IDIOTYPE PROFILE OF AN IMMUNE RESPONSE

I. Contrasts in Idiotypic Dominance between Primary and Secondary Responses and between IgM and IgG Plaque-forming Cells*

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Numerous antigens are now known that can induce antibodies bearing similar or identical variable region determinants (idiotypes) in all individuals of one or more strains of inbred mice (1–3). In many cases, the immune response to such antigens is of a highly restricted character, as judged by isoelectricfocusing (IEF)[1] of induced antibodies or other criteria. A curious feature is that the fraction of antibodies bearing a particular cross-reactive idiotype (Id) varies markedly between the known systems. Characteristic values indicating the extent of idiotypic dominance in the various systems can be tabulated (4). For example, ~30–35% of induced antibodies to group A streptococcal carbohydrate in A/J mice bear a common Id, designated A5A (5). In contrast, the response of BALB/c mice to the phosphorylcholine (PC) determinant, presented on T-independent (6) or T-dependent (7) carriers, is almost entirely (≥90%) dominated by antibodies bearing the T15 Id. Many of the factors that determine the extent of dominance of a paricular Id in a given response are obscure, although the clonal heterogeneity of B cells capable of responding to the epitope is likely to be important.

The enumeration of characteristic values of clonal dominance implies a certain degree of stability. However, situations are known in which the representation of particular Ids changes considerably during the course of a response or during a multiple immunization regimen. MacDonald and Nisonoff (8) reported that cross-reactive idiotypic specificities, present in the sera of individual rabbits early in the course of a hyperimmunization schedule with a *p*-azobenzoate conjugate, were replaced, at 2–4 mo, by a new set of cross-reactive specificities. A better-defined system, the NP-b Id present on anti-(4-hydroxy-3-nitrophenyl)-acetyl (NP) antibodies of C57BL/6 mice, has been analyzed by Mäkelä and Karjalainen (9) and Jack et al. (10) and Imanishi-Kari et al. (11). Here, primary anti-NP antibodies reacted with an

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[1] *Abbreviations used in this paper:* ABA, *p*-azobenzenearsonate; BL, bacterial levan; C, complement; C_H, heavy chain constant; CDA, complete Difco adjuvant H37 Ra; CGG, chicken gamma globulin; CRI, major cross-reactive idiotype; EBSS, Earle's balanced salt solution; ELISA, enzyme-linked immunosorbent assay; HB, *p*-hydroxybenzamidate; Id, idiotype; IEF, isoelectricfocusing; KLH, keyhole limpet hemocyanin; NP, (4-hydroxy-3-nitrophenyl)-acetyl; PBS, phosphate-buffered saline; PC, phosphorylchloline; PFC, plaque-forming cells; RAT, L-tyrosine p-azobenzenearsonate; SRC, sheep erythrocytes; T_h, T helper; TNP, trinitrophenyl(ated); V, variable; V_H, heavy chain variable; V_L, light chain variable.

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anti-Id serum to the extent of ≥85%, the proportion dropping with repeated immunization. This change correlated with temporal alterations in IEF patterns of specific antibodies obtained from mice at different times during hyperimmunization. Primary anti-NP antibodies display four spectrotypic bands (9) common to all C57BL/6 mice. The patterns, however, show an increasing dominance of distinctive unshared bands when individual hyperimmunized mice are compared. Karjalainen (12) has determined that the expression of a different Id, NP-a in BALB/c mice responding to NP-chicken gamma globulin (CGG), runs a parallel course and shows several other similarities to the NP-b system.

Our paper deals mainly with characterization of the primary response of A/J mice to the antigen *p*-azobenzenearsonate-keyhole limpet hemocyanin (ABA-KLH). The secondary and hyperimmune humoral responses of such mice include antibodies that bear a major cross-reactive Id (CRI), which has been extensively studied by Nisonoff et al. (13). The proportion of antibodies which are CRI^+ from individual mice varies between 20 and 70%. The mean value is in the range of 35–50%, based on indirect precipitation of anti-ABA antibodies with anti-CRI (14, 15). It has not previously been clear whether this level of idiotypic dominance was also characteristic of the primary response because, to our knowledge, this response had not been analyzed. We wished to determine if there was any apparently consistent difference in idiotypic preponderance between primary, secondary, and hyperimmune responses. In addition, it was of interest to compare the levels of the Id in IgM and IgG classes during the course of the response. Plaque-forming cell (PFC) methodology seemed most appropriate for these purposes. We report here that an interesting trend does indeed appear: the CRI is more dominant in the primary than in secondary or hyperimmune responses, and thus resembles the NP-b and NP-a systems in that respect. Furthermore, the kinetics of the primary response were consistent with the idea that IgG PFC originate from IgM PFC by isotype switching. However, it appeared that there was a clonal preference in the switching step, and that CRI^+ IgM PFC were preferentially switched to give rise to a striking Id dominance in early IgG PFC.

