

# HETEROGENEITY OF THE BALB/c ANTIPHOSPHORYLCHOLINE ANTIBODY RESPONSE AT THE PRECURSOR CELL LEVEL\*

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The immune response to most antigenic determinants is characterized by the production of a heterogeneous array of antibody molecules. Previous work in this laboratory has demonstrated that this heterogeneity results from the stimulation by an antigenic determinant of a variety of bone marrow-derived antibody-forming cell precursors (B cells),<sup>1</sup> each of which gives rise to a clone of cells producing a homogeneous product which contributes to a heterogeneous composite (1). The phosphorylcholine (PC) determinant found on pneumococcal strain R36A is exceptional in that the immune response to it in BALB/c mice has been reported to be markedly restricted, both in immunoglobulin class and variable region antigenicity (2, 3). In addition, several independently derived plasmacytomas producing homogeneous immunoglobulins with specificity for PC possess similar antigenic determinants or idiotypes (4). Two of these, TEPC 15 and HOPC 8, show identity by sequencing through the first hypervariable region (5). Antibody raised in A/He mice that is specific for the variable region of these myeloma proteins appears to react with the naturally occurring antibody produced in BALB/c mice in response to immunization with R36A bacteria (3, 6).

Thus, immunization of BALB/c mice with R36A, or PC as a hapten-carrier conjugate, generally results in an IgM antibody response that has unique binding characteristics for PC and shares idiotypic specificity with the TEPC 15 and HOPC 8 myeloma proteins (3, 6, 7). Furthermore, anti-TEPC 15 serum can specifically suppress both the *in vivo* and *in vitro* immune response to PC, suggesting that all B cells responsive to PC bear receptors with the TEPC 15 idiopeptide and that no other B cells can be stimulated by this antigen, even in the absence of the predominant clone (6, 8). This evidence has led to the conclusion that the response to the PC determinant in BALB/c mice is restricted to the TEPC 15 idiopeptide and IgM class, and may actually be monoclonal.

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<sup>1</sup> *Abbreviations used in this paper:* BAC, bromoacetyl cellulose; BBS, 0.05 M borate-0.15 M NaCl; B cell, bone marrow-derived antibody-forming cell precursor; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DPPC, *p*-diazonium phenylphosphorylcholine; HAc, acetic acid; Hy, *Limulus polyphemus* hemocyanin; PBS, 0.02 M phosphate-0.15 M NaCl, pH 7.4; PC, phosphorylcholine; PPC-TGG-Hy, 3-(*p*-azophenylphosphorylcholine)-*N*-acetyl-L-tyrosylglycylglycine Boc Hydrazide-hemocyanin.

Past analyses have tended to confirm this notion in that attempts to raise IgG antibodies and antibodies with a different idiotype in BALB/c mice have failed. These attempts included the use of PC on a carrier protein as an immunogen injected into nonimmune or carrier-immunized mice (7, 8). While the immune response to PC was slightly enhanced by this procedure, the results of both primary and secondary immunization yielded no detectable serum IgG antibody nor indirect plaque-forming cells. Furthermore, a vast majority of cells capable of binding PC after immunization with either the bacteria or hapten-carrier complexes appear to have receptors with the TEPC 15 idiotype (3, 8).

In view of the vast array of specificities comprising the B-cell repertoire, the conclusion that only a single clone is able to respond to a given determinant has important implications. A definitive answer to this question is not only desirable but should be available in the context of stimulation in splenic fragment cultures containing isolated B cells which, as previously reported, maximizes B-cell detection (9).

