

IDIOTYPIC ANALYSIS OF POTENTIAL
AND AVAILABLE REPERTOIRES IN THE ARSONATE
SYSTEM

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The discovery of the individual antigenic specificity of myeloma proteins (1, 2) and of the idiotypy of antibodies (2-4) has opened fascinating ways to investigate the fundamental problems of immunology (5, 6).

We and others (7-11) have shown that it is possible, using an idiotypic cascade (antibodies against antibodies against antibodies . . .) to manipulate, in a specific way, the immune system towards a predetermined goal, i.e., the induction of a normally silent idiootype. These results and others (12) indicate that the potential repertoire of one individual is larger than the functional repertoire (the one which is selected after antigen immunization).

It has been suggested (13) that available repertoires are strictly germline determined and are T cell independent. Although this may be the case in some systems, we show in this paper that in the arsonate (Ars)¹ system the selection of available repertoires depends on complex regulatory pathways.

The cross-reactive idiootype (CRI), CRI_A, is a large family of nonidentical but highly homologous immunoglobulins (14). This idiootype is encoded by one or few germline genes in the A/J strain. The heavy chain, variable region (V_H) segments have no identical counterparts in the BALB/c genome (15). Several reports (16-18) have suggested that BALB/c mice can nevertheless synthesize CRI_A-like idiotypes. The appearance of such idiotypes can be regularly induced in all BALB/c mice by the idiotypic cascade (18). We have also identified five distinct idiotopes on the germline product of A/J mice. Four of these idiotopes are also displayed by anti-Ars monoclonal antibodies (MAb), prepared from BALB/c mice that have been manipulated to induce the expression of CRI_A-like idiotypes (19). Amino acid sequences have shown that idiootype-positive anti-Ars antibodies from A/J and BALB/c use similar light chains, similar D segments,

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¹ *Abbreviations used in this paper:* Ab, antibody; Ars, arsonate; CRI, cross-reactive idiootype; HP, hybridoma protein; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MAb, monoclonal antibody; SE, sheep erythrocyte; TMV, tobacco mosaic virus; V_H, variable region heavy chain.

but different V_H segments. In this report, we have used the limiting dilution analysis (20, 21), after polyclonal activation *in vitro*, to measure the frequencies of B lymphocytes able to secrete Ars-binding antibodies displaying the CRI_A , in nonimmune and immune A/J mice. Similar studies were performed with non-immune, immune and manipulated BALB/c mice.

Materials and Methods

Mice. BALB/c mice were obtained from C.E.N. (Mol), Belgium. A/J mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Immunizations. Mice immunized with Ars-keyhole limpet hemocyanin (KLH) received an intraperitoneal injection of 200 μ g Ars-KLH in complete Freund's adjuvant (primary response) and were injected according to the same protocol at 2-wk intervals for secondary and tertiary responses.

The preparation of the rabbit anti- CRI_A antisera has been described (18). E4 and H8 anti- CRI_A antiidiotypic MAb were obtained as follows. Briefly, adult BALB/c mice were hyperimmunized with affinity-purified A/J anti-Ars serum antibodies cross-linked to KLH. 3 d before fusion, animals were boosted intravenously with the same preparation. Spleen cells from two mice and the SP2₀ Ag:14 myeloma cell line were used to prepare hybridomas according to Köhler and Milstein (22) as modified by J.-D. Franssen et al. (23). Culture supernatants were assayed on day 14 for anti- CRI_A antibodies by solid-phase radioimmunoassay using polyvinyl microtiter trays, coated with predetermined amounts of purified A/J anti-Ars serum antibodies. Bound anti- CRI_A antibodies were scored by measuring the subsequent uptake of I^{125} -labeled 36-65 anti-Ars CRI_A^+ MAb (γ 1). Cells from positive wells were cloned twice on soft agar. Large amounts of MAb were produced as ascitic fluids in mice pretreated with pristane. Each monoclonal anti- CRI_A was first prepared by ammonium sulfate precipitation (50% saturation) and then either fractionated on DEAE cellulose with a gradient of phosphate buffer or on Sepharose columns coupled with 36-65 MAb and eluted with glycine HCl at pH 2.2. Anti-antiidiotypic antibodies (Ab3) were obtained as described (18).

Mitogens. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 was obtained from Difco Laboratories Inc., Detroit, MI.