Materials and Methods

Mice. Female A/J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine and used at age 6–16 wk.

Antigens and Immunizations. ABA-KLH was prepared by dissolving 2 g KLH (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) in 50 ml borate-buffered saline (16), pH 9.1. 2 mmol of ABA diazonium salt in solution was added, with stirring, over a 15-min period while the pH was maintained at 9.1 with 4 N NaOH. The reaction was allowed to proceed on ice for 2 h, followed by dialysis against phosphate-buffered saline (PBS) and storage at −20°C. The ABA diazonium salt solution was prepared by dissolving 2 mmol (434 mg) *p*-arsanilic acid (Eastman Kodak Co., Rochester, N. Y.) in 6 ml of 1 N HCl at 0°C, followed by addition of 2 ml of 1 M $NaNO_2$ in saline in three aliquots over a 3-min period, and then 2 ml of H_2O at 0°C. ABA was coupled to CGG and human myeloma protein according to the method of Tabachnick and Sobotka (17). Trinitrophenylated CGG (TNP-CGG) and TNP-sheep erythrocytes (SRC) were prepared according to the method of Garvey et al. (16).

Conjugates were typically dissolved in PBS to 10 mg/ml or 5 mg/ml, emulsified in an equal volume of complete Difco adjuvant H37 Ra (CDA), and administered i.p. in 0.1- or 0.2-ml vol, as given elsewhere.

Anti-CRI Sera. Rabbit anti-CRI sera were produced according to the method of Ju et al. (18) by repeated immunization of rabbits with affinity-purified A/J anti-ABA antibodies from hyperimmune ascites. Antisera were rendered Id specific by passage over columns of Sepharose

4B coupled with normal A/J Ig [$(NH_4)_2SO_4$-precipitated proteins from A/J hyperimmune ascites induced by CDA alone]. Anti-CRI sera were judged to be Id specific by an enzyme-linked immunosorbent assay (ELISA) (19) analysis: the antisera that bound to CRI that was adsorbed to plastic microtiter dish wells was detected with protein A coupled with alkaline phosphatase. Binding was inhibited ≥94% in the presence of excess free CRI but not >10–15% by excess normal A/J Ig. A specificity analysis of the anti-CRI sera was also performed in the plaque assay (Results).

Hemolytic Plaque Assay. ABA was conjugated to SRC via an intermediate imidoester linkage as described by Isakson et al. (20). In this protocol, *p*-arsanilic acid was first diazotized and coupled to methyl *p*-hydroxybenzimidate (HB) (Pierce Chemical Co., Rockford, Ill.). The resulting compound was then reacted overnight with SRC. The ABA-HB-SRC prepared this way were shown in preliminary assays to disclose 5–10 times as many PFC as ABA-SRC prepared by direct diazotization.

A modification of the original Jerne plaque assay (21) was employed in these studies. A 0.5% solution of Bacto-Agar (Difco Laboratories, Detroit, Mich.) in Earle's balanced salt solution (EBSS), containing 400 μg/ml DEAE-dextran (Sigma Chemical Co., St. Louis, Mo.), was prepared and distributed in 0.5-ml aliquots into small tubes in a 46°C water bath. 50 μl of a 1:4 suspension of ABA-HB-SRC in PBS was then added along with 100 μl of washed splenic lymphocytes at an appropriate dilution in EBSS (to give 100–800 PFC/plate). 10 μl of goat anti-mouse IgM (N. L. Cappel Laboratories Inc., Cochranville, Pa.) was also added to some tubes for indirect PFC determination (i.e., to inhibit IgM PFC). For analysis of CRI content of PFC, 6–10 μl of rabbit anti-CRI was added to some tubes. Each mixture was then gently vortexed and spread in a circular motion on the surface of a plastic 13- × 100-mm petri dish (1001; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) to cover about two-thirds of the area, allowed to harden, and then incubated in a humid box at 37°C for 1 h. 2 ml of a 1:40 dilution of guinea pig complement (C) (Grand Island Biological Co., Grand Island, N. Y.) in cold EBSS was then layered over the agar. To develop IgG plaques, rabbit anti-mouse IgG (N. L. Cappel Laboratories Inc.) was added to the C solution at an appropriate dilution for optimal indirect PFC development (1:200–1:800). No indirect plaques developed without addition of this antiserum. Dishes were then further incubated for 2 h at 37°C, followed by counting of PFC.