We report an analysis of the immune response to PC in BALB/c mice which has incorporated: (a) extremely sensitive radioimmunoassays for both anti-PC antibody and antibody of the TEPC 15 idiotype, (b) immunization in carrier-primed mice with PC attached to the carrier through a tripeptide spacer which appears to maximize carrier integrity and recognition, and (c) the isolation of B cells in splenic fragment cultures derived from carrier-primed recipients. The results indicate that (a) the majority of B cells in BALB/c mice that are specific for PC yield clones whose antibody is of the TEPC 15 idiotype, (b) clones making anti-PC antibody of other idiotypes represent from 2 to 50% of the total clonal response in nonimmune mice, (c) anti-PC antibody of the IgG1 immunoglobulin class is detectable in monoclonal antibodies of the TEPC 15 idiotype and in monoclonal antibodies of other idiotypes, and (d) the serum of *Limulus polyphemus* hemocyanin (Hy)-primed mice immunized with PC coupled to Hy through a tripeptide spacer contains anti-PC antibody with the TEPC 15 idiotype of both IgG and IgM classes.

## Materials and Methods

**Animals.** 8-wk old BALB/cJ mice were obtained from Carworth Div., Becton, Dickinson & Co., New City, N. Y. BALB/cAnN and A/He strains were acquired through the Institute for Cancer Research, Philadelphia, Pa., and maintained in our mouse colony. 8-wk old germfree BALB/c mice were purchased from ARS/Sprague-Dawley Div., The Mogul Corp., Madison, Wis., and conventionally reared C57BL/6 mice from Jackson Laboratories, Bar Harbor, Maine.

**Antigens.** *Diplococcus pneumoniae* strain R36A, obtained from Dr. R. Austrian, University of Pennsylvania, Philadelphia, Pa. was heat killed at 90°C for 60 min. *p*-diazonium phenylphosphorylcholine (DPPC) was prepared according to Chesebro and Metzger (10). 10 mg of DPPC was coupled to 100 mg of Hy (11) in a vol of 4 ml in 0.02 M phosphate-0.15 M NaCl buffer at pH 8.9 in ice. After stirring for 18 h at 4°C, the solution was dialyzed and passed through a Sephadex G-50 column. The ratio of coupling for the PC-Hy compound was 20 mol of PC/100,000 g of Hy, using a molar absorbency of 475 nm of 12,600 in 0.1 N NaOH to determine the DPPC coupling in the protein; protein concentration was measured by the method of Lowry et al. (12). The preparation of 3-(*p*-azophenylphosphorylcholine)-*N*-acetyl-L-tyrosylglycylglycine Boc Hydrazide-hemocyanin (PPC-TGG-Hy), a phosphorylcholine-tripeptide-spacer conjugate, is described in another paper;<sup>2</sup> the antigen contained 10 mol of hapten/ml of Hy.

<sup>2</sup>Sigal, N., P. Gearhart, and N. Klinman. The frequency of phosphorylcholine-specific B cells in conventional and germfree BALB/c mice. Manuscript submitted for publication.

*Immunizations.* BALB/c mice received an intraperitoneal injection of 0.1 mg of Hy in complete Freund's adjuvant (CFA) and were used as recipients in cell transfer studies 6-8 wk later. Some of these carrier-primed mice were injected with 0.1 mg of PPC-TGG-Hy in CFA and bled at weekly intervals after immunization to provide serum antibody to PC. Sera was also obtained from mice injected intravenously with three injections of  $10^9$  bacteria during 1 wk; this schedule was repeated at monthly intervals, with bleedings 7-10 days after the final injection.

*Immunoabsorbents.* Bovine serum albumin-bromoacetyl cellulose (BSA-BAC) was made according to Robbins et al. (13). 3 g of wet weight BAC was washed in 0.2 M  $\text{NaH}_2\text{PO}_4$  and 0.8 g BSA was added. The pH was brought to 3.9 with 1 M citric acid and stirred overnight at room temperature. After centrifugation, the supernate was discarded and 25 ml of 0.1 M  $\text{NaHCO}_3$ , pH 8.9, was added to the BAC. The pH was adjusted to 8.9 and the mixture stirred at 4°C overnight. The BSA-BAC was washed several times with 0.02 M phosphate-0.15 M NaCl, pH 7.4 (PBS), and then with 0.05 M borate-buffered saline (BBS), pH 8.0, and suspended to 10 ml in BBS. The pH was brought to 8.9 with 2 N NaOH in ice, and 75 mg of DPPC was added dropwise. After shaking in the dark at 4°C for 24 h, the PC-BSA-BAC was washed with PBS and then shaken for 15 min with 25 ml of 1 N acetic acid (HAc). The BAC was centrifuged and the acid wash repeated, followed by several washes in PBS.

