+} HP were quite similar in their effectiveness as

### Table II

| Unlabeled inhibitor | Presence of | Anti-CRI |
|---------------------|-------------|----------|
|                     | CRI         | R7B7.10  | 2F6.4  | 1F2.1  |
| nMIgG               | -           | >5,000 (17) | >5,000 (10) | >5,000 (0) | >5,000 (6) |
| HIS serum‡          | -           | >5,000 (48) | 5,000 (50) | >5,000 (16) | >5,000 (49) |
| A/J anti-Ar serum   | +           | 23 (100) | 47 (100) | 67 (100) | 25 (100) |
| Purified serum      | +           | 28 (99) | 57 (100) | 82 (97) | 25 (100) |
| A/J anti-Ar HP      |             |          |          |          |          |
| R16.7               | +           | 15 (93) | 23 (100) | 22 (100) | 21 (100) |
| R20.4               | +           | 22 (81) | 72 (97) | 61 (96) | 75 (98) |
| R9.3                | +           | 367 (62) | 21 (97) | 25 (93) | 60 (97) |
| R10.8               | +           | 445 (34) | 39 (96) | 76 (93) | 64 (100) |
| R17.5               | +           | 500 (61) | 25 (93) | 38 (93) | 36 (98) |
| R23.2               | +           | 500 (56) | 100 (95) | 64 (95) | 100 (100) |
| R24.6               | +           | 1,000 (55) | 22 (93) | 78 (92) | 78 (98) |
| R22.4               | +           | 2,000 (96) | 100 (95) | 56 (93) | 68 (99) |
| 123E6               | +           | ND§      | >5,000 (27) | 85 (91) | >5,000 (41) |
| 124E1               | +           | ND§      | >5,000 (31) | >5,000 (7) | >5,000 (33) |
| R18.11II           | -           | >2,000 (13)** | >5,000 (34) | >5,000 (1) | >2,000 (9)** |
| R19.9II            | -           | >5,000 (26) | >5,000 (22) | >5,000 (1) | >5,000 (10) |
| 21.10II           | -           | >5,000 (21) | >5,000 (15) | >5,000 (7) | >5,000 (15) |

Wells of polyvinyl chloride microtiter trays were coated with anti-CRI HP or purified rabbit anti-CRI antibody. Various amounts of unlabeled inhibitor proteins (anti-Ar) were added, followed by 10 ng \textsuperscript{125}I-labeled, purified A/J serum anti-Ar antibodies.

* Values in parentheses show the percent inhibition by 5,000 ng or where indicated (**), by 2,000 ng unlabeled inhibitor.
‡ Hyperimmune suppressed mice were immunologically suppressed by pre-inoculation of rabbit anti-CRI serum followed by hyperimmunization with KLH-Ar. The serum contains a high titer of anti-Ar antibody but lacks the CRI.
§ Not done.
II Representative of the "minor" A/J cross-reactive idiotype (4).
TABLE III
Inhibition of Binding of Labeled, R16.7 HP(CRI+) to Immobilized Anti-CRI Preparations by Various Unlabeled Anti-Ar Preparations