Culture from the Limiting Dilution Assay. Frequency analysis was performed with LPS *in vitro*, according to Andersson et al. (20). Graded numbers of spleen cells to be analyzed were cultured in Iscove's modified Eagle's medium supplemented with glutamine, antibiotics, 10% fetal calf serum, and 2×10^{-5} M 2-mercaptoethanol, in the presence of rat thymocytes (3.2×10^6 /ml) as filler cells and LPS (50 μ g/ml) and incubated in a 96-well plate. At each cell dose, 24–48 replicate cultures were set up and assayed at day 5 for Ig-secreting plaque-forming cells, using the staphylococcal protein A reverse plaque assay (24). Alternatively, cultures were continued until day 14 and the supernatants were assayed for the presence of idiotopes or Ars-specific activity. Each experiment presented was made on two to five different individuals.

Titration of Idiotoxes in Culture Supernatants. Sheep erythrocytes (SE) were coupled by the chromium chloride method with affinity-purified anti- CRI_A MAb in saline (0.9%) and mixed in V-shaped microtiter plates with 25 μ l culture supernatants, at a final concentration of 0.5%. After 1-h incubation at room temperature, the number of positive wells was scored.

Each culture supernatant was tested on Ars-bovine serum albumin in an enzyme-linked immunosorbent assay as described previously (18) and on H8- and E4-coated SE. SE coated with an unrelated antiidiotypic hybridoma protein (HP) of the same subclass as H8 and E4 (γ 1) were used as controls to avoid detection of autoantiallotypic antibodies. Positive wells in the hemagglutination test using either E4- or H8-coated SE were tested in the presence of *p*-azophenylarsonate, at a concentration (4×10^{-2} M) determined to cause specific inhibition of the hemagglutination test using E4- or H8-coated SE with 36–65 HP, but to be ineffective in the same test using the MAb2 (A11) and its homologous

anti-tobacco mosaic virus (TMV) MAb (C1). The frequencies were calculated according to C. Taswell (25).

Results

Description of Antiidiotypic Antibodies Used in these Studies. In the experiments reported here we have used two anti-CRI_A MAb as well as a polyclonal rabbit antiidiotypic serum raised against purified anti-Ars antibodies from A/J mice. The rabbit antiserum bound 70% of A/J anti-Ars antibodies while the MAb2 called E4 and H8 bound 36 and 25% respectively. The two MAb2, raised against 36–65, recognize distinct idiotopes since we have found anti-Ars MAb displaying one idiotope without the other. We have used those two anti-CRI_A MAb2 to screen, in a direct hemagglutination test, the reactivity of culture supernatants from limiting dilution analysis of splenic B cells under LPS activation *in vitro*.

Frequencies of CRI_A-producing B Cells in Naive and Ars-KLH-primed A/J Mice. The frequency of splenic B cells that could produce Ig after LPS stimulation (LPS-reactive cells) was assessed as described by Andersson et al. (14). The approximate frequencies of BALB/c or A/J spleen cells reactive to LPS activation *in vitro* are similar in both strains: 1 cell out of 15 B cells is able to secrete Ig.

The main question we planned to investigate was whether the clonal dominance of CRI_A⁺ anti-Ars-specific B cells is already established before any immunization with Ars. For this purpose, we measured the frequencies of CRI_A⁺ B cells in the spleens of A/J nonimmune and A/J Ars-KLH-primed individuals. As shown in Fig. 1A, the frequency of E4⁺ LPS-reactive B cells in A/J nonimmune individuals is low (1 cell out of nearly 1.2×10^4 LPS-reactive spleen cells). Those CRI_A⁺ B cells represent a very small part of the Ars-specific B cells (nearly 0.5%), in agreement with N. Sigal's frequency determination using the splenic focus assay (17).

Interestingly, the injection of Ars-KLH to A/J mice enhanced dramatically the frequency of CRI_A⁺ LPS-reactive B cells (1 cell out of 20 LPS-reactive B cells) in such a way that 70% of Ars-specific B cells are CRI_A⁺ (Fig. 1B). Thus, when the proportion of CRI_A⁺ LPS-reactive B cells among the total Ars-specific B cells in nonimmune and Ars-KLH-primed A/J mice is compared, a preferential expansion of B cells secreting Ig-bearing CRI_A idiotopes in A/J mice immunized with Ars-KLH is observed (Table I).