PC-glycyltyrosine-Sepharose 4B was prepared according to Chesebro and Metzger (10). The agarose gel was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

The plasmacytoma MOPC 460 was obtained from Dr. Michael Potter, National Institutes of Health, Bethesda, Md. The 460 myeloma protein was purified from ascites fluid using an immunoabsorbent of dinitrophenyl-lysine-Sepharose following the procedure of Goetzl and Metzger (14). The purified protein was then coupled to cyanogen-activated Sepharose 4B (15).

Immunoabsorbents of IgM, IgG1, and IgA were prepared using the following immunoglobulin preparations. IgM was purified by fractionation of normal mouse sera on a Sephadex G-200 column,  $180 \times 3.4$  cm, in PBS. IgG1 protein was obtained from the purification of MOPC 31c myeloma protein on DEAE-cellulose as previously described (16). Purified TEPC 15 was used as a source of IgA. The proteins were added to 0.3 g wet weight BAC in a ratio of 1:30 and the coupling continued following the procedure described above for BSA-BAC. These adsorbents were extensively washed by centrifugation with 0.5 N HAc and PBS until the absorbance of the supernate at 280 nm was 0.

Bacterial immunoabsorbents were prepared by growing a culture of strain R36A bacteria overnight. The culture fluid was centrifuged and the bacteria washed in PBS several times; then the organisms were heat killed at 90°C for 60 min. The bacteria were suspended to one-half of the original volume, and 0.3 ml of this suspension was used as an immunoabsorbent in the radioimmunoassay.

*Purification of Myeloma Proteins.* The PC-binding myeloma proteins TEPC 15 and MOPC 603 (both  $\alpha$ ,  $\kappa$ ) (4) were purified from the ascites fluids of mice transplanted with the corresponding plasmacytomas (obtained from M. Potter, National Institutes of Health, Bethesda, Md.). The ascites fluid was reduced with 0.005 M dithiothreitol at pH 8.6 in Tris and alkylated with 0.011 M iodoacetamide (10). This solution was diluted 1:2 with BBS and 50 ml was added to a 20-ml column of PC-glycyltyrosine-Sepharose. The column was washed extensively with BBS until the effluent had an absorbance of less than 0.05 at 280 nm. The protein was eluted with 0.5 N HAc and immediately dialyzed against 0.2 M phosphate, pH 8.0, and then against PBS, pH 7.4, for 2 days. Approximately 1 mg of protein/ml of TEPC 15 ascites was recovered; the yield was less from MOPC 603 ascites (0.3 mg/ml).

Purified protein from the W3129 myeloma tumor was a gift from M. Weigert, Institute for Cancer Research, Philadelphia, Pa. The IgM from the ascites fluid of mice with the MOPC 104E plasmacytoma (M. Potter) was purified by G-200 Sephadex gel filtration.

*Anticlass-Specific Antibody.* Goat antimouse IgM, IgG1, and IgA antisera were purchased from Meloy Laboratories, Inc., Springfield, Va. 2 ml of each antiserum was precipitated with 45%  $(\text{NH}_4)_2\text{SO}_4$  and resuspended to 1 ml. These were added to the appropriate heavy-chain class BAC immunoabsorbents described above, i.e. IgM-BAC, IgG1-BAC, and IgA-BAC. The antisera and immunoabsorbents were shaken at room temperature in a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.) for 1 h and then washed with cold PBS until the absorbance at 280 nm was less than 0.01. The antibody was eluted with the addition of 0.5 ml of 0.5 N HAc (17), and the BAC shaken for 15 min at room temperature and centrifuged. This was repeated with another portion of acid. The acid eluates were combined and quickly recentrifuged at 18,000 g for 10 min and dialyzed in

thin-walled dialysis tubing against 0.2 M phosphate, pH 8.0, followed by several changes in PBS. Generally, 0.3–0.9 mg of antibody is recovered from 2 ml of serum; a portion of this is labeled with  $^{125}\text{I}$  as described previously (18) and used in the radioimmunoassay to detect antiphosphorylcholine antibodies of the  $\mu$ -,  $\gamma$ 1-, and  $\alpha$ -chain class. The labeled antisera were determined to be class specific by the criteria summarized in Table II.