| Unlabeled inhibitor | Presence of CRI | Anti-CRI | 7B7.10 | 2F6.4 | IF2.1 |
|---------------------|----------------|----------|--------|--------|--------|
|                     |                | RaCRI    |        |        |        |
| nMIgG               |                | >5,000 (29) | >5,000 (0) | >5,000 (0) | >5,000 (21) |
| HIS serum‡          |                | 2,000 (65) | >5,000 (33) | >5,000 (13) | >5,000 (27) |
| A/J anti-Ar serum   | +              | 40(100)   | 53(100) | 53(100) | 52(100) |
| Purified serum      | +              | 34(100)   | 57(100) | 52(100) | 88(100) |
| A/J Anti-Ar         |                 |          |        |        |        |
| HP R16.7            | +              | 17(100)   | 9(100) | 14(100) | 20(100) |
| HP R20.4            | +              | 27(86)    | 51(96) | 35(100) | 44(100) |
| HP R9.3             | +              | 285 (63)  | 5 (97) | 12 (99) | 24 (100) |
| HP R10.8            | +              | 500 (59)  | 8 (95) | 24 (99) | 24 (100) |
| HP R17.5            | +              | 500 (62)  | 9 (95) | 21 (98) | 21 (99) |
| HP R23.2            | +              | 500 (60)  | 22 (96) | 38 (99) | 61 (100) |
| HP R24.6            | +              | 1,000 (54) | 23 (93) | 32 (100) | 24 (99) |
| HP R22.4            | +              | 2,000 (55) | 18 (91) | 24 (99) | 66 (99) |
| HP 123E6            | +              | ND§       | >5,000 (13) | 78 (99) | >5,000 (33) |
| Hp 124E1            | +              | ND§       | >5,000 (12) | >5,000 (0) | >5,000 (42) |
| HP R18.1I‡          | -              | >2,000 (28)** | >5,000 (18) | >5,000 (8) | >5,000 (38) |
| HP R19.9‡           | -              | >5,000 (33) | >5,000 (18) | >5,000 (7) | >5,000 (47) |
| HP R21.10‡          | -              | >5,000 (20) | >5,000 (21) | >5,000 (14) | >5,000 (38) |

Wells of polyvinyl chloride microtiter trays were coated with anti-CRI HP or purified rabbit anti-CRI antibody. Various amounts of unlabeled inhibitor proteins (anti-Ar) were added, followed by 10 ng labeled R16.7 HP.

* Values in parentheses show the percent inhibition by 5,000 ng, or, where indicated (**) by 2,000 ng unlabeled inhibitor.
† As in Table II.
§ Not done.

Effect of Hapten on the Interaction between Anti-CRI HP and the CRI+ HP R16.7. Further evidence concerning the fine specificity of the monoclonal anti-CRI antibodies is presented in Table IV, which shows the effects of hapten on the binding of labeled inhibitors when any of the three anti-CRI HP was used as the immobilized preparation. Although some variation in inhibitory capacity is noted, the degree of variation is small as compared to that observed with rabbit anti-CRI as the immobilized antibody. These results support the conclusion that each of the monoclonal anti-CRI antibodies recognizes a determinant that is shared by 8 of the 10 CRI+ HP tested; the results are thus consistent with the presence of a highly conserved determinant found in these eight CRI+ HP. Serum anti-Ar antibodies were roughly equivalent in inhibitory capacity to the monoclonal CRI+ HP, indicating that the same or a very similar determinant is present on a large proportion of the serum antibodies.

The protocol for the experiments of Table III is identical to that of Table II, with the exception that the CRI+ HP R16.7 rather than serum anti-Ar was used as the labeled ligand. The results are very similar to those of Table II. Unlabeled CRI+ HP vary markedly, and, with the same order of effectiveness as in Table II, as inhibitors of binding to rabbit anti-CRI, but 8 of 10 are strong inhibitors of binding of labeled HP R16.7 to each of the three monoclonal anti-CRI antibodies.
monoclonal anti-CRI, or labeled purified rabbit anti-CRI, to immobilized CRI\(^+\) HP R16.7. Each derivative of arsanilate inhibited binding of each monoclonal anti-CRI antibody; the strongest inhibitor is the 2-ring hapten, Ar-HPP. Among the arsanilate compounds, the order of inhibitory capacity is \((p > m > 0)\). These results are consistent with relative binding affinities of haptens observed with rabbit anti-\(p\)-azobenzearsonate antibodies (24). The data indicate that each of the monoclonal anti-CRI antibodies reacts with an idiotypic determinant that is part of or very close to the hapten-binding site of the anti-Ar HP R16.7.

With rabbit anti-CRI as the labeled ligand, only Ar-HPP was an effective inhibitor of binding at the concentrations tested. The weak inhibitory capacity of the smaller haptens might be attributable either to a higher affinity for idiotype of the rabbit anti-CRI, as compared to the monoclonal anti-CRI antibodies or to greater steric interference by the haptens with the interactions of the monoclonal anti-CRI antibodies.

