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Immunology

Idiotypic manipulation in mice: BALB/c mice can express the crossreactive idiotype of A/J mice

(regulation network/somatic variants)

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ABSTRACT **The response of A/J mice to arsonate-coupled keyhole limpet hemocyanin is characterized by a crossreactive idiotype (CRI_A). CRI_A^+ antibodies are restricted to the Igh-1^e haplotype and are never expressed in BALB/c mice after immunization with antigen. Studies at the DNA level suggest that the gene encoding the CRI_A heavy chain in A/J mice is probably absent in the genome of BALB/c mice. Despite this, using the immunization cascade tool, we have been able to induce the expression of CRI_A^+ antibodies in BALB/c mice. These studies led to an apparent paradox, whose understanding will provide new insights into the regulatory mechanisms of the immune system. We suggest that clones secreting CRI_A-like Igs in BALB/c mice are "somatic variants" that could arise from gene conversion events.**

The discovery (1–3) and study of idiotypy has opened new insights into the most fascinating problems of immunology: the origin of antibody diversity and the unravelling of regulatory mechanisms underlying the immune response. Idiotypy refers to the fact that different individuals from the same species use different immunological repertoires when confronted with the same antigen. Exceptions to this rule have been extensively studied (4). Public or recurrent idiotypes are regularly expressed in all members from some strains of mice immunized with a given antigen.

Broadly, immune regulatory mechanisms concern two major problems (5): (*i*) the building up of available repertoires (natural tolerance, idiotypy, and the immune response phenomenon) and (*ii*) the control of ongoing immune responses (increase and decrease in binding affinity, isotype switching, feedback mechanisms, . . .). We do not yet understand the rules by which newborn B lymphocytes constantly released from bone marrow to periphery are positively or negatively selected. We have shown that it is possible to modify expressed repertoires and to guide the immunological system towards a predetermined goal. This was achieved by obtaining sequential sets of anti-idiotypic antibodies (6–9). When randomly chosen rabbits are pretreated with anti-idiotypic antibodies (designated Ab2 or second-order antibodies), they respond by the synthesis of anti-anti-idiotypic antibodies (designated Ab3 or third-order antibodies), some of which do not bind antigen but share idiotopes with the starting idiotype (designated Ab1). When immunized with antigen, nearly all manipulated rabbits synthesize antibodies designated Ab1′. A large fraction of these Ab1′ are strongly idiotypically crossreactive with the starting idiotype (designated Ab1). Therefore, it seems that a large part of the potential immune repertoire is silent and that some idiotypic determinants could be the target of specific regulatory mechanisms, as expected if the immune system is a functional idiotypic network.

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Similar results have been obtained in mice (9–12). The exact mechanisms responsible for the modulation of available repertoires are still poorly understood. The immunization cascade could relieve one idiotype from suppression and allow the activation of a silent gene or could furnish a positive selection pressure to promote the induction of a given idiotype. Therefore, it is of great potential interest to perform such studies in systems where detailed structural information at the protein and DNA levels is now at hand.

A favorable approach for such an analysis is the arsonate (Ars) system (13–15). When A/J mice are immunized with arsonate-coupled keyhole limpet hemocyanin (Ars-KLH), all mice express a crossreactive idiotype (Ars-CRI). Such CRI_A^+ antibodies appear only in mice belonging to the Igh-1^e and Igh-1^d haplotypes. All the other strains, such as BALB/c, are usually negative for CRI_A expression. The CRI_A^+ Ig constitutes a large family of closely related members: there is 90–95% of homology between the amino acid sequences of CRI_A^+ antibodies (16, 17). Only one or very few germ-line genes seem to be involved in the encoding of the variable region of the heavy chain (V_H) (15, 18, 19). Recent evidence seems to indicate that no corresponding germ-line genes exists in negative strains such as BALB/c. We demonstrate here that it is possible to induce CRI_A^+ anti-Ars antibodies in BALB/c mice, which never express this idiotype after immunization with only the antigen.

MATERIAL AND METHODS

Immunizations. *Ab1.* BALB/c mice were obtained from Centre d'Energie Nucléaire (Mol, Belgium). A/J mice were purchased from The Jackson Laboratory. Mice immunized with Ars-KLH received an intraperitoneal injection of 200 μg of Ars-KLH in complete Freund's adjuvant (CFA) (primary response) and an intravenous injection of 100 μg of Ars-KLH in saline one month later (secondary response).