*Splenic Focus Technique.* Spleens from unimmunized BALB/c mice were homogenized in Dulbecco's modified Eagle's medium using a Teflon tissue homogenizer (9).  $20 \times 10^6$  cells from this suspension were injected intravenously into Hy carrier-primed recipients that had been irradiated with 1600 R 6 h earlier.

Fragment cultures of spleens of recipient mice were prepared 16 h after cell transfer, as described previously (19). The fragments were individually stimulated in culture with  $5 \times 10^{-7}$  M hapten on the tripeptide carrier (PPC-TGG-Hy), and culture fluids were changed every 2–3 days. Fluids collected 10–15 days after stimulation were assayed for antihapten antibody, class of antibody, and the presence of the TEPC 15 idiotype.

*Radioimmunoassay.* The radioimmunoassay of culture fluids for mouse antihapten antibody has been described previously (18). 20  $\mu\text{l}$  of culture fluid was added to 30  $\mu\text{g}$  of PC-BSA-BAC, and bound antihapten antibody was detected by the subsequent binding of  $^{125}\text{I}$ -labeled purified rabbit antimouse Fab fragment antibody (18). The assay was standardized for quantitation by the addition of 1, 5, 10, and 20 ng of purified TEPC 15. Similarly, an analysis of antibody class was performed by detecting antibody from 20  $\mu\text{l}$  of culture fluids bound to PC-BSA-BAC with  $^{125}\text{I}$ -labeled anti- $\mu$ , anti- $\gamma$ 1, or anti- $\alpha$ .

*Anti-TEPC 15 Idiotype Sera.* A/He mice were immunized with purified TEPC 15 myeloma protein according to the method of Lieberman and Humphrey (20). Only sera with precipitating antibody to the myeloma in an Ouchterlony reaction were used. Approximately 1 ml of pooled sera, taken from several bleedings of an individual mouse, was diluted to 10 ml with PBS and added to a 15 ml MOPC 460-Sepharose column and passed slowly through to remove antibody to the constant region determinants of TEPC 15. This absorbed serum did not bind any other IgA proteins, including MOPC 603 (Fig. 1).

*Anti-Idiotype Assay.* Culture fluids positive for anti-PC antibody were tested for the presence of TEPC 15 idiotype in a solid-phase radioimmunoassay (21), following the procedure of Carson and Weigert (22). 1 ml of a 1:8,000 dilution of anti-idiotype serum in PBS, pH 7.4, was added to  $10 \times 75$ -mm polystyrene test tubes (Falcon Plastics, Div. Becton, Dickinson, & Co., Oxnard, Calif.) and incubated for 4 h at room temperature. The serum was withdrawn and the tubes washed with 1 ml of PBS; 1 ml of PBS containing 1% BSA (BSA-PBS) was added and the tubes incubated for 30 min. The BSA-PBS was removed and various amounts of unlabeled inhibitor proteins or 20  $\mu\text{l}$  of culture fluids were added, followed by the addition of 1.1 ml of  $^{125}\text{I}$ -labeled TEPC 15, containing about 25,000 cpm (2 ng of TEPC 15) in BSA-PBS plus 0.1% rabbit serum. Purified TEPC 15 in BSA-PBS was labeled by a chloramine T procedure (23) and had a sp act of 18.6  $\mu\text{Ci}/\mu\text{g}$  protein. After incubation at 37°C for 18 h, the tubes were washed three times with PBS and counted in a gamma counter. Nonspecific binding was measured with tubes to which BSA-PBS was added and represented 0.1–0.2% of the added counts.