Inhibition of the Interaction of \(^{125}\text{I}-\text{labeled Anti-CRI HP with Anti-Ar Antibody or HP R16.7 by Unlabeled Anti-CRI HP.}\) The data in Table V provide further evidence that the three monoclonal anti-CRI antibodies combine with the same region of CRI\(^+\) serum anti-Ar antibodies or of CRI\(^+\) HP R16.7. The table shows the results of experiments in which various unlabeled anti-CRI preparations were allowed to compete with labeled purified anti-CRI antibodies for binding to immobilized serum anti-Ar antibody or HP R16.7.

Two conclusions may be drawn from the data. First, there is mutual competition between each pair of anti-CRI HP or between each HP and rabbit anti-CRI for binding either to serum anti-Ar or to HP R16.7. Second, HP 2F6.4 appears to bind with higher affinity to the idiotypic determinant on CRI\(^+\) molecules. The possibility of higher affinity is supported by the fact that 2F6.4 not only competes more effectively than the other anti-CRI HP against labeled 2F6.4 but also is more effective than unlabeled 7B7.10 or 1F2.1 in displacing labeled 7B7.10 or labeled 1F2.1, respectively. Similarly, the rabbit anti-CRI might bind with a higher affinity than 7B7.10 or 1F2.1, as shown by its superior capacity to displace 2F6.4 from binding to serum anti-Ar or to HP R16.7.

| Hapten                | Labeled anti-CRI tested |
|-----------------------|-------------------------|
|                       | 7B7.10 | 2F6.4 | 1F2.1 | RaCRI |
| \(p\)-Arsanilate      | 0.12   | 2.1   | 0.13  | >25 (31%) |
| \(m\)-Arsanilate      | 0.16   | 3.5   | 0.48  | >25 (19%) |
| \(\epsilon\)-Arsanilate| 2.4   | >20   | 2.9   | >25 (0%) |
| Ar-HPP                | 0.0006 | 0.004 | 0.0006 | 0.6 |
| \(p\)-Aminobenzoate   | 12     | >20   | 10    | >25 (0%) |

Wells of polyvinyl chloride microtiter trays were coated with the CRI\(^+\) HP R16.7 at a concentration of 29 \(\mu\)g/ml, then incubated for 3 h at room temperature with various concentrations of hapten inhibitors before the addition of 10 ng \(^{125}\text{I}-\text{labeled anti-CRI HP or}^{125}\text{I}-\text{labeled specifically purified rabbit anti-CRI (RaCRI).}\)

* Values in parentheses show percent inhibition at 25 mM hapten concentration.

\(m\)azobenzene-\(p\)-arsonate-(\(p\)-hydroxyphenyl)-propionic acid.
TABLE V
Competition of Various Anti-CRI Preparations for Binding to CRI* Anti-Ar Antibody or to a CRI* HP

| Experiment | Coating of wells | 125I-labeled anti-CRI | Unlabeled inhibitor (anti-CRI or nM1gG) | ng inhibitor required for 50% inhibition§ |
|------------|------------------|-----------------------|---------------------------------------|----------------------------------------|
| 1          | Purified A/J      | 7B7.10                | 2F6.4*                                | 1F2.1*                                 |
|            | Anti-Ar antibody  | 2F6.4                 | 1,300 (69)                            | 15 (100)                               |
|            |                  | 1F2.1                 | 18 (99)                               | 3 (94)                                 |
|            | RaCRI, nM1gG      |                       | 19 (100)                              | 18 (99)                                |
|            |                  |                       | 25 (100)                              | 10 (98)                                |
|            |                  |                       | >5,000 (14)                           | >5,000 (0)                             |
| 2          | Anti-Ar HP, R16.7 | 7B7.10                | 2F6.4                                 | 1F2.1                                 |
|            |                  | 2F6.4                 | 500 (70)                              | 27 (99)                                |
|            |                  | 1F2.1                 | 9 (99)                                | 11 (93)                                |
|            |                  |                       | 18 (99)                               | 65 (99)                                |
|            |                  |                       | 10 (98)                               | 40 (96)                                |
|            |                  |                       | >5,000 (0)                            | >5,000 (0)                             |

Wells of polyvinyl chloride microtiter trays were coated with purified A/J anti-Ar antibody (6 μg/ml) (experiment 1) or with the CRI* HP, R16.7 (10 μg/ml) (experiment 2). Various concentrations of unlabeled inhibitor proteins were added followed by 10 ng labeled anti-CRI HP.