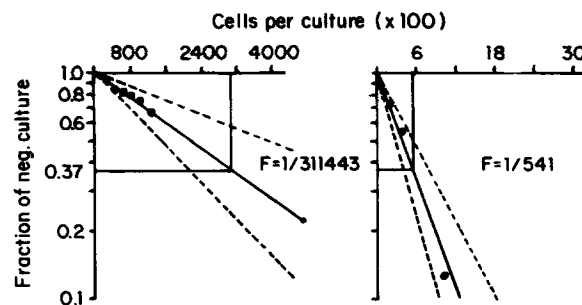


FIGURE 1. Precursor frequencies of E4⁺ spleen B cells in adult normal A/J (A) or in Ars-KLH-immune A/J (B). The numbers are those given by the computer program. F, frequency per total input cells.

TABLE I
Precursor Frequencies in Spleen from Nonimmune A/J and BALB/c Mice

Strain	Treatment	LPS-reactive cells/ spleen cells	Ars + cells/ LPS-reactive cells	E4 ⁺ cells/LPS-reactive cells	CRI _A ⁺ cells/ Ars ⁺ cells
					%
A/J	None	1:29	1:70	1:1.2 × 10 ⁴ (90%)*	0.5
A/J	Two injections of Ars-KLH	1:29	1:13	1:18 (90%)	72
BALB/c	None	1:32	1:125	1:1.8 × 10 ⁴ (50%)	0.4
BALB/c	Two injections of Ars-KLH	1:32	1:58	1:3 × 10 ⁴	0.2

* The numbers in parentheses correspond to the percentage of supernatants that are hapten inhibitable.

Since MAb2 can bind to Ab1-like antibodies (CRI_A⁺) or to anti-antiidiotypic antibodies against MAb2 that are unrelated to the CRI_A family, the screening with E4- and H8-coated SE was performed in the presence or absence of the Ars hapten. The hapten concentration used was determined to cause specific inhibition of the hemagglutination test using E4- or H8-coated SE with 36–65, but to be ineffective in the same test using an unrelated monoclonal antibody and its homologous MAb2 (see Materials and Methods). This test is able to identify the CRI_A⁺ Ars-specific clones but fails to measure the CRI_A⁺ clones that are not specific for arsonate (Id⁺Ag⁻). Table I shows the proportion of Ars-inhibitable culture supernatants reactive with E4-coated SE.

To confirm the discrimination between the CRI_A⁺ clones and the anti-MAb2 clones in our test, the reactivity of each culture supernatant with both MAb2 known to recognize distinct idiotopes of the CRI_A family was measured. Surprisingly, up to 90% of the positive wells in direct hemagglutination and virtually 100% of the double positive clones (E4⁺H8⁺) were hapten inhibitable in nonimmune and Ars-KLH-primed A/J individuals. The very weak proportion of anti-MAb2 clones (10% maximum) suggests that the frequency of anti-antiidiotypic B cell clones is very small in A/J mice in contrast with BALB/c mice (see below).

Frequencies of Idiotope-producing B Cells in Nonmanipulated BALB/c Mice. We have examined whether the lack of expression of CRI_A⁺ Ig in the BALB/c anti-Ars response is due to the lack of relevant precursors or might be the result of regulatory mechanisms acting negatively on CRI_A⁺ B cells, since we have been able (18) to induce CRI_A⁺ Ars-binding antibodies in BALB/c mice.

The frequency of E4⁺ LPS-reactive B cells in naive (nonmanipulated) BALB/c mice is low (Table I). However, this frequency can be estimated to at least 1 cell out of 2–3 × 10⁴ LPS-reactive B cells. This frequency is not significantly different from that scored in A/J nonimmune individuals, although a larger individual variability was observed. Moreover, an average of 50% of those E4⁺ wells are hapten inhibitable; this suggests that the frequency of anti-anti-CRI_A B cell clones is significantly higher in a CRI_A⁻ (BALB/c) than in a CRI_A⁺ (A/J) strain. BALB/c mice, when primed with Ars-KLH, showed an increase in the

frequency of Ars-specific, LPS-reactive B cells, but not that of E4⁺ B cells. The proportion of E4⁺ Ars-specific B cell clones among the total Ars-specific clones reaches 0.2% in BALB/c Ars-KLH-primed individuals (Table I). Thus, while the frequencies of E4⁺ LPS-reactive B cells are similar in nonimmune A/J and BALB/c mice, inoculation with Ars induced a very clear preferential expansion of CRI_A⁺ B cell clones in A/J mice and did not modify their clonal size in BALB/c mice (a CRI_A⁻ strain).

