

## Sudden appearance of anti-protein IgG1-forming cell precursors early during primary immunization

(B cells/immunoglobulin variable region genes/somatic mutation/isotype switch)

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Contributed by G. J. V. Nossal, March 23, 1989

**ABSTRACT** The anti-keyhole limpet hemocyanin (KLH) B-cell repertoire of unimmunized adult mice was examined by culture of splenocytes (generally 100–3000) at limiting dilution. Cells were polyclonally stimulated with *Escherichia coli* lipopolysaccharide (LPS) and an interleukin-4-containing lymphokine mixture in the presence of 3T3 fibroblast filler cells. After 7 days of culture, supernatants were examined for their content of anti-KLH IgM and IgG1 antibody by an enzyme-linked immunosorbent assay (ELISA). Parallel cultures of smaller numbers (generally 1–15) of splenocytes were examined to determine the cloning efficiency of B cells in terms of total IgM and IgG1 production. Whereas one spleen cell in 370 produced clones secreting anti-KLH IgM, only 1% of these produced IgG1 that could bind to KLH, despite the fact that about half of the clones switched to IgG1 production with these stimuli. In mice immunized with KLH, this situation did not change until day 5, when there was a sudden, explosive emergence of B cells that could form clones secreting anti-KLH IgG1. The absolute number of such cells in the spleen was found to rise by a factor of 350 between days 3 and 7 of immunization. Moreover, the median amount of IgG1 antibody formed per clone and binding to KLH also rose markedly. In contrast, neither the numbers nor the median KLH-binding antibody content of anti-KLH IgM clones changed significantly after immunization. The results show that the repertoire of anti-protein B cells detected through IgM formation in ELISA consists chiefly of cells producing antibody of low avidity and of doubtful *in vivo* significance. Assuming that the small proportion of these cells making antibody that is of sufficient avidity to bind as the IgG1 isotype are the ancestors of the many such cells found on day 7 of the primary immune response, one would have to postulate a very high recruitment and/or division rate to account for the increase in numbers and avidity that occurs. It is possible that the anti-KLH IgG1 precursors that suddenly emerge are the results of early variable region gene (*V*) mutations in B cells. Moreover, it is not excluded that they represent products of a subset of B cells different from those that give rise to the primary *in vitro* anti-KLH IgM response. The findings have implications for theories of B-cell tolerance.

When the preimmune B-cell repertoire of murine spleen with antibody-forming potential against autologous intracellular structures was recently examined in our laboratory (1), it was noted that more than 3% of the repertoire was autoreactive when using polyclonal stimulation of IgM production as the test. However, when an interleukin 4-containing lymphokine mixture was used to induce nearly half of these cells to switch to IgG1 production, the resultant bivalent antibody could no longer bind to the target cells in an enzyme-linked immunosorbent assay (ELISA) in 99.99% of cases. This indicated that the number of high-affinity anti-self B cells must be very low.

Given the considerable literature that exists on repertoire analysis using IgM-secreting clones (e.g., refs. 2–8), it seemed important to determine whether the preimmune repertoire to a typical protein antigen displayed similar features—i.e., capacity to bind as an IgM, but inactivity as an IgG1. Furthermore, we asked whether the repertoire generated during an *in vivo* primary immune response could be explained simply through a clonal expansion of some of the preimmune cells, or not. These questions have been made more cogent through recent work of Linton *et al.* (9). They have produced evidence (i) that a secondary immune response depends not on the progeny of the B cells that cause primary antibody production but on a separate B-cell subset, and (ii) that the members of this subset pass through a window-of-tolerance susceptibility shortly after being stimulated by antigen, presumably in order to silence such cells as undergo variable region gene (*V*) mutation, fortuitously leading to high-affinity anti-self reactivity.