* Anti-CRI HP.
‡ RaCRI, purified rabbit anti-CRI antibody.
§ Values in parentheses indicate percent inhibition with 5,000 ng inhibitor.

TABLE VI
Suppression of the Major Cross-Reactive Idiotype in Adult A/J Mice by RaCRI or by Anti-Idiotypic HP

| Number of mice | Anti-CRI | Dose | Anti-Ar titer mean (range) | Anti-Ar antibody required for 50% inhibition* |
|----------------|----------|------|----------------------------|-----------------------------------------------|
| 5              | None     | —    | 0.53(0.44-1.8)             | 13, 98, 22, 24, 54                           |
| 4              | RaCRI    | 100 μg IBC‡ | 1.3(0.9-1.5)            | 1,300, 1,200, 80, 700                        |
| 4              | 7B7.10   | 300 μg | 2.8(2.0-4.4)              | 1,000, 170, 300, 1,300                       |
| 4              | 100 μg   | 2.4(1.0-3.1) | 160, 260, 130, 230     |                                               |
| 5              | 10 μg    | 2.2(0.7-3.4) | >2,000, 1,800, >4,000 |                                               |
| 5              | 1 μg     | 3.3(1.0-5.2) | 840, 780, 490, >4,000 |                                               |
| 4              | 2F6.4    | 300 μg | 1.9(0.9-2.9)              | 3,200, 4,000, 3,000, 2,100                    |
| 4              | 100 μg   | 1.7(0.9-4.5) | 4,000, 2,000, 3,900 |                                               |
| 6              | 10 μg    | 4.0(2.2-7.9) | 4,000, 150, 400, 58, 42, 75 |                                               |
| 6              | 1 μg     | 3.0(0.18-8.9) | 180, >4,000, 70, 600 |                                               |
| 3              | 1F2.1    | 100 μg | 1.5(1.0-2.2)              | 400, 100, 100                                |
| 5              | 10 μg    | 2.6(1.0-3.9) | 63, 29, 31, 23, 28     |                                               |
| 6              | 1 μg     | 3.7(1.9-4.6) | 89, 23, 92, 76, 39, 500 |                                               |

Adult A/J were inoculated with various anti-CRI preparations i.p. on days −3 and 0, immunized on days 14 and 28 with 125 μg KLH-Ar emulsified in complete Freund’s adjuvant, and bled on day 40.
* Each test utilized 10 ng labeled A/J anti-Ar antibody and slightly less than an equivalent amount of rabbit anti-CRI. Immune complexes were precipitated with goat anti-rabbit Fc.
‡ IBC, idiotype-binding capacity as determined by radioimmune assay. The values for the anti-CRI HP are weights of protein injected.

Suppression of CRI Formation by Monoclonal Anti-CRI. The results in Table VI show that two of the three anti-CRI HP, 2F6.4 and 7B7.10, are capable of suppressing CRI formation when inoculated into adult mice (8-14 wk of age) before immunization with KLH-Ar. (The protocol is given in a footnote to Table VI). Suppression of idiotype was observed in a significant percentage of the mice when as little as 1 μg of anti-CRI HP was inoculated; smaller doses were not tested. There was no reduction
MONOClonAL ANTI-IDiotYPIC ANTiBodies

of total anti-Ar titers associated with the inoculation of the anti-CRI antibodies. The reason for the ineffectiveness of HP 1F2.1 in suppressing the CRI is not known.

Discussion

The present experiments further define a family of molecules that constitute the major cross-reactive idioype associated with A/J anti-Ar antibodies. Serological and amino acid sequence analysis have shown (8–13) that molecules expressing the idioype exhibit microheterogeneity. Amino acid sequence variations occur both in framework and hypervariable regions; however, there appears to be at least 90% homology of sequence between the H or L chains of any pair of CRI* HP studied so far. At the serological level, microheterogeneity is shown by the variation in inhibitory capacities of CRI* HP in the radioimmune assay for CRI and by the presence of "private" idioype determinants on individual CRI* HP that are not detectable on other CRI* HP (12, 25). (Such private determinants are, however, generally detectable at low levels in pooled A/J anti-Ar serum).