The formula for the percent CRI^+ PFC used was: [1 − PFC (with anti-CRI)/PFC (without anti-CRI)] × 100% = the percent CRI^+ PFC. Nonspecific PFC, as determined by plaquing against unconjugated SRC, were always subtracted beforehand.

Hapten Inhibition of PFC. ABA-L-tyrosine (RAT) was prepared as described previously (22) and dissolved in EBSS in half-log concentration intervals. 3 ml of the indicated concentration of hapten solution was overlayed on the agar immediately after hardening in individual petri plates. After 1 h incubation at 37°C, guinea pig C and rabbit anti-mouse IgG were added directly to the hapten solution followed by 2 h of further incubation. EBSS alone was overlayed on control plates.

Hybridoma AK-2.2. The hybridoma used in the hapten inhibition studies was prepared according to the method of Oi and Herzenberg (23) by fusing NS-1 myeloma cells with spleen cells from an A/J mouse hyperimmunized with ABA-KLH. The spleen donor was given a single intravenous boost of 50 μg ABA-KLH 4 d before fusion. Hybridoma AK-2.2 (IgG,κ) was selected with hypoxanthine-aminopterin-thymidine medium, cloned twice by limiting-dilution, and grown in Dulbecco's modified Eagle's medium (DME) H21 with 10% fetal calf serum. It was CRI^+ by anti-CRI inhibition of PFC.

Statistical Analysis. The difference in levels of CRI in IgM and IgG PFC was compared for significance using the Student's *t* test for paired samples (24).

Results

Specificity and Titration of Anti-CRI. Analysis of the specificity of anti-CRI sera by ELISA has been described in Materials and Methods. Specificity was also checked in the plaque assay, and titrations were performed to determine the optimal quantity of anti-CRI to be used per plate. Such a study for one anti-Id serum, designated 3c, on

primary and secondary PFC, is shown in Fig. 1. The titration results are essentially as expected. Inhibition of PFC increases to a plateau level with increasing anti-CRI. The plateau is typically not entirely flat, but shows a slight upward slope, probably a result of nonspecific inhibitory activity of the antiserum. Because it was observed that some normal rabbit sera could inhibit plaques at >10 μl per plate, only those anti-CRI giving maximal inhibition at ≤10 μl were used. Based on these results, 3c was routinely used at 10 μl per plate. At this concentration, 3c caused little inhibition of A/J primary anti-TNP and secondary anti-SRC PFC (Fig. 1). Another anti-CRI serum used in some of the experiments given here, IIb, gave similar satisfactory results (data not shown) and was used at 6 μl per plate.

Also shown in Fig. 1 A is a titration with a rabbit antiserum prepared against a CRI^+ hybridoma antibody, R16.7 (25), kindly supplied by Dr. A. Nisonoff, Brandeis University, Waltham, Mass. This antiserum gave substantial and almost equivalent inhibition of the direct and indirect primary PFC. CRI^+ IgM PFC are readily detectable in both the A/J response to ABA-KLH and to the T independent antigen, ABA-*Brucella abortus* (26).

Kinetics of the A/J Primary Response to ABA-KLH. The kinetics of the primary response of A/J mice to a single injection of 500 μg ABA-KLH, in terms of total PFC per spleen and CRI^+ proportion of these, are shown in Fig. 2. The results of two separate experiments are given. Some of the differences between the two studies may be attributable to differences in immunization protocol (see legend to Fig. 2). In spite of this, some interesting points of concordance are apparent.

First, the IgM PFC show a biphasic course. Such oscillatory kinetics have been reported in other responses to different antigens (27, 28). The situation with respect to IgG PFC is less certain (compare Fig. 2, studies 1 A and 2 A). Both direct and indirect PFC per spleen are low on day 18 (Table I, line 2) and peaks were not detected after day 13.

A second point of particular interest is the very high dominance of the CRI in both the IgM and IgG PFC populations which surpass 80% CRI^+ in the course of the primary response (Fig. 2). This finding did not correlate well with previously reported lower values for hyperimmune antibodies (14, 15), and there existed some uncertainty

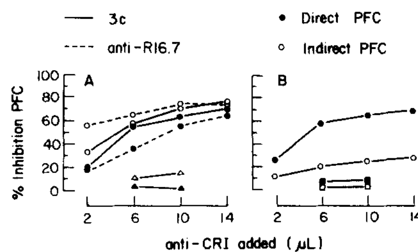

FIG. 1. An example of a specificity and tiration analysis of two anti-CRI sera. Two A/J mice received a single injection each of 500 μg ABA-KLH in CDA and were plaqued 12 d later (A). Control mice (triangles) were injected with 100 μg TNP-CGG and plaqued against TNP-SRC. Alternatively, mice received a priming injection and were boosted 5 wk later and plaqued at day 10 postchallenge (B). Control mice (squares) were undergoing a secondary anti-SRC response. Two anti-CRI sera were checked with primary PFC: 3c, a rabbit antiserum to pooled A/J anti-ABA antibodies; and anti-R16.7, a rabbit antiserum to a CRI^+ hybridoma antibody (see text). Solid symbols, direct PFC; open symbols, indirect PFC.