Accordingly, we stimulated murine splenocytes at limiting dilution with *Escherichia coli* lipopolysaccharide (LPS) and a supernatant from stimulated EL-4 thymoma cells (EL-BGDF, a lymphokine mixture including B-cell growth and differentiation factors) and searched for anti-keyhole limpet hemocyanin (KLH) clones. Many anti-KLH IgM-formers were encountered, and half of these could be induced to switch to IgG1 formation, but in most cases this IgG1 failed to bind to KLH. When mice were immunized with KLH, B cells capable of forming anti-KLH IgG1 clones after *in vitro* stimulation suddenly appeared on day 5 after immunization, and their numbers rose rapidly until day 7. Furthermore, this population included a proportion of clones of much higher antigen-binding capacity than that of the preimmune repertoire. The possibility exists that these are the first cases of cells selected for higher affinity to antigen following immunoglobulin *V* gene hypermutation.

### MATERIALS AND METHODS

**Animals, Antigen, and Immunization.** Specific pathogen-free male CBA/CaH WEHI mice 6 to 10 weeks old were used. Some were immunized intraperitoneally with 100  $\mu$ g of alum-precipitated KLH and 10<sup>9</sup> *Bordetella pertussis* organisms (Commonwealth Serum Laboratories, Melbourne, Australia).

**Cell Preparation and Culture.** Spleen cell suspensions were prepared as described (10, 11) including erythrocyte and dead-cell removal steps. Flat-bottom, 96-well microtiter trays (Disposable Products, Adelaide, Australia) were used for the 150- $\mu$ l cultures. Up to 5000 cells per well (usually 100–3000 for anti-KLH determinations and 1–15 for total immu-

Abbreviations: AFCP, antibody-forming cell precursor(s); EL4-BGDF, a lymphokine mixture, including B-cell growth and differentiation factors, present in a thymoma culture supernatant; KLH, keyhole limpet hemocyanin; LPS, *E. coli* lipopolysaccharide; *V*, variable region gene.

noglobulin synthesis studies) were cultured in RPMI 1640 medium supplemented with 5–10% (vol/vol) fetal calf serum and 100  $\mu$ M 2-mercaptoethanol in the presence of 5000 BALB/c 3T3 fibroblast cells (Commonwealth Serum Laboratories). Polyclonal mitogen LPS B from *E. coli* 0111:B4 (Difco) was used at 75  $\mu$ g/ml together with 5% (vol/vol) of a 5 $\times$ -concentrated conditioned medium from concanavalin A-stimulated thymoma EL4 (EL4-BGDF), which contains interleukin 4 and other B-cell growth and differentiation factors. Cultures were incubated at 37°C in an atmosphere of humidified air containing 10% CO<sub>2</sub> for 7 days. Normally, 60 replicate cultures of each of three or four different input cell numbers were studied, two spleens being pooled for each observation.

**Assessment of Antibody Production.** Antibody formation was assessed by using a sensitive ELISA procedure as described (12). An appropriate dilution of supernatant from each well was individually transferred into the wells of a U-bottom, 96-well, poly(vinyl chloride) tray (Dynatech) pre-coated with affinity-purified sheep anti-mouse immunoglobulin (Silenus Laboratories, Dandenong, Australia) at 4  $\mu$ g/ml, or, for antigen-specific assays, 10  $\mu$ g of KLH per ml, for a final volume of 50  $\mu$ l. The diluent for the assay was mouse tonicity phosphate-buffered saline (0.02 M, pH 7.2), containing 0.3% skim milk powder, 0.05% Tween 20, and 1% (vol/vol) newborn bovine serum. The plates were kept at room temperature (RT) overnight and washed extensively before the addition of the horseradish peroxidase-coupled second reagent, a goat antibody with specificity for either mouse IgM or IgG1 isotypes (Southern Biotechnology Associates, Birmingham, AL), for a further 4 hr at RT. The plates were again washed extensively before the addition of 100  $\mu$ l of the enzyme substrate 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) at 0.55  $\mu$ g/ml in 0.1 M citric acid with 0.1% H<sub>2</sub>O<sub>2</sub>. Absorbance was read 1 hr later by using a Titertek Multiskan ML (Flow Laboratories) at 414 nm, with a reference wavelength of 492 nm.