Frequency Determination in Manipulated BALB/c Mice. Treatment of BALB/c mice with rabbit anti-CRI_A or MAb2 (H8) without subsequent injection of Ars-KLH led to a significant enhancement in the frequency of CRI_A⁺ LPS-reactive B cells (Fig. 2, Table II). Treatment with rabbit anti-CRI_A antibodies significantly enhanced the frequency of B cell clones secreting Ig bearing the E4 and H8

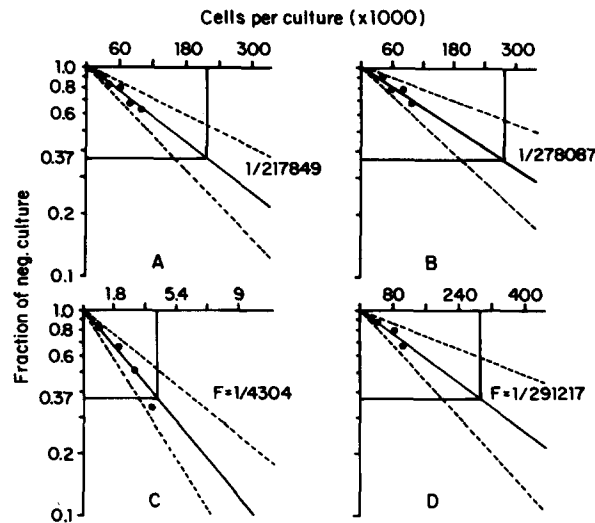


FIGURE 2. Precursor frequencies in manipulated BALB/c mice. (A and B) Mice that have been injected with rabbit anti-CRI_A. (C and D) Mice injected with MAb2 H8. The readout in A and C was done with SE coated with MAb2 H8, the readout in B and D with SE coated with MAb2 E4.

TABLE II
Precursor Frequencies in Spleen from Manipulated BALB/c Mice

Strain	Treatment	LPS-reactive cells/spleen cells	ARS ⁺ cells/LPS-reactive cells	E4 ⁺ /LPS-reactive cells	H8 ⁺ /LPS-reactive cells	E4 ⁺ /Ars ⁺	H8 ⁺ /Ars ⁺
BALB/c	Rabbit anti-CRI _A	1:32	1:99	1:8.9 × 10 ⁵ (75%)*	1:6.8 × 10 ⁵ (80%)	0.9	1.2
BALB/c	MAb2-H8	1:32	1:86	1:9.3 × 10 ⁵ (80%)	1:134 (50%)	0.8	32

* The numbers in parentheses correspond to the percentage of supernatants that are hapten inhibitable.

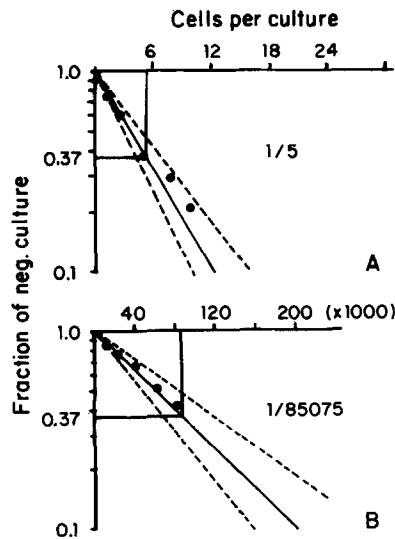


FIGURE 3. Normal (C57BL/6 \times BALB/c) F_1 spleen cells were assayed on day 5 for Ig-secreting cells using the protein A plaque assay (A) or on day 14 for $E4^+$ cells using SE coated with E4 MAb2 (B).

idiotypes (1 cell out of 7×10^3 LPS-reactive spleen cells). Most of the positive wells were hapten inhibitable. Treatment of BALB/c mice with H8 MAb2 gave rise to a very high increase in the frequency of B cell clones secreting Ig reactive with MAb2 H8 (Figs. 2C and D). Since 50% of the positive wells were hapten inhibitable, H8 MAb2-treated BALB/c mice are able to produce H8 $^+$ E4 $^-$ Ars-specific Ig.

Since LPS only acts on 1 splenic B cell out of 16 in BALB/c mice, we compared the frequencies recorded using LPS in BALB/c and (C57BL/6 \times BALB/c) F_1 mice. (C57BL/6 \times BALB/c) F_1 mice are characterized by a high level of LPS-reactive spleen cells (1 splenic B cell out of 2.5) (Fig. 3A). As shown in Fig. 3B, the frequency of $E4^+$ LPS-reactive B cells in the CRI_A^- (C57BL/6 \times BALB/c) F_1 strain (1 out of 1.7×10^4 LPS-reactive B cells) is very similar to that scored in BALB/c mice (1 out of 2×10^4 LPS-reactive B cells).