Ab2. For heterologous Ab2, rabbits received subcutaneous injections of 200 μg of affinity-purified A/J anti-Ars antibodies (A/J Ab1; obtained from pooled ascites fluids). The first injection of A/J anti-Ars antibodies emulsified in CFA was followed 2 wk later by one injection in incomplete Freund's adjuvant (IFA). Subsequent injections were made in saline at weekly intervals. BALB/c Ab2 were prepared as follows. Affinity-purified A/J anti-Ars antibodies (50 μg) were injected subcutaneously in CFA, followed 2 weeks later by a second injection in IFA. Antibodies were then injected weekly in saline. Simultaneously, ascitic fluids were induced in the same mice.

Abbreviations: CRI, crossreactive idiotype; Ars, arsonate; KLH, keyhole limpet hemocyanin; Ars-KLH, KLH coupled to *p*-arsanilic acid; V, variable regions of Igs; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

These Ab2 antisera were rendered specific for idiotypic determinants by repeated passages over Sepharose columns coupled with normal A/J globulins, followed by adsorption on a column of Sepharose coupled to BALB/c anti-Ars antibodies (see results).

Ab3. BALB/c mice received subcutaneously one injection of purified rabbit Ab2 emulsified in CFA, followed 2 wk later by a second injection in IFA; this in turn was followed 2 wk later by three weekly injections in saline.

Radioimmunoassays. (*i*) Antisera were tested for anti-Ars antibody on bovine serum albumin-Ars-coated polyvinyl microtiter plates. The antisera were incubated 2 hr at 20°C before the addition of ^{125}I-labeled purified goat anti-mouse antibodies (2 hr at 20°C).

(*ii*) The content of CRI_A or anti-anti-CRI_A antibodies (Ab1 and Ab3, respectively) in serum samples was determined by a solid-phase radioimmunoassay. The rabbit Ab2 binds 70% of a pool of purified radio-labeled A/J anti-Ars antibodies. In inhibition assays, trays are coated overnight with 0.2 μg of purified Ab2 able to bind 75% of CRI_A^+ antibodies. Putative inhibitors are added simultaneously with labeled ligand and incubated 4 hr at 20°C. Each assay was carried out in the presence of normal mouse serum (2%) and normal rabbit serum (4%). All dilutions were done in phosphate buffer containing 0.5% bovine serum albumin.

(*iii*) The percentage of anti-Ars antibodies bearing the CRI_A idiotype was measured. The binding of unfractionated anti-Ars antiserum on unsolubilized antigen was inhibited by anti-CRI_A antiserum. The antisera were incubated overnight (at 4°C) with 5 μl of anti-idiotypic antibodies purified from rabbit or BALB/c Ab2 antiserum and then adsorbed 2 hr at 20°C on Ars-bovine serum albumin-coated polyvinyl microtiter plates. Bound anti-Ars antibodies were detected by the uptake of labeled purified goat anti-mouse antibodies. CRI_A^+ antibodies were quantified by measuring the inhibition of the binding of ^{125}I-labeled goat anti-mouse Ig.

Monoclonal Antibodies. The monoclonal antibodies 36-65 and 36-60, 93G7, and R16.7 were kindly given by M. Gefter, D. Capra, and A. Nisonoff, respectively.

RESULTS AND DISCUSSION

We shall follow the order of the immunization cascade. A/J Ab1 (pooled affinity-purified anti-Ars antibodies) were used to induce Ab2 (rabbit anti-CRI_A). These anti-idiotypic second-order antibodies were then injected into naive BALB/c mice, which then make Ab3 (anti-Ab2 antibodies or third-order antibodies). These mice will be denoted anti-idiotype-treated mice. The same mice were then injected with antigen (Ars-KLH). They synthesized Ab1′ antibodies, and they will be called manipulated mice.

The specificity of the Ab2 was confirmed in a competitive radioimmunoassay. Only antisera of A/J mice immunized with Ars-KLH inhibited the binding of ^{125}I-labeled CRI by the rabbit anti-CRI antibodies (Ab2). Moreover, monoclonal antibodies defined as CRI_A^+ (93G7, 36-65, R16.7) were optimal inhibitors in this reaction, whereas 36-60 (Ab1 CRI_A^-) was not.

To ensure a rigorous specificity, the rabbit Ab2 was further adsorbed on a column of Sepharose coupled to BALB/c Ab1, and an excess amount of BALB/c Ab1 antiserum was added in the various inhibition assays.

All of the 20 BALB/c mice antisera tested failed to inhibit the binding of A/J Ab1 by the adsorbed rabbit antiserum, whereas all of the 20 A/J mice tested completely inhibited the same reaction (data not shown).