**ELISA Analysis.** Computerized analysis of the ELISA was performed with software developed by A. P. Kyne at The Walter and Eliza Hall Institute. Duplicate, serial 1:1 dilutions of the myeloma proteins MOPC 104E (IgM) and MOPC 21 (IgG1) on each test plate were used to standardize the assays. A cubic spline curve-fitting technique was used to fit a curve through all points of the standard curves, and the antibody content of individual wells was computed from this. Poisson analysis (13) was used to determine the frequency of immunoglobulin-secreting precursors by using a maximal-likelihood estimator for linear regression analysis (14).

## RESULTS

**Both IgM and IgG1 Anti-KLH Clones Can Be Detected by Limiting Dilution Culture and Obey Poisson Statistics.** Mice were injected with KLH, administered a booster, rested for several months, administered a booster again, and killed 3 weeks later. From 125 to 1000 cells were cultured in the presence of LPS, EL4-BGDF, and 3T3 filler cells, and supernatants were examined for IgM and IgG1 anti-KLH antibody (Fig. 1). The logarithm of the fraction of negative wells was linearly related to the number of spleen cells per culture in both cases, indicating that only one cell type—i.e., a clonable anti-KLH antibody-forming cell precursor(s) (AFCP) was limiting in the assay. The hyperimmune mice showed  $40.8 \pm 7.5$  IgM AFCP and  $2.7 \pm 1$  IgG1 AFCP per  $10^4$  splenocytes.

**Cloning Efficiency of B Cells in Terms of IgM and IgG1 Production.** In each experiment, small numbers of spleen cells (typically 1–15) were cultured with LPS, EL4-BGDF, and filler cells to determine the cloning efficiency after polyclonal stimulation as assessed by IgM and IgG1 antibody

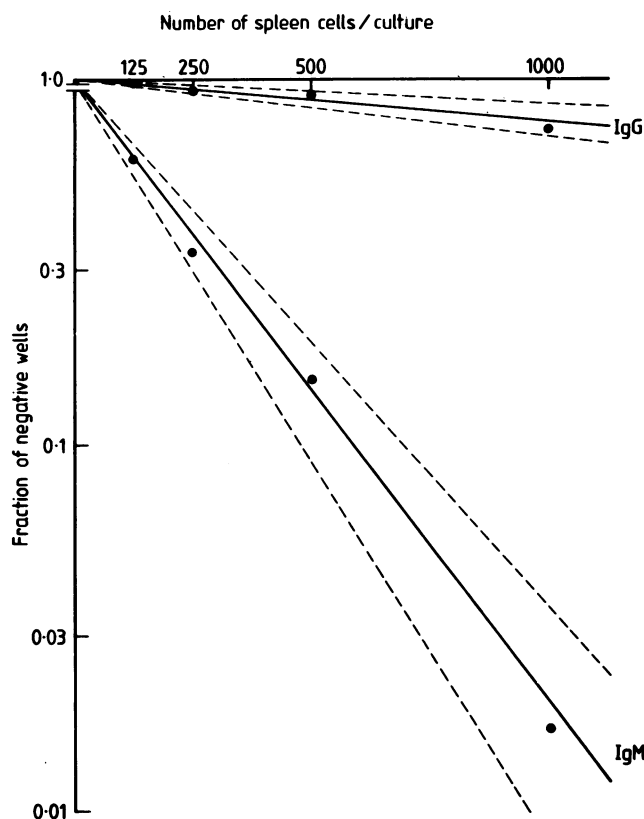


FIG. 1. Limiting-dilution analysis of anti-KLH AFCP among spleen cells from mice hyperimmunized against KLH. Cultures contained 125–1000 cells. Dashed lines represent 95% confidence limits.

formation, regardless of specificity. For this purpose, a polyvalent anti-murine Ig antibody was used as the capture layer in the ELISA. It was noted that the mean cloning efficiency did not vary significantly with immunization status, though considerable interexperiment variation was encountered, perhaps in part because this lengthy series of experiments spanned nearly 2 years. In fact,  $23.5 \pm 16.4\%$  of splenocytes formed IgM and  $13.0 \pm 8.2\%$  formed IgG1. On the assumption that approximately half of the splenocytes were B cells, this indicated that nearly half of the B cells formed IgM and about half of them switched to IgG1 production, consistent with previous experience (1, 15).