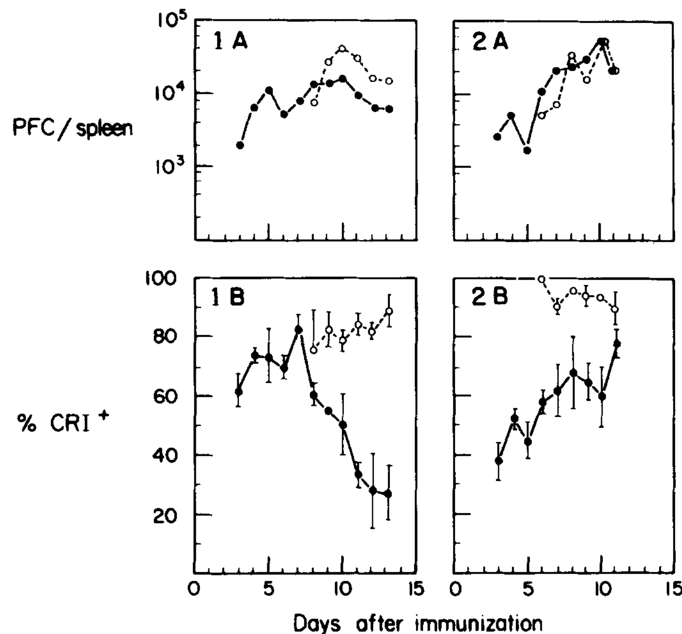

FIG. 2. Two kinetic studies (1 A and B; 2 A and B) of the primary responses of A/J mice to ABA-KLH. (●) direct PFC; (○) indirect PFC. A large group of mice received a single injection each of 500 μg ABA-KLH in PBS and emulsified 1:1 in CDA in a total vol of 0.1 ml (study 1) or 0.2 ml (study 2) on day 0. Mean values for four mice (study 1) or three mice (study 2) per day are given. IgG PFC are shown beginning on their first day of appearance. A number of control mice were injected with 500 μg KLH in CDA and checked for anti-ABA PFC; no more than 700 PFC/spleen were ever detected. In studies 1 A and 2 A, geometric mean PFC/spleen are given; SE limits are omitted for clarity. Mean SEM for study 1 A = 1.33; for study 2 A = 1.28 (geometric mean ×/÷ 1 SEM). In studies 1 B and 2 B, arithmetic mean of the percent CRI⁺ PFC ± 1 SEM is indicated.

TABLE I

PFC and Id Profile at Various Times in Anti-ABA Responses

	Immunization protocol		Day§	PFC/spleen*			
	Prime‡	Challenge		Direct	CRI⁺	Indirect	CRI⁺
					%		%
1	—	A-K‖	10	29,823(1.31)	55	46,838(1.30)	87
2	—	A-K	18	2,043(1.22)	30	3,247(1.28)	56
3	A-K	A-K	3	7,735(1.13)	68	27,752(1.22)	36
4	A-K	A-K	10	249,319(1.37)	64	423,185(1.24)	34
5	A-K¶	A-K (PBS)	10	124,590(1.73)	70	217,262(1.18)	39
6	—	A-CGG	10	2,607(1.11)	60	5,063(1.06)	72
7	A-CGG	A-CGG	10	105,342(1.48)	55	439,880(1.35)	41

* Data presented as geometric mean ×/÷ SEM. All results are pooled from two or more experiments.
‡ 500 μg antigen (ABA-KLH [A-K]) in CDA injected i.p. Challenge was 5 wk after priming.
§ Postchallenge.
‖ From Fig. 2, studies 1 A and 2 A.
¶ Hyperimmunized, five or more injections.

as to whether the difference was a result of the differing methodologies employed. To investigate this point, a group of A/J mice that had been hyperimmunized with ABA-KLH in CDA (five or more injections) was boosted with 50 μg ABA-KLH in PBS and plaqued 7 d later. The results (Table I, line 5) showed an approximate four- to fivefold increase in direct and indirect PFC over the primary response. More importantly, the IgG PFC were only 39% CRI^+. IgM PFC showed a much higher Id content— ~70%. If analyzed serologically, the overall CRI^+ percentage would undoubtedly be dictated mainly by the larger quantity of IgG present. A clearcut idiotypic maturation had thus occured in the IgG PFC population and this resulted in good agreement between the present and previous data (14, 15). It should be pointed out (Table I, line 4) that animals undergoing a secondary response, induced 5 wk after an initial injection of ABA-KLH with the same antigen and checked on day 10, showed an Id profile like that of hyperimmune animals (IgG PFC only 34% CRI^+). It thus seemed likely that the extreme Id dominance in the primary response was real.