Antibody of the TEPC 15 idiotype was quantitated by the amount of inhibition of binding of the labeled TEPC 15 to the anti-idiotype-coated plastic tubes. 1, 3, 6, 10, and 20 ng of purified TEPC 15 was added with 20  $\mu\text{l}$  of Dulbecco's media to standardize the quantitation of inhibition. The specificity of inhibition was demonstrated by the lack of inhibition with MOPC 603 (Fig. 1) and serum from conventionally reared C57BL/6 or germfree BALB/c mice (Table IV).

*Serum Antibody Analysis.* 1 ml of serum from a pooled day 21 bleeding of Hy-primed BALB/c mice immunized with PPC-TGG-Hy was applied to a  $127 \times 1.3$ -cm column of Sephadex G-200 equilibrated with PBS, pH 7.2. The absorbance at 280 nm was measured for each 2 ml eluted fraction; in addition an assay of anti-PC activity was performed with 20  $\mu\text{l}$  from each fraction in the radioimmunoassay, using  $^{125}\text{I}$ -labeled anti-Fab, anti- $\mu$ , or anti- $\gamma$ 1.

To obtain IgG antibody uncontaminated with IgM, a DEAE-cellulose fractionation of the serum was done. DEAE-cellulose was equilibrated with 0.01 M phosphate, pH 8.0; a 50%  $(\text{NH}_4)_2\text{SO}_4$  precipitate of 1 ml of day 21 serum was extensively dialyzed against the buffer. The serum was applied to a  $1 \times 12$ -cm column of DEAE-cellulose and washed with 0.01 M  $\text{PO}_4$ , pH 8.0, until the absorbance was 0.01 at 280 nm. A higher ionic strength buffer, 0.02 M  $\text{PO}_4$ -0.05 M NaCl, pH 8.0, was

then used to elute the IgG protein. This peak, fraction 2, which had a 280/250 nm ratio of 2.3, was assayed for anti-PC antibody of the  $\mu$ - and  $\gamma$ 1-class in the radioimmunoassay and for the presence of the TEPC 15 idiootype.

## Results

*Specificity and Sensitivity of the Radioimmunoassay for Anti-PC Antibody.* The radioimmunoassay used in these studies has previously been demonstrated to quantitate as little as 0.5 ng of murine antibody against a variety of antigenic determinants (23). In addition, through the use of  $^{125}\text{I}$ -labeled purified anti- $\mu$  and anti- $\gamma$ 1 antisera, the amount of antibody of these immunoglobulin classes can be quantified. Table I summarizes data obtained from analyses of several sera and immunoglobulin preparations for anti-PC activity as detected by  $^{125}\text{I}$ -labeled anti-Fab, anti- $\mu$ , and anti- $\gamma$ 1 antibodies. It can be seen that the serum of BALB/c mice immunized with R36A bacteria has as much as 760  $\mu\text{g/ml}$  of anti-PC antibody, mostly of the IgM class. Conventionally reared, nonimmune BALB/c mice have 25–110  $\mu\text{g/ml}$  of antibody, whereas the same strain raised in a germfree environment has negligible amounts of antibody to PC. A comparison between serum antibody from mice immunized with PPC-TGG-Hy and those receiving PC-Hy clearly shows the difference between these two antigens in stimulating IgG1 antibody production. Nonimmune serum from

TABLE I  
*Class and Quantity of Anti-PC Antibody in Nonimmune and Immune Serum*

Serum source*	$\mu\text{g/ml}$ detected by:‡		
	Anti-Fab	Anti- $\mu$	Anti- $\gamma$ 1
BALB/c, nonimmune	64 (25–110)§	32	18
BALB/c, immunized with R36A	400 (155–760)	208	28
BALB/c, immunized with PPC-TGG-Hy¶	125	10	110
BALB/c, immunized with PC-Hy**	30	12	22
BALB/c, germfree	0–1	1	0
C57BL/6, nonimmune	0–1	1	0

\* 0.02  $\mu\text{l}$  of serum was added to PC-BSA-BAC, incubated 1 h, and washed once with PBS-0.1% horse serum (PBS-HS). 0.1 ml of labeled antibody containing 2 ng was added to the centrifuged pellet and incubated at 4°C overnight. The tubes were then washed and centrifuged four times in PBS-HS and the pellet counted in a gamma counter.