**Anti-KLH AFCP Among Unimmunized and KLH-Immunized Splenocyte Populations.** Spleen cells from unimmunized mice showed  $27 \pm 15$  cells per  $10^4$  splenocytes that could be stimulated to form clones producing anti-KLH IgM. Thus, the representation of anti-KLH clonotypes by this measure was 1 in 370 splenocytes or approximately 1 in 185 B cells. With the cloning efficiency of 23.5% splenocytes considered, the representation of anti-KLH IgM-forming AFCP in the preimmune repertoire is 1 in 87 clonable IgM-forming B cells. However, when supernatants were examined for anti-KLH IgG1, very few positives were found, and those that were encountered showed low optical density in the ELISA. In fact, only  $0.27 \pm 0.13$  anti-KLH IgG1 AFCP were detected per  $10^4$  splenocytes—1% of the number of anti-KLH IgM AFCP. As half of the latter switched to IgG1 production, this represents only 2% of the possible candidates. The other 98% of anti-KLH AFCP must have been making antibody of too low an affinity for KLH to bind to KLH as a bivalent IgG1 molecule.

Fig. 2 shows the results following the primary immunization of mice with KLH. As the total number of recovered splenocytes increased from *ca.*  $6 \times 10^7$  in unimmunized mice

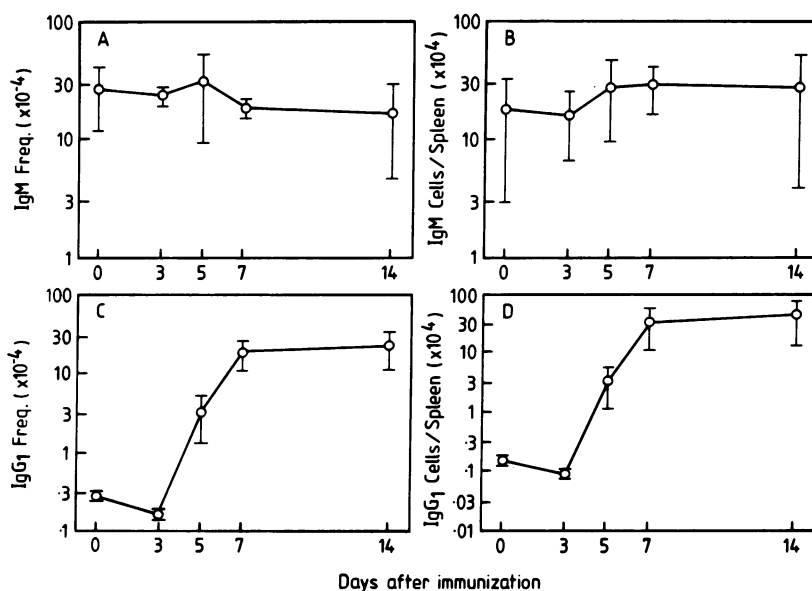


FIG. 2. Frequency (A and C) or total number (B and D) of anti-KLH AFCP before or after a single intraperitoneal immunization with KLH + pertussis. (A and B) IgM AFCP. (C and D) IgG1 AFCP. Each point is the mean of three or four experiments. Vertical bars represent standard deviations.

to *ca.*  $16 \times 10^7$  at day 7, the results have been presented as both frequencies and total numbers per spleen. Interestingly, neither the proportion nor the total number of anti-KLH IgM AFCP increased significantly after immunization. Had the immunogen stimulated a significant proportion of the observed AFCP in the preimmune repertoire, one might have expected some increase *in vivo* soon after immunization. In contrast, the frequency and absolute number of anti-KLH IgG1 AFCP increased sharply between days 3 and 7. In fact, the mean number per spleen increased by a factor of 37 between days 3 and 5 and by a further factor of 10 between

days 5 and 7, after which it appeared to plateau. At the height of the primary response, the numbers of IgM and IgG1 anti-KLH AFCP were approximately equal—namely, *ca.*  $3 \times 10^5$  per spleen.