A third point worthy of note in the kinetic data is that the Id dominance of the CRI does not appear to be quantitatively equal between IgM and IgG PFC, even for the earliest-appearing IgG PFC. A statistical analysis of IgM and IgG PFC in individual mice on the first day of appearance of IgG PFC (day 8 in study 1 and day 6 in study 2 of Fig. 2) showed that IgG PFC were significantly higher than IgM PFC in terms of proportion of CRI^+ PFC ($P < 0.025$ in study 1; $P < 0.001$ in study 2 of Fig. 2). This is strikingly illustrated on day 6, Fig. 2B, where 100% of the IgG PFC were CRI^+, whereas the mean for IgM PFC was only ~60%. This result, with earliest IgG PFC being 100% CRI^+, was also seen in another study (data not shown). The first indirect PFC in study 1 were only 76% CRI^+. However, this value increased to 90% by day 13, i.e., a slow, steady increase occurred. During this time, though, the IgM PFC decreased steadily in terms of CRI content from 62 to <30% (Fig. 1B). A mechanism that could explain this data is that CRI^+ IgM PFC were being preferentially switched to CRI^+ IgG PFC.

Other Time Points and Different Carriers. After peaking on day 10, both direct and indirect PFC declined steadily as indicated by the data for day 18 of the primary response (Table I, line 2). The percentage of IgG PFC which were CRI^+ also had dropped from a peak of 80–100% to only 56%. It was of interest to determine whether the Id made a transient recovery in early secondary IgG PFC. This apparently was not the case because the Id profile at day 3 already resembled that at day 10 in the secondary or hyperimmune responses, i.e., IgG PFC at 30–40% CRI^+ with IgM PFC higher at ~70% CRI^+ (Table I, line 3).

The carrier protein KLH, coupled with ABA, was not unique in inducing a strong Id dominance in the primary response. ABA-CGG gave a comparable pattern including the idiotypic maturation of IgG PFC (Table I, lines 6 and 7). The clonal dominance does not appear as great in this case, but a complete kinetic analysis was not done and so this may not represent the CRI peak. Another T dependent antigen, ABA coupled to a human myeloma protein, and administered in CDA, did not induce a detectable primary response.

Evaluation of Relative Heterogeneities and Avidities by Hapten Inhibition of PFC. Inhibition

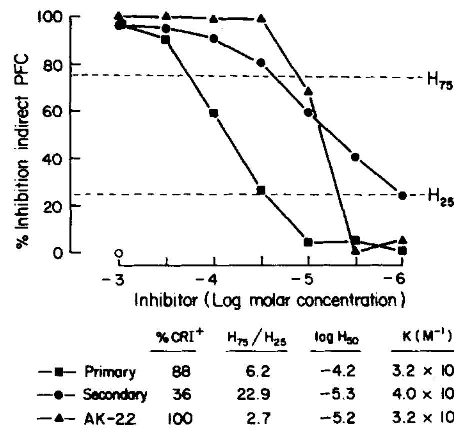

FIG. 3. Data derived by hapten inhibition of indirect PFC. Primary IgG PFC were checked on day 10 after an i.p. injection of 500 μg ABA-KLH and secondary IgG PFC on the same day after a boost of 500 μg ABA-KLH which was 5 wk after priming (same quantity of the same antigen). Results for six spleens are pooled per point. Plaquing was as described in the Materials and Methods section, except that the agar was overlayed with 3 ml of inhibitor (RAT in EBSS) of the indicated concentration. AK-2.2 are hybridoma cells secreting IgG anti-ABA antibodies (CRI^+ by anti-CRI inhibition). The single open circle at 10^{-3} M RAT indicates the absence of inhibition of secondary anti-SRC PFC. Median association constant (K) was calculated using the formula $K = 2/H_{50}$ (19).