Ab3. Five BALB/c mice were then injected with such purified Ab2 anti-idiotypic antibodies. After five successive immunizations, these mice were tested for Ab3 activity. As shown in Fig. 1*A*, all of the injected mice significantly inhibited the binding of ^{125}I-labeled CRI_A (A/J Ab1) to the rabbit Ab2. This finding is consistent with the presence of Ab3 directed against the idiotypic determinants expressed by rabbit Ab2. The same bleedings were tested for specific anti-Ars activity. The results of binding assays (Fig. 2*A*) show that these antisera specifically bind the antigen, in contrast to the sera of nonimmune BALB/c mice or mice injected with normal rabbit Igs. Moreover, the antisera of these Ab2-treated mice contain higher concentrations of anti-Ars antibodies (Ab1) than do the antisera of control BALB/c mice primed with Ars-KLH only. In our previous studies on the immunization cascade (for review, see ref. 20), we have suggested that Ab3 contains three subsets: anti-Ab2 antibodies that just recognize the immunogen; Ab3 that do not recognize the antigen but share idiotypic specificities with Ab1; and a very

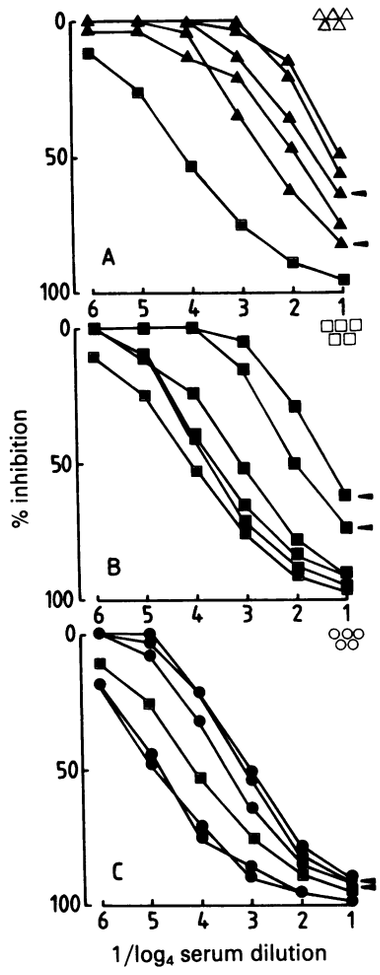

FIG. 1. CRI_A^+ assay of manipulated and control BALB/c mice. Inhibition of binding of labeled, purified A/J anti-Ars antibodies to rabbit anti-CRI_A serum. In each assay, the standard inhibition curve of a pool of hyperimmune anti-Ars A/J sera is presented (▣). Unlabeled inhibitors were as follows. (*A*) BALB/c Ab3 antisera (i.e., mice immunized with purified rabbit anti-idiotypic antibodies (▲); BALB/c antisera from mice immunized with normal rabbit Ig (△). (*B*) Primary BALB/c Ab1′ antisera (i.e., Ab3-producing mice immunized once with Ars-KLH) (■); primary anti-Ars response of untreated BALB/c mice (□). (*C*) Secondary BALB/c Ab1′ antisera (i.e., Ab3-producing mice immunized twice with Ars-KLH) (●); secondary BALB/c anti-Ars response of untreated mice (○). Arrowheads indicate group II animals.

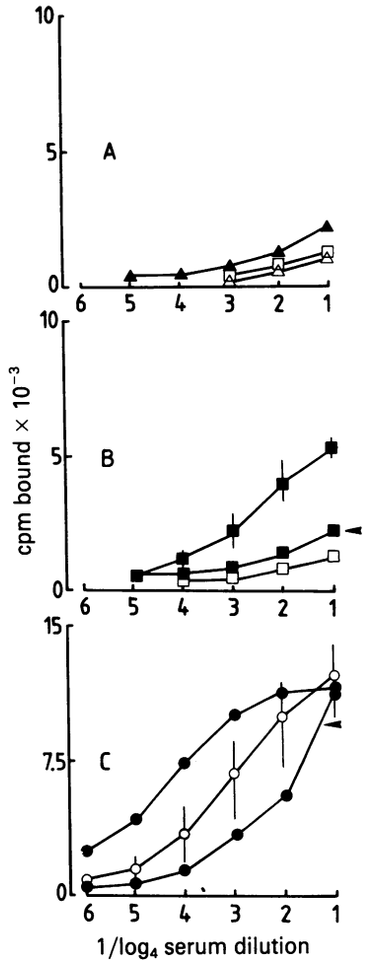

FIG. 2. Antigen binding capacity of manipulated and control BALB/c mice. Individual mice were tested for anti-Ars antibodies with a solid phase radioimmunoassay. (*A*) BALB/c Ab3 antisera (▲); BALB/c antisera from mice hyperimmunized with normal rabbit Ig (△). (*B*) Primary BALB/c Ab1′ antisera (■); primary anti-Ars antisera from untreated BALB/c mice (□). (*C*) Secondary BALB/c Ab1′ antisera (●); secondary anti-Ars antisera from untreated BALB/c mice (○). Arrowheads indicate group II animals.