**Amounts of Anti-KLH-Bindable Antibody per Clone as a Function of Time After Immunization.** Throughout these experiments, the amount of antibody that bound to KLH in the ELISA varied considerably from clone to clone, ranging between a few picograms to >1 ng. Fig. 3 shows results of individual clonal supernatants from ELISA trays in which the proportion of positives was sufficiently small to render clonal

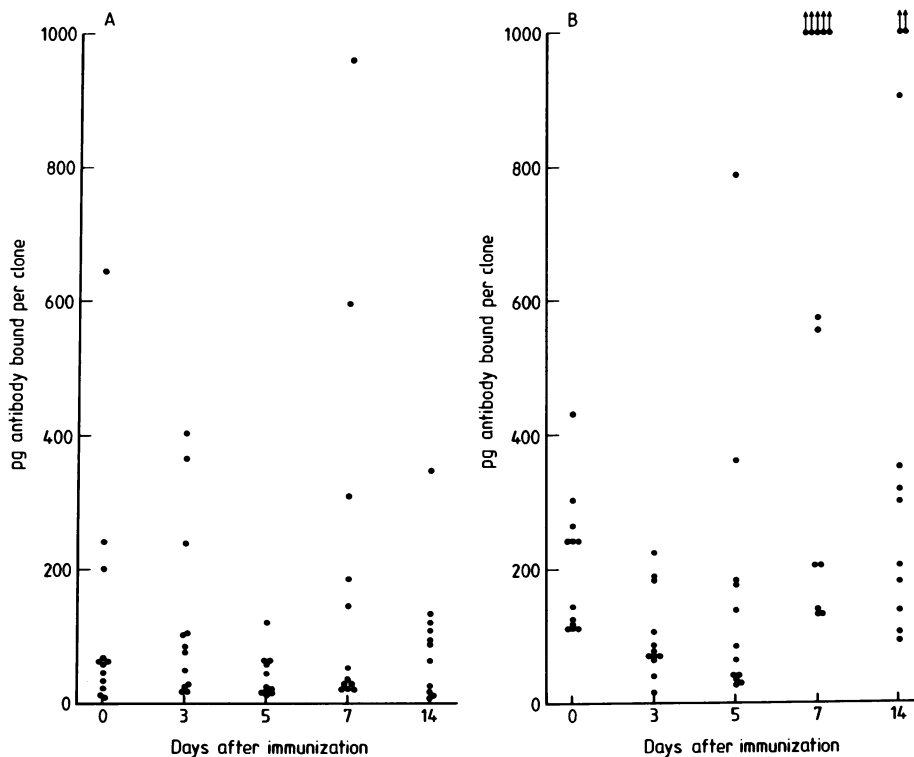


FIG. 3. Quantitation of antibody bound to KLH in ELISA of clonal supernatants from unimmunized or KLH-immunized mice. Each dot represents one clone, as results were selected from trays where clonal overlap was negligible. (A) IgM antibody. (B) IgG1 antibody.

overlap negligible (13). For the IgM AFCP, it is evident that there was no systematic change with time after immunization. In the case of IgG1 AFCP, a significant proportion of the clones from day 5 on, but not before, showed binding of  $>0.5$  ng per clone to KLH.

**Coincidence or Independence of IgM and IgG1 Clones.** When small numbers of cells were cultured to determine cloning efficiency, the ELISA capture layer being anti-murine immunoglobulin antibody, it was noted that those clones that formed IgG1 were usually also positive for IgM. This was true whether the mice had been immunized or not. Coincidence of IgM and IgG1 anti-KLH AFCP could not readily be studied in unimmunized mice or in mice up to 5 days after immunization, because culture trays yielding reasonable numbers of IgG1 AFCP were essentially confluent for IgM AFCP. However, when cells from mice immunized 7 to 14 days previously were examined, it became clear that the majority of anti-KLH IgG1 AFCP did not yield IgM anti-KLH *in vitro* (Table 1). This suggests that such AFCP had undergone an isotype-switch gene rearrangement *in vivo*. In fluorescence-activated cell sorting of anti-KLH IgG1 AFCP from immunized mice with isotype-specific antibodies (P. Lalor and G.J.V.N., unpublished data), it appears that about 80% of such cells are surface IgG1-positive, IgM-negative.