of PFC with free hapten can provide estimates of avidity and heterogeneity at the cellular level (29, 30). This technique was used to determine if the primary response represents a more restricted population of antibody-secreting cells than the secondary response, as suggested by anti-Id inhibition. Relative heterogeneity in these experiments was based on the slope of the inhibition line between H_{75} and H_{25} (molar concentration of inhibitor causing 75% and 25% inhibition of PFC, respectively). It can be seen that the data points describe a nearly straight line in this region with the primary response giving a steeper slope (lower H_{75}/H_{25}) than the secondary response (Fig. 3). For purposes of comparison, and also to verify that this method probably was detecting avidity distribution, AK-2.2 hybridoma cells, secreting IgG anti-ABA, were plaqued. This hybridoma was derived by fusion of A/J anti-ABA-KLH hyperimmune spleen cells and NS-1 myeloma cells, and is CRI^+ by anti-Id inhibition of PFC, although it has not unequivocally been shown to express the major idiotope(s) associated with the CRI. A steep slope was obtained with H_{75}/H_{25} equal to 2.7. The data thus suggest that primary IgG PFC approach a monoclonal population in terms of avidity distribution, with secondary PFC being considerably more heterogeneous.

The data are also informative as to avidity changes. Using the simple relation worked out by Jerne et al. (21), $K = 2/H_{50}$, where K is the median association constant, the values given in Fig. 3 were obtained. The results suggest that a 10-fold increase in avidity occurred between the primary and the secondary response in IgG PFC. (IgM PFC were not tested by this method because a very high concentration of free hapten was required to give any inhibition.) The association constant for secondary PFC which was derived here is surprisingly close to that determined by Kapsalis et al. (31) for the ABA system, using hyperimmune antibodies and essentially the same ligand, RAT, in equilibrium dialysis. Because the primary PFC were 88%

CRI$^+$ whereas the secondary were only 36% CRI$^+$, idiotypic maturation occurred concurrently with avidity maturation in this PFC population, but they showed an inverse correlation (CRI declined with increasing avidity).

Discussion

The A/J CRI is one of the best characterized of the CRI systems. Studies that utilized mainly radioimmune competition assays, with affinity-purified hyperimmune serum or ascitic anti-ABA antibodies, have revealed numerous interesting features (13). The CRI shows genetic linkage to heavy chain constant region (C_H) allotype (32) and also to an Ly-3 polymorphism which is closely linked to the κ light chain locus (33). It is found on 20–70% of hyperimmune anti-ABA antibodies in individual mice and is associated mostly with the IgG_1 class. On IEF, bands with isoelectric points (pI) of 6.7 and 6.9 were highly enriched for the Id. Analysis of serum antibodies suggested a single heavy chain variable region (V_H) sequence in CRI$^+$ molecules (34). More recent analysis of CRI$^+$ hybridoma antibodies, however, demonstrated considerable heterogeneity, both in the light chain variable region (V_L) and V_H (35, 36). CRI$^+$ hybridomas that secrete IgM or any of the subclasses of IgG have now been reported (25), and serological studies revealed both cross-reactive (public) and individual (private) Id determinants on many of them (25, 36). It is likely that serum antibodies also express this heterogeneity (37).

The present studies were undertaken to characterize the primary response of A/J mice to ABA-KLH, which was not detected in earlier studies (38). Current success may be a result, at least in part, of the use of the more sensitive ABA imidoester-coupled SRC (20) instead of the directly diazo-coupled indicator cells used formerly in the plaque assay. However, the possibility has not been ruled out that minor variations in properties of the antigen preparation, such as extent of ABA substitution or degree of aggregation of the KLH, might dramatically influence the outcome of a primary immunization. Such minor variations may be of particular importance in responses dominated by a major Id. In support of this are the results reported by Claflin et al. (39) that PC-KLH induced a fairly weak, exclusively IgM PFC response in BALB/c mice, even after repeated immunizations. In contrast, Bottomly et al. (7), using the same mouse strain and antigen in adoptive-transfer experiments, obtained much stronger responses, which included IgG PFC.

We used PFC methodology throughout these experiments, with inhibition by anti-CRI to determine the proportion of plaques which were CRI$^+$. It was important first to titrate the anti-Id to ensure that inhibition was on a plateau level for both primary and secondary PFC (Fig. 1). Thus, all CRI$^+$ PFC should have been detected and the differences in Id content which were observed were not likely a result of changes in other properties, such as secretion rate. Inhibition with an antiserum prepared against a CRI$^+$ hybridoma antibody, R16.7, was also checked with primary PFC. It gave inhibition of both IgM and IgG PFC comparable with that obtained with the rabbit anti-CRI. Although this result seems surprising, it should be noted that R16.7 has somewhat unusual properties compared with other CRI$^+$ hybridoma antibodies (25): it inhibits the binding of rabbit anti-CRI to labeled CRI (pooled A/J anti-ABA antibodies) almost as well as does unlabeled CRI, and in excess causes almost 100% inhibition of binding in this system. Most other hybridoma antibodies caused only

fractional degrees of inhibition (25). On the basis of these observations, it has been suggested (25) that pooled A/J anti-ABA antibody may contain multiple distinct CRI determinants, and that R16.7 may carry most of these on a single molecule. If so, it might be expected that rabbit anti-CRI and anti-R16.7 would have similar inhibitory properties.