The present data demonstrate that 8 of 10 CRI* HP tested express a common idioype determinant reactive with each of three monoclonal anti-idioype antibodies; these CRI* HP vary greatly in their quantitative inhibitory capacity in the radioimmune assay for CRI when heterologous anti-CRI antibodies are used (12). The presence of a common or shared idioype determinant was shown in the present study by inhibition tests in which the anti-Ar HP were allowed to compete with labeled purified A/J anti-Ar antibodies or with a labeled CRI* HP for binding to monoclonal anti-CRI. 8 of 10 of the anti-Ar HP proved to be strong inhibitors of binding, and the quantitative variation (weight required for 50% inhibition) was relatively small. When high concentrations of anti-Ar HP were used, the displacement of the labeled serum antibody or CRI* HP was virtually complete. The fact that monoclonal anti-Ar HP were about equivalent to unlabeled serum anti-Ar in inhibitory capacity is particularly informative because it suggests conservation of the determinant in the bulk of the serum idioype population and supports the conclusion that the anti-Ar HP are representative of serum antibodies. It should be noted that the three monoclonal anti-CRI antibodies were derived from different clones. Evidence for this was the variation in electrophoretic mobility despite the fact that all three were IgG. Also, one of the monoclonal anti-CRI antibodies has a distinctly higher affinity for CRI than the other two.

There appears to be some microheterogeneity, even with respect to the relatively conserved determinant. One of ten CRI* HP was a poor inhibitor of binding of each of the three monoclonal HP and another inhibited poorly in two of the three systems. Evidence for microheterogeneity in the serum antibody population was the fact that the monoclonal anti-CRI antibodies bound a smaller proportion of the labeled serum anti-Ar than was bound by rabbit anti-CRI directed against a CRI* HP. The most effective monoclonal anti-CRI, 2F6.4, bound 60% of the CRI* population defined by the rabbit antiseraum. It was shown that the anti-Ar bound by the monoclonal anti-CRI is a subpopulation of those molecules that are bound by the rabbit anti-CRI; immobilized rabbit anti-CRI removed all of the labeled anti-Ar reactive with 2F6.4.

The idioype reactive with each of the three monoclonal anti-CRI antibodies appears to involve the combining region of anti-Ar antibody. Derivatives of arsanilate inhibited the interaction of each anti-CRI HP with the CRI* HP, R16.7, and the
order of inhibitory capacities for different haptens corresponded with the expected affinities for anti-Ar antibody. Thus, the para-amino derivative was a more effective inhibitor than the ortho or meta-amino derivative of benzene arsonate, and a compound comprising two rings linked by an azo group (as in the immunogen) was much more effective than the single-ring molecules.

Further, although less definitive, evidence that the three monoclonal anti-CRI antibodies combined with the same region of A/J anti-Ar antibody was derived from competition experiments, which showed that the three monoclonal anti-CRI antibodies compete for the same antigenic region of A/J serum anti-Ar or of a CRI+ HP. One of the anti-CRI HP as well as rabbit anti-CRI combined with the idiotypic region with considerably greater affinity than the other two anti-CRI HP.

Two of the three anti-CRI HP described in this report are able to prevent the production of CRI+ anti-Ar antibody in A/J mice when administered before antigen; no marked effect on the total synthesis of anti-Ar antibody was noted. The inability of one of the anti-CRI HP to suppress formation of CRI+ molecules is not understood. It does not seem to be related to antibody class, as all three anti-CRI HP are IgG1K. Other factors involved might be affinity, dose of anti-CRI, time of administration, etc. Nevertheless, because the anti-CRI HP are of murine origin, their ability to suppress the production of the CRI is consistent with a regulatory role for spontaneously formed anti-CRI antibodies (26–30). In addition, recent studies (31) have indicated that an anti-CRI HP can substitute for antigen priming in vivo in the induction of delayed-type hypersensitivity or cell-mediated lympholysis against the Ar-hapten (M. I. Greene, personal communication). Thus, an anti-CRI HP can modulate responses at the T cell level.

The large degree of suppression of CRI caused by administration of the anti-CRI HP is also of interest. The fact that the CRI was reduced to almost undetectable levels in some suppressed mice is consistent with the presence of a shared idiotype within the CRI+ family of molecules.