small subset that displays the idiotypic properties of Ab1 and the antigen binding capacity. This third subset is clonally expanded after antigen stimulation. In fact, this third subset was not clearly demonstrated in the previous experiments (6–8). Further experiments were performed to quantitate the anti-Ars response of manipulated and control BALB/c mice. As can be seen from Table 1, Ab2-treated BALB/c mice produce significant amounts of anti-Ars antibodies. The results shown here clearly suggest the existence of this third subset.

Ab1′. Two (group I) or 3 (group II) months after the last injection of Ab2, the BALB/c mice synthesizing Ab3 were immunized with Ars-KLH. The antisera, collected after a single injection of antigen, contained more anti-Ars antibodies than did control mice making a primary response (Fig. 2*B*). Surprisingly, group I individuals are better responders to Ars-KLH than are group II mice. The antisera of the same mice were then tested after a second challenge with Ars-KLH. The results are depicted in Fig. 2*C*.

Thus, it seems likely that the injection of rabbit Ab2 has primed the Ab3-producing mice for specific anti-Ars activity. The anti-Ars antibodies synthesized in the primary and secondary responses of these mice should then carry idiotypic determinants related to the major A/J idiotype.

The antisera of the manipulated BALB/c mice were then tested for their capacity to inhibit the binding of ^{125}I-labeled CRI_A by the rabbit Ab2. The sera of the five Ab1′ mice completely inhibited this reaction; moreover, we observed a clear correlation between the concentration of anti-Ars antibodies in an antiserum and its inhibitory activity (Fig. 1 *B* and *C*). This finding is consistent with the presence, in the antisera of Ab1′ BALB/c mice, of anti-Ars antibodies bearing the major idiotype of A/J mice (CRI_A).

To be certain that the antibody molecules responsible for inhibition in this assay were indeed specific for the Ars hapten, we performed the following experiment. The binding of unfractionated A/J anti-Ars antiserum on the insolubilized antigen can be specifically inhibited by increasing concentrations of rabbit anti-idiotypic antibodies. In a similar experiment performed with BALB/c mice (Table 2), we could show that only the anti-Ars antibodies of manipulated (Ab1′) mice interact with rabbit anti-CRI_A antibodies.

The same results have been obtained by using a polyclonal BALB/c anti-CRI_A antiserum as inhibitor of the same reaction (Table 3). This provides further evidence for the presence in the antiserum of the BALB/c Ab1′ mice of anti-Ars antibodies bearing idiotypic determinants of the A/J CRI^+ family.

Finally, spleen cells of one of the Ab1′ mice (group I) were injected intravenously together with Ars-KLH into five syngeneic BALB/c recipients sublethally irradiated. The binding of the sera of all recipient mice is strongly inhibited by both anti-CRI_A antisera (data not shown).

When we compare the results that are described here with those from Capra (15) and Gefter (19), we are faced with an apparent paradox. On one hand, we demonstrate the induction of a silent idiotype in BALB/c mice; on the other hand, the DNA analysis studies suggest that the germ-line V_H gene, coding for

Table 1. Anti-Ars titer of manipulated and control BALB/c mice

Group*	Treatment	Anti-Ars titer, μg/ml
1 (5)	None	<0.005†
2 (5)	Normal rabbit Ig	<0.005†
3 (5)	Rabbit Ab2 anti-CRI_A	0.092, 0.117, 0.050, 0.383, 0.132
4 (5)	Rabbit Ab2 followed by KLH-Ars	0.213, 0.153, 2.92, 5.96, 3.43
5 (10)	KLH-Ars	0.082 (0.02–0.273)†

The anti-Ars titer was determined by a solid phase radioimmunoassay.
*The number of mice is in parentheses.
†Mean values; range is in parentheses.

Table 2. CRI_A^+ anti-Ars antibodies assay

	Anti-Ars antiserum by no., % of inhibition				
Mouse strain	1	2	3	4	5
A/J	36.1	46.3	—	—	—
BALB/c untreated	7.9	5.4	1.2	1.6	5.0
BALB/c Ab1′	19.2	63.1	73.8	45.5	21.1

The binding of unlabeled anti-Ars antibodies to insolubilized Ars-bovine serum albumin was inhibited by the rabbit anti-CRI_A antiserum. CRI_A^+ antibodies are quantified by measuring the inhibition of the uptake of ^{125}I-labeled goat anti-mouse Ig. Results are expressed as % of inhibition.