Our data disclose a regular pattern of temporal variation in CRI dominance between the primary and the secondary or hyperimmune responses of A/J mice to the ABA epitope. As in the NP-a and NP-b systems (9, 12), the major Id is more dominant in the early than in the later responses, at least among IgG PFC. A day-by-day kinetic analysis of Id dominance in the primary response and at selected time points in the secondary and hyperimmune responses revealed the following scenario. At day 3 in the primary response, IgM PFC were 40–60% CRI$^+$ and this percentage increased to 70–85% by day 7. From then until day 11 or 13, the CRI content of IgM PFC either remained fairly stable at this level or decreased sharply to ~30%, depending on the immunization protocol. IgG PFC first appeared at day 6 or 8 and were significantly higher in CRI$^+$ proportion than IgM PFC on that day. From a peak of 85–100% CRI$^+$, IgG PFC had declined to 50–60% CRI$^+$ by day 18. Although increased in numbers, IgG PFC were only 30–40% CRI$^+$ in the secondary (day 3 or 10) or hyperimmune (day 10) responses. This idiotypic maturation in indirect PFC correlated inversely with an approximate 10-fold increase in median binding avidity.

The same type of idiotypic maturation found here, and known to occur with NP-b and NP-a, may be characteristic of some other systems as well. For example, BALB/c mice respond to the T-independent antigen bacterial levan (BL) with production of inulin-binding antibodies, the great majority of which bear an Id shared with the U61 myeloma protein (40). No apparent maturation of this response is seen, by IEF analysis, on repeated immunization with BL (41). However, antibodies produced by mice in response to inulin-KLH clearly become more heterogenous after multiple immunizations (see Fig. 3 of [41]); primary, but not secondary antibodies, are dominated by five invariant shared bands. An Id analysis might show that U61 Id is similar to CRI, NP-b, and NP-a with respect to the dynamics of Id dominance during the course of the response. Many other Id, however, are unlikely to fall in the same category. As one example, it was reported recently (42) that a major Id present on murine antibodies to hen egg-white lysozyme from hyperimmune animals was not present on primary antibodies to the same antigen. Furthermore, whereas the similarity of the CRI and NP-b systems has been stressed, the two are likely to show some major differences. In particular, the λ to κ light chain shift seen with NP-b (9) is not likely to occur in the CRI system, because the latter is dependent on κ chains for its expression (33), at least on antibodies.

The hapten-inhibition data tend to support conclusions derivable from the anti-Id analysis concerning relative heterogeneity of primary and secondary PFC responses. They also indicate that secondary IgG PFC show a median avidity which is ~10-fold higher than that of primary IgG PFC. Although the association constant calculated for secondary IgG PFC agreed quite well with that derived by Kapsalis et al. (31) who used equilibrium dialysis, the comparative data introduce a problem. They reported that the avidity of CRI$^+$ antibodies in the hyperimmune response was only slightly less than the mean avidity of hyperimmune antibodies in general, whereas the data in Fig. 3 indicate that primary IgG PFC, which are almost entirely CRI$^+$,

have a median avidity 10 times lower than secondary PFC. To reconcile these observations, one would have to postulate that avidity maturation occured within the CRI$^+$ population, and this would require a heterogeneity of binding sites in that population. Such a postulate is tenable in view of the evidence for structural heterogeneity of CRI$^+$ molecules mentioned earlier, but our findings are inconclusive inasmuch as the CRI$^-$ population in hyperimmune sera could account for the observed avidity differences. Moreover, because objections to the use of hapten inhibition of PFC for avidity estimates have been raised on both theoretical (43) and practical (44) grounds, it would be necessary to directly compare primary and secondary antibodies by equilibrium dialysis before reaching any firm conclusions.