## DISCUSSION

While the preimmune B-cell repertoire, as revealed by an analysis of mitogen-activated B-cell clones or hybridomas derived from them, consists chiefly of cells expressing unmutated germ-line immunoglobulin *V* genes, secondary and hyperimmune responses to antigens are dominated by B cells that have been subjected to *V* gene hypermutation (16–20). The primary IgM response to antigens consists almost exclusively of antibodies coded for by unmutated germ-line *V* genes and, with certain immunogens, is much more restricted in *V* gene usage than might have been expected from the observed binding specificities of polyclonally activated clonotypes (21, 22). The question arises whether the higher-affinity, *V*-gene-mutated B cells seen in secondary responses are the direct descendants of B cells activated in the primary response or are generated in some other way. Arguments have been presented (9, 18, 23) to suggest that the somatic immunoglobulin *V* gene hypermutation occurs not in a pri-

mary or secondary immune response as such but after activation by antigen of a separate set of B lymphocytes that are responsible for generating memory B cells, probably chiefly through multiplication in germinal centers. If this is correct, it is important to characterize this set of B cells and, if possible, the earliest mutants arising after immunization.

The starting point of the present studies was a growing concern that the IgM antibodies produced by LPS stimulation of murine B cells and analyzed by ELISA might not be telling us much about the realities of *in vivo* immune responses. Repertoire representation against individual antigens, particularly haptens, can be very high, as can cross-reactivities, both depending greatly on the details of the ELISA method. Given that virgin B-cell-derived clones can now readily be switched to IgG1 production *in vitro* through the use of interleukin 4, it seemed to us that analyzing the repertoire by using an IgG1 readout might give us a tool of greater relevance to immune activation, tolerance, and autoimmunity. However, we were not prepared for the drastic difference in clonotype frequencies actually observed when the window was changed from an IgM ELISA to one dependent on IgG1. With intracellular antigens (1) and xenogeneic serum proteins (unpublished data), the preimmune B-cell repertoire had so few cells of the requisite affinity that their frequency was actually unmeasurable ( $<1$  per  $10^6$  spleen cells). This raises the issue of whether *V* gene mutation is required not just for affinity maturation but, indeed, for any IgG antibody of physiological relevance. For this reason, we chose an antigen of high molecular weight and known strong immunogenicity, KLH possessing numerous diverse B-cell epitopes, which did react with the IgG1 product of a few virgin B cells, representing  $\approx 1$  in 5000 of the repertoire. The physiologic relevance of these few clones to an *in vivo* anti-KLH response can still be questioned because it was only after immunization that significant numbers of clones were identified that made antibody bind strongly to KLH (Fig. 3). The sudden appearance and rapid rise in numbers of IgG1 anti-KLH AFCP stands in contrast with the failure of immunization to raise the numbers or affinity spectrum of anti-KLH IgM AFCP (Figs. 2 and 3). The latter finding casts into further doubt the relevance of IgM-based repertoire screening to antigen-driven *in vivo* responses.

The derivation and characteristics of the IgG1 AFCP cannot be determined by kinetic observations alone. The simplest postulate is that infrequent high-affinity AFCP in the virgin repertoire begin to multiply soon after antigen injection and that the AFCP seen on day 5 and thereafter are the direct progeny of those cells. If affinity considerations were set aside, the kinetics of Fig. 2 could just accommodate that view. More than six divisions would be required in 48 hr, or fewer if extensive recruitment of AFCP from bone marrow and other sites into the spleen were postulated. On the other hand, it is intriguing that day 5 after immunization, the day when IgG1 AFCP are first seen in greater numbers than before immunization, corresponds exactly to the time that it takes for antigen to induce a new germinal center (24). It is tempting to speculate that the day 5 IgG1 AFCP might represent the first somatically mutated B cells. Be that as it may, it is of interest that cells harvested 3 days after immunization, or cells taken from unimmunized mice, could generate so few high-affinity clones *in vitro*. This argues against the idea (25) that somatic mutants can be rapidly generated *in vitro* during a primary response and more in favor of the view (18, 23) that hypermutation occurs *in vivo* by a process separate from that generating the first antibody-forming cells.