Summary

Previous reports have shown that A/J anti-p-azophenyl arsonate (anti-Ar) antibodies that share a major cross-reactive idiotype (CRI) comprise a family of closely related but nonidentical molecules. Serological studies with CRI+ monoclonal anti-Ar antibodies have suggested the presence of a conserved idiotypic determinant within the family. The present study utilized monoclonal anti-idiotypic antibodies to define further the nature of the conserved idiotypic determinant. It was found that 8 of 10 CRI+ monoclonal antibodies possess an idiotypic determinant reactive with each of three monoclonal anti-idiotypic antibodies. In addition, ~60% of CRI+ serum anti-Ar antibodies reacted with one of the monoclonal anti-idiotypic preparations. The monoclonal anti-idiotypic antibodies react with an idiotope in the region of the hapten-binding site, as indicated by the ability of free haptens to inhibit idiotype-anti-idiotype interactions. Finally, two of three monoclonal anti-idiotypic antibodies suppressed the subsequent production of CRI+ serum anti-Ar antibodies when administered before antigen, without significantly affecting the total anti-Ar response.

Received for publication 7 July 1981.
References

1. 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.

2. Gill-Pazaris, L. A., A. R. Brown, and A. Nisonoff. 1979. The nature of idiotypes associated with anti-p-azophenylarsonate antibodies in A/J mice. *Ann. Immunol.* (Inst. Pasteur) 130C: 199.

3. Brown, A. R., P. Estess, E. Lamoyi, L. Gill-Pazaris, P. D. Gottlieb, J. D. Capra, and A. Nisonoff. 1980. Studies of genetic control and microheterogeneity of an idiotype associated with anti-p-azophenylarsonate antibodies of A/J mice. In Membranes, Receptors, and the Immune Response. E. P. Cohen and H. Köhler, editors. Alan R. Liss, Inc., New York.

4. Gill-Pazaris, L. A., E. Lamoyi, A. R. Brown, and A. Nisonoff. 1981. Properties of a minor cross-reactive idiotype associated with anti-p-azophenylarsonate antibodies of A/J mice. *J. Immunol.* 126:75.

5. Brown, A. R., and A. Nisonoff. 1981. An intrastrain cross-reactive idiotype associated with anti-p-azophenylarsonate antibodies of BALB/c mice. *J. Immunol.* 126:1263.

6. Brown, A. R., E. Lamoyi, and A. Nisonoff. 1981. Relationship of idiotypes of the anti-p-azophenylarsonate antibodies of A/J and BALB/c mice. *J. Immunol.* 126:1268.

7. Ju, S.-T., A. Gray, and A. Nisonoff. 1977. Frequency of occurrence of idiotypes associated with anti-p-azophenylarsonate antibodies arising in mice immunologically suppressed with respect to a cross-reactive idiotype. *J. Exp. Med.* 145:540.

8. Estess, P., A. Nisonoff, and J. D. Capra. 1979. Structural studies on induced antibodies with defined idiotypic specificities. VIII. NH2-terminal amino acid sequence analysis of the heavy and light chain variable regions of monoclonal anti-p-azophenylarsonate antibodies from A/J mice differing with respect to a cross-reactive idiotype. *Mol. Immunol.* 16:1111.

9. Estess, P., E. Lamoyi, A. Nisonoff, and J. D. Capra. 1980. Structural studies on induced antibodies with defined idiotypic specificities. IX. Framework differences in the heavy and light chain variable regions of monoclonal anti-p-azophenylarsonate antibodies from A/J mice differing with respect to a cross-reactive idiotype. *J. Exp. Med.* 151:863.

10. Alkan, S. S., R. Knecht, and D. G. Braun. 1980. The cross-reactive idiotype of anti-4-azobenzenearsenonate hybridoma-derived antibodies in A/J mice constitutes multiple heavy chains. *Hoppe-Seyler's Z. Physiol. Chem.* 361:191.