Discussion

The purpose of this study was to compare available or functional immune repertoires with potential repertoires. The potential repertoire is here experimentally defined by the frequency of B lymphocytes able to secrete a given antibody (anti-Ars) or a given idiotypic (CRI_A^+ , the major intrastrain cross-reactive idiotypic of A/J mice immunized with Ars coupled to KLH) after LPS activation. Our results only concern the LPS-reactive subset and the limitations of this kind of analysis are obvious (25).

It is important to stress that the idiotypic readout was done with two antiidiotypic MAb, which recognize two distinct idiotopes of the germline-encoded antibody 36-65 (from strain A/J). Only those wells that contained antibodies able to bind Ab2 and that were hapten inhibitable can be considered without

ambiguity. This is to avoid the detection of the Ab3 subset, which is made up of antiidiotypic antibodies to Ab2 and which have nothing to do with the CRI_A idio type.

Our main results can be summarized as follows: (a) In both nonimmune BALB/c and A/J mice, the frequency of Ars-positive precursors was very high. By contrast, the frequency of CRI_A⁺ precursors was only a minor subset (0.5%) of the Ars subset. There was no tremendous difference between the CRI_A expressor strain (A/J) and the nonexpressor strain (BALB/c). (b) When A/J mice were immunized with KLH-arsenate, the minor CRI_A subset was selectively amplified (100×); no such enhancement was observed in BALB/c mice similarly treated. (c) CRI_A-like antibodies were easily induced by the idiotypic cascade in BALB/c mice. We recently prepared monoclonal anti-Ars antibodies from such Ab1' mice and showed that four distinct idiotopes (out of five) are shared by 36–65 (germline from strain A/J) and by CRI_A-like MAb from manipulated mice (19). In antiidiotype-treated BALB/c mice (Ab3 mice), the frequency of CRI_A⁺ anti-Ars precursors was roughly fivefold higher than in nonimmune or antigen-immunized BALB/c mice.

A much higher enhancement was observed in BALB/c mice pretreated with the MAb2 H8. Similar frequencies were found in spleen and bone marrow cells (data not shown), in agreement with the results obtained by others (13, 21).

Although our data concern only LPS-reactive B cells, we think that our results can be generalized. The main reason for this statement is that our data for nonimmune and immune mice are in strong agreement with those of Sigal (17). Sigal has used a radically different method, the splenic focus assay. In this method, developed by Klinman and Press (26), the differentiation of B cells into antibody-secreting cells is induced by carrier-primed radioresistant T helper cells. Finally, (C57BL/6 × BALB/c) F₁ mice were used that possess a high level of LPS-reactive splenic B cells (1 out of 2.5) and very similar frequencies were observed (see Fig. 3).

We are therefore faced with the following problem. The CRI_A idio type is encoded by one or a few germline genes in A/J strain. The BALB/c germline DNA does not seem to contain an identical counterpart (21). Nevertheless, the BALB/c potential immune repertoire contains B lymphocytes able to secrete anti-Ars antibodies that share some idiotopes with the A/J germline product 36–65. In fact, we have shown that the CRI_A that is recurrent in the A/J strain behaves as an idio type in the sense defined by J. Oudin (3), in the BALB/c strain.

Interestingly, the CRI_A repertoire is just a very small subset of the potential anti-Ars repertoire of A/J mice (0.5%). Similar frequencies are found in nonexpressor strains, like BALB/c. Upon immunization, there is selective enhancement of this repertoire in A/J but not in BALB/c mice. In some way, the A/J mice are programmed to express, in a dominant manner, the CRI_A idio type despite the fact that this idiotypic dominance is not found in the potential repertoire of nonimmune A/J mice. Such a programming can be induced in BALB/c mice by using the idiotypic cascade. It seems therefore tempting to suggest that the programming in A/J mice is also due to idiotypic regulatory pathways. It is hard to believe that antigen alone could be the inducer of the program. It is well-known that the Ars-positive CRI_A⁺ repertoire contains antibodies that have

higher affinity than 36-65 (27). The most obvious candidate as an inducer of the program leading to idiotypic dominance is a helper T cell displaying autoantiidiotypic receptors. It is important to note that such cells were first described in the Ars system by Woodland and Cantor (28). Since then, the occurrence of such T cells has been investigated and confirmed by the groups of Adorini et al. (29) and Gleason and Köhler (30). Concomitantly, the occurrence of suppressor T cells specific for the CRI_A^- repertoire in A/J and for the CRI_A^+ repertoire in BALB/c mice should be considered. Such idio-type-specific suppressor T cells have been found in the MOPC 460 system (31).