Table 3. CRI_A^+ anti-Ars antibodies assay

Mouse strain	Anti-Ars antiserum by no., % of inhibition				
	1	2	3	4	5
A/J	19.5	27.0	—	—	—
BALB/c untreated	0.0	0.0	0.0	0.0	0.0
BALB/c Ab1′	14.6	57.0	44.0	19.6	4.5

The binding of unlabeled anti-Ars antibodies to Ars-bovine serum albumin was inhibited by BALB/c anti-CRI_A antiserum. For additional information, see Table 2.

the variable region of the heavy chain CRI in A/J mice, seems to be absent in BALB/c mice. These DNA studies support the straightforward interpretation that the CRI_A is expressed when the gene is present, and it is absent when there is no corresponding gene. This simple idea does not ascribe a role to regulation in the expression of the CRI_A. What might then be the mechanisms that induce the appearance of anti-Ars antibodies displaying the CRI of A/J mice in BALB/c mice?

(*i*) We should first consider the possibility that the DNA studies are incomplete or that our results are misleading. For the time being, we see no obvious reason to take this interpretation into account. Indeed, the DNA data provide conclusive evidence for genetic polymorphism between germ-line A/J and BALB/c DNA. On the other hand, our data show that "we are fishing the same fish": the CRI_A we have studied is the same as that defined by the "canonical" criteria of Nisonoff and Capra. It is still possible that our manipulations with anti-idiotypic antisera have induced the synthesis of Igs bearing idiotopes similar to those of the A/J anti-Ars antibodies, but that individual molecules do not carry the complete set of idiotopes. This problem can only be solved by the study of monoclonal antibodies. However, it should be stressed that it has already been shown that distinct idiotopes that are on the same molecule in one bleeding can be found on different Igs in subsequent bleedings from the same animal (2).

(*ii*) If we accept both sets of results, several fascinating perspectives emerge. The two sets of studies give information at different levels. The DNA and amino acid sequence studies bring unidimensional information, whereas our serological studies concern the three-dimensional structure of Igs. It is not easy to extrapolate from one level to the other, but this is a key point in immunology. After all, antigens and anti-idiotypic antibodies do not read nucleotide or amino acid sequences. They recognize the spatial structures of epitopes and idiotopes. A first possibility might be that different amino acid sequences could give rise to Igs displaying very similar idiotypic specificities.

However, if we exclude the data obtained with some Ab2, which behave like an internal image of the antigen (21–23), all data so far obtained clearly show a close relationship between idiotypy and amino acid sequences (15).

A second proposal would be that the genes responsible for the expression of CRI_A in manipulated mice are indeed absent from the germ line, as suggested by the DNA analysis studies, but are present in some lymphocyte clones in the manipulated mice. The CRI_A genes could stem from somatic modifications of other genes.

The injection of anti-idiotypic antibodies could provide a strong pressure for the selection of these somatic variants displaying the CRI_A idiotype.

Against this proposal are the recent results of Lucas and Henry (ref. 24; see also ref. 25), who observed the appearance of CRI_A^+ Ig in BALB/c mice upon injection of arsonate coupled to *Brucella Abortus* (thymus-independent antigen) in BALB/c mice. In that case, no way of idiotypic selection is apparent.

To explain all of the data, we suggest that a process of gene conversion between two or several genes of BALB/c DNA is responsible for the appearance of a "somatic variant" displaying the CRI_A properties; this process would be strongly amplified by the immunization cascade.

All of these data clearly suggest that idiotypic regulation is operating in the selection of available repertories (26). There is a basic functional idiotypic network that could be conserved during evolution despite the fact that multigenic families of *V* genes evolve constantly and are in a state of dynamic equilibrium. As we already have pointed out, the idea that a functional idiotypic network can provide an internal selective pressure for conserving genes that are apparently useless does not imply that every *V* gene is "frozen" once and forever. All that is needed is the conservation of *V* genes coding for families of idiotypically interacting molecules.

More complicated possibilities can be envisaged, but we think that it is useless to discuss them at this stage of our work. A partial answer to the paradox described in this paper might come from a structural analysis of the monoclonal Ab1′ that we have obtained from manipulated BALB/c mice that produce CRI_A^+ anti-Ars antibodies.

Note Added in Proof. We have generated monoclonal Ab1′ antibodies from manipulated BALB/c mice. These antibodies bind arsonate and display the CRI_A idiotopes on the same molecule.

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