11. Marshak-Rothstein, A., A. M. Siekevitz, M. N. Margolies, M. Mudgett-Hunter, and M. Gefter. 1980. Hybridoma proteins expressing the predominant idiotype of the anti-azo-phenylarsonate response of the A/J mice. *Proc. Natl. Acad. Sci. U. S. A.* 77:1120.

12. Lamoyi, E., P. Estess, J. D. Capra, and A. Nisonoff. 1980. Heterogeneity of an intrastrain cross-reactive idiotype associated with anti-p-azophenylarsonate antibodies of A/J mice. *J. Immunol.* 124:2834.

13. Lamoyi, E., P. Estess, J. D. Capra, and A. Nisonoff. 1980. Presence of highly conserved idiotypic determinants in a family of antibodies that constitute an intrastrain cross-reactive idiotype. *J. Exp. Med.* 152:703.

14. Daugharry, H., J. E. Hopper, B. MacDonald, and A. Nisonoff. 1969. Quantitative investigations of idiotypic antibodies. I. Analysis of precipitating antibody populations. *J. Exp. Med.* 130:1047.

15. Kohler, G., and C. Milstein. 1976. Derivation of specific antibody tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* 6:511.

16. Gefter, M. L., D. Margolies, and M. D. Scharff. 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. *Somatic Cell Genet.* 3:231.

17. Shulman, M., C. D. Wilde, and G. Kohler. 1978. A better cell line for making hybridoma secreting specific antibodies. *Nature (Lond.).* 276:269.
18. Littlefield, J. W. 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science (Wash. D.C.)* 141:709.

19. Klinman, N. R., A. R. Pickard, N. H. Sigal, P. J. Gearhart, E. S. Metcalf, and S. K. Pierce. 1976. Assessing B cell diversification by antigen receptor and precursor cell analysis. *Ann. Immunol. (Paris)* 127C:489.

20. Potter, M. 1972. Immunoglobulin-producing tumors and myeloma proteins of mice. *Physiol. Rev.* 52:631.

21. Hart, D. A., A.-L. Wang, L. Pawlak, and A. Nisonoff. 1972. Suppression of idiotypic specificities in adult mice by administration of anti-idiotypic antibody. *J. Exp. Med.* 135:1293.

22. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* 245:3059.

23. Hunter, R. 1970. Standardization of the chloramine-T method of iodination. *Proc. Soc. Exp. Biol. Med.* 135:909.

24. Pauling, L., D. Pressman, and A. L. Grossberg. 1944. The serological properties of simple substances. VII. A quantitative theory of the inhibition by hapten of the precipitation of heterogeneous antiserum with antigens, and comparison with experimental results for polyanptic simple substances and for azoproteins. *J. Amer. Chem. Soc.* 66:784.

25. Marshak-Rothstein, A., J. D. Benedetto, R. L. Kirsch, and M. L. Gefter. 1980. Unique determinants associated with hybridoma proteins expressing a cross-reactive idiotype: frequency among individual immune sera. *J. Immunol.* 125:1987.

26. Jerne, N. K. 1974. Towards a network theory of the immune system. *Ann. Immunol. (Paris)* 125C:373.

27. Rodkey, L. S. 1974. Studies of idiotypic antibodies. Production and characterization of antoantidiotypic antiserum. *J. Exp. Med.* 139:712.

28. Kluskens, L., and H. Kohler. 1974. Regulation of immune response by autogenous antibody against receptor. *Proc. Natl. Acad. Sci. U. S. A.* 71:5083.

29. Cosenza, H. 1976. Detection of anti-idiotype reactive cells in the response to phosphorylcholine. *Eur. J. Immunol.* 6:114.

30. Schrater, A. F., E. A. Goeldl, G. J. Thorbecke, and G. W. Siskind. 1979. Production of auto-anti-idiotype antibody during the normal immune response to TNP-Ficol. I. Occurrence in AKR/J and BALB/c mice of hapten-augmentable, anti-TNP plaque-forming cells and their accelerated appearance in recipients of immune spleen cells. *J. Exp. Med.* 150:138.

31. Thomas, W. R., G. Moreahan, I. D. Walker, and J. F. A. P. Miller. 1981. Induction of delayed-type hypersensitivity to azobenzeneauronate by a monoclonal anti-idiotype antibody. *J. Exp. Med.* 153:743.