It is clear that available and potential repertoires are different. Available repertoires are not just those which are germline encoded. We would like to stress this point by taking examples from outside our work. Neonate repertoires seem to be restricted. These repertoires are similar in different individuals from a given strain, but different from one strain to the other. F_1 animals display another functional repertoire than the one expressed by the parental strains (32). Also, private idiotypes are not inherited in a Mendelian fashion. The phenomenon of original antigenic sin (33) and the deep maternal influence (34) on the available repertoire of the offspring should also be considered.

J. Cerny and R. Cronkhite (12) showed that some idiotopes seem to be helped, some are suppressed, and some are left autonomous. This underlines the danger of extrapolating too much from one system to another. At first glance, our conclusions for the arsonate system do not agree with those of recent results in the 4-hydroxy-3-nitrophenyl acetyl (NP) and the MOPC 460 systems (13, 25). These authors have compared the frequencies of precursors in spleen cells and in bone marrow depleted from B cells displaying surface Ig. Very similar frequencies were found. This could imply that, in fact, available repertoires are strictly germline encoded. There is no positive or negative selection during the release of precursors to the periphery. It is commonly believed that pre-B cells are devoid of surface receptors and are not subject to positive or negative signals from the environment. This statement could not be entirely correct insofar as some data indicate precommitment at the prereceptor B cell pool level and a higher frequency of the so-called pre-B cells (Ig^- cells) in immunized animals (35).

We think that the apparent paradox between the conclusions of different authors stems from our poor understanding of memory in the immune system. We have already stressed the importance of short-lived lymphocytes in secondary responses (36). Briefly, when primed mice are infused with tritiated thymidine before a boosting injection of TMV, a large proportion of plasma cells appearing in the spleen during a secondary response are labeled. The use of double transfer experiments allowed us to exclude thymidine reutilization as the explanation. Many plasma cells that appear in secondary responses are recruited from highly dividing B lymphocytes, devoid of detectable surface Ig at the time of thymidine infusion. We suggest that immune memory can be partly explained in the following way. During a primary response, some so-called memory cells are selected. These memory cells return to a microenvironment that allows the perpetuation of stem cells (bone marrow). We suggest that the memory cells transform into memory stem cells, displaying a much lower number of surface

receptors (in a way, these memory stem cells can ignore positive or negative signals).

The establishment and maintenance of a long-lived immune memory could be due to repeated asymmetric divisions of memory stem cells. At each division, the memory stem cell gives rise to a new memory stem cell and to a B memory lymphocyte that can migrate to the periphery. This B lymphocyte dies in a few days if antigen is absent but will be replaced continuously by the next division of memory stem cells. This suggestion implies that the so-called prereceptor B cell pool (Ig⁻ pool) is in fact heterogeneous and contains not only the real precursors but also lymphocytes that have been already "educated". This scheme will be developed in more detail in a subsequent paper.

Summary

We have shown that, by suitable idiotypic manipulation, BALB/c mice can express the major cross-reactive idotype (CRI) of A/J mice in response to azophenylarsonate (Ars). In order to know if the CRI_A idotype is present in the potential repertoire of BALB/c before any intentional selection, we used polyclonal activation in vitro and limiting dilution analysis. The readout was done with two monoclonal anti-CRI_A antibodies that recognize distinct idiotopes on a CRI_A⁺ A/J germline-encoded monoclonal antibody. We studied the frequency of CRI_A⁺ lipopolysaccharide (LPS)-reactive cells in the spleens of nonimmune and immune A/J mice and in the spleens of naive and manipulated (i.e., producing CRI_A⁺ antibodies) BALB/c mice. A/J and BALB/c naive individuals presented very high frequencies of Ars-specific B cells while the frequency of CRI_A⁺ B cells was only a minor subset (0.5%) of the total Ars-specific subset in the two strains. When A/J mice were immunized with Ars-keyhole limpet hemocyanin, a clear preferential expansion of the CRI_A⁺ minor subset of A/J mice was observed (100×). No such enhancement was observed in BALB/c mice similarly treated. Manipulated BALB/c mice presented a higher frequency of CRI_A⁺ anti-Ars B cells than naive or antigen-immunized BALB/c individuals.

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