Most of the IgG1 AFCP appearing 1 week after immunization have undergone isotype-switch gene recombination *in vivo* prior to culture (Table 1 and unpublished data). This observation should prove practically useful for the isolation of such cells through flow cytometry and the subsequent

Table 1. Coincidence or independence of clones positive for IgM or IgG1

Culture tray	Capture reagent	Wells positive for IgM and/or IgG1, no.		
		IgM + IgG1	IgM only	IgG1 only
1	Anti-globulin	6	11	0
2	Anti-globulin	8	9	2
3	Anti-globulin	7	14	1
4	Anti-globulin	12	7	2
5	KLH	3	14	11
6	KLH	0	15	4
7	KLH	1	7	4
8	KLH	2	8	9

Culture supernatants from 60-well trays of cloning-efficiency studies, with very small input cell numbers, or from studies determining the numbers of anti-KLH AFCP, with more cells per well, were bound to anti-globulin or KLH-coated ELISA trays, respectively, so that an aliquot to be tested for IgM binding was placed in the corresponding well to an aliquot to be tested for IgG1 binding. In this manner, the question of whether particular clonal cultures had secreted IgM antibody, IgG1 antibody, both, or neither could be determined.

analysis of their immunoglobulin *V* genes. However, it begs the question of whether the cells result from divisions (and perhaps mutations) in the same AFCP that give rise to the primary response or whether they derive from a separate B-cell lineage (9).

If most or all antigen-induced IgG antibodies are made by cells with *V* gene mutations, this has implications for theories of immunologic tolerance and autoimmunity. Until recently, our laboratory (26, 27) and Klinman and co-workers (9, 28) have been the strongest supporters of the view that tolerance can exist within the B-cell compartment and is not due only to T-cell regulation. Studies on transgenic mice have made this concept more popular (29). Our own work has concentrated on tolerance induction among virgin B lymphocytes as assessed by primary immunization *in vitro*. It has not dealt with the issue of what might happen if a B cell, responding correctly to a foreign antigen, were to undergo a somatic mutation that rendered it reactive to a self antigen. If such an antigen were present in a germinal center, it could cause further proliferation of the mutant anti-self clone. While the absence of T-cell help might militate against the later activation of such forbidden clones, the possibility of polyclonal activation (for example, through lymphokines or bacterial mitogens) could pose a threat. This has prompted Linton *et al.* (9) to suggest a second window of tolerance susceptibility for the precursors of memory B cells, soon after they begin to respond to antigen by division and somatic mutation. If the high-affinity IgG1 AFCPs identified in the present study indeed represent antigen-selected mutated B cells, adult tolerance models could be constructed in which their abrogation by antigen could represent a sharp tool to examine this claim. Moreover, most pathogenic autoantibodies show evidence of numerous *V* gene mutations (reviewed in ref. 30). This suggests a dependence on antigenic stimulation and, thus, perhaps a failure of the postulated post-antigenic tolerance mechanism.

The ready clonability *in vitro* of the IgG1 AFCP from immunized mice should help their further phenotypic characterization. It will be of interest to determine in detail their dependence on lymphokines, exact cloning efficiency, degree of isotype fixation, and responsiveness to T-independent and T-dependent signaling. As our knowledge of functional B-cell subsets is rather limited, any new approach to the analysis of memory cells and their precursors is worthy of exploration.

The outstanding technical assistance of Ms. Marita Walmsley is warmly acknowledged. This work was supported by the National Health and Medical Research Council (Canberra, Australia); by Grant AI-03958 from the National Institute of Allergy and Infectious Diseases; and by the generosity of a number of private donors to The Walter and Eliza Hall Institute.

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