One of the most interesting findings in our studies was the difference in dominance of the Id in IgM and IgG classes. Except for the period in the primary response when substantial numbers of IgM PFC were presumably switching to IgG, the fraction of CRI$^+$ IgM PFC remained high. In contrast, the proportion of Id$^+$ PFC of the IgG class decreased considerably after peaking in the early primary response. It is noteworthy that this course of events parallels that which has been observed elsewhere in several studies of avidity maturation (45, 46): a clearcut avidity maturation (increase) in IgG but little, if any, in IgM. Assuming that both IgM and IgG PFC populations express comparable variable (V)-region diversity, the mechanisms, currently unknown, for the differential avidity maturation (if such is indeed occurring here) may be equivalent to those responsible for the differential Id maturation, especially if these have a cause-and-effect relationship. That is, low-avidity CRI$^+$ PFC may be eclipsed, in the course of the response, because of the more rapid expansion of higher-avidity CRI$^-$ PFC among IgG, but not IgM, PFC. Although some avidity maturation might occur within the CRI$^+$ population, this may be more limited than that possible in the Id$^-$ population. One would predict, on this basis, that major Id that have no avidity advantage, such as the anti-ABA CRI, would peak early in the response, whereas Id with high avidity for antigen might become increasingly dominant as the response progresses. A late-appearing Id has recently been described (42), but no avidity data are available as yet. As an alternative to a purely selective mechanism, one might postulate that IgG PFC contain more V-region diversity than IgM PFC, which would require somatic generation of diversity during or after the isotype switch. Although such a mechanism would readily explain the differential maturation phenomena, there is no solid evidence to support it.

The kinetic data on the primary response suggest that a preferential isotype switch, based on idiotype, is effected, and this raises the question of T cell involvement. The role of T cells in promoting the IgM to IgG shift is still controversial, but the following two points seem well established: T cells may not be absolutely required for inducing the switch in at least some B cells, because this shift has been observed in cultures of "purified" B cells activated by lipopolysaccharide (47, 48). However, T cells can at least greatly facilitate the switch (49). The theory that we currently favor for the high Id dominance in the primary response, as well as the preferential switching, is that CRI$^+$ IgM-secreting B cells receive more T cell help than CRI$^-$ ones, and, thus, they differentiate faster. The extra help might derive from an Id-specific T helper (T_h) cell for which we have preliminary evidence (our unpublished data). There is ample evidence, in several systems, for the existence of naturally occurring T_h cells that recognize autologous immunoglobulin determinants, including idiotypic ones (50).

Woodland and Cantor (51) have already demonstrated the requirement, in the A/J CRI system, for an Id-specific, CRI complementary T_h cell to activate CRI^+ B memory cells in adoptive secondary responses. This same cell may be causing the effects described here in the primary response. However, the possibility has not been excluded that anti-ABA B cell precursors in unprimed A/J mice might be mainly CRI^+, thus accounting for the Id dominance in the primary response, but not the selective isotype switching. These questions are currently being investigated.

Summary

The primary response of A/J mice to *p*-azobenzenearsonate-keyhole limpet hemocyanin (ABA-KLH) was investigated. A day-by-day analysis at the plaque-forming cell (PFC) level was performed, with inhibition by anti-cross-reactive idiotype (CRI) serum to determine percentage of CRI^+ PFC. A regular pattern in the dynamics of Id (idiotype) dominance was observed. Just as in the NP-b and NP-a systems (9, 12), the major Id (CRI) is more dominant in primary than in secondary or hyperimmune responses. This trend is more apparent in IgG PFC which are generally 80–95% CRI^+ at day 10 in the primary response but only 30–40% CRI^+ at day 10 in secondary or hyperimmune responses. A somewhat different pattern is seen with IgM PFC. These may reach a peak of 85% CRI^+ in the primary response, but secondary or hyperimmune IgM PFC, which are lower in numbers than IgG PFC, remain high in CRI content at ~70%. The PFC data on extent of id dominance in secondary or hyperimmune responses is fully compatible with previously reported serological data by others. Analysis of IgG PFC by hapten inhibition indicated that heterogeneity was in the order secondary PFC > primary PFC > hybridoma AK-2.2 PFC with H_{75}/H_{25} values of 22.9, 6.2, and 2.7, respectively; where H_{75} and H_{25} are the hapten concentrations required to give 75% and 25% of inhibition of PFC, respectively. Hapten inhibition data also suggested that secondary IgG PFC were 10 times higher in median binding avidity for ABA-L-tyrosine than primary IgG PFC.

The kinetic analysis strongly indicated that CRI^+ IgM PFC were preferentially switched to IgG PFC in the primary response. In both studies, the CRI content of the earliest-appearing IgG PFC was significantly higher than that of IgM PFC on that day. For example, in one case IgM PFC were 60% CRI^+ on day 6 whereas IgG PFC were 100% CRI^+. The high Id dominance and selective isotype switching may have either a B or a T cell basis.

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