‡ Quantitation of antibody detected with iodinated anti-Fab was standardized with known concentrations of the purified TEPC 15 protein. The quantity of antibody of the IgM and IgG1 class was determined by comparing the amount of antibody detected by anti-Fab from monoclonal foci of solely IgM or IgG1 class and the corresponding counts detected by anti- $\mu$  or anti- $\gamma$ 1.

§ The numbers in parentheses represent the range of natural antibody found in various bleedings of nonimmune mice. The proportion of IgM and IgG1 is relatively constant within this range.

|| Individual sera from five mice bled 5–7 days after immunization with strain R36A were analyzed.

¶ This analysis was performed on a pool of sera from eight Hy-primed mice immunized with PPC-TGG-Hy and bled 21 days later.

\*\* This analysis was performed on a pool of sera from eight Hy-primed mice immunized with PC-Hy and bled 18 days later.

a different strain of mice, C57BL/6, is essentially devoid of natural anti-PC antibody.

The specificity of the antiheavy-chain labels used in the assay is demonstrated in Table II by the binding of TEPC 15 myeloma protein only by anti-Fab and anti- $\alpha$  antibodies, and the reactivity of clonal antibodies with anti-Fab and one of the heavy-chain labels but not with the others. Table III summarizes the specific inhibition observed with high concentrations of phosphorylcholine and PC-Hy.

TABLE II  
*Specificity of Antiheavy-Chain Antibody in the Radioimmunoassay of Anti-PC Antibody*

Protein‡	cpm detected by:*			
	Anti-Fab	Anti- $\mu$	Anti- $\gamma$ 1	Anti- $\alpha$
20 ng of TEPC 15	2,158	34	74	8,286
Clone P8bH7	1,996	40	5,854	22
Clone P11hH8	540	800	0	26

\* Background counts representing nonspecific binding were subtracted.

‡ TEPC 15 protein and 20  $\mu$ l of culture fluids from two clones were assayed as described in Table I and detected with various labeled antibodies.

TABLE III  
*Specific Inhibition of the Radioimmunoassay for Anti-PC Antibody*

Protein*	Anti-Fab‡	Inhibition of control binding
	cpm	%
20 ng TEPC 15	2,286	—
20 ng TEPC 15 + $1 \times 10^{-3}$ M PC	614	73
20 ng TEPC 15 + $1 \times 10^{-4}$ M PC	1,650	28
20 ng TEPC 15 + $2 \times 10^{-4}$ M PC-Hy	800	65
20 ng TEPC 15 + $2 \times 10^{-5}$ M PC-Hy	1,190	48

\* Purified, reduced, and alkylated TEPC 15 protein was assayed as described in Table I. PC inhibitors were incubated with TEPC 15 protein for 30 min before it was added to the immunoadsorbent.

‡ Nonspecific binding to the immunoadsorbent was subtracted.

*Specificity and Sensitivity of the Radioimmunoassay for the TEPC 15 Idiotype.* Rigorous immunization of A/He mice with TEPC 15 protein produced precipitating antibody to TEPC 15 in 70% of the mice. The data presented in this paper have been obtained using the serum of one mouse. Even though the serum did not react to MOPC 603 protein in an Ouchterlony agar slide, there was some nonspecific binding to IgA myelomas in the radioimmunoassay. This activity was removed after passage through a MOPC 460 Sepharose column. Although it has been reported that A/He anti-TEPC 15 serum does not contain antiallotype

activity (24), we routinely absorbed the anti-idiotypic serum in order to increase the sensitivity of the inhibition assay.

As seen in Table IV, binding of TEPC 15 to this anti-idiotypic serum is not inhibited by  $5 \times 10^{-3}$  M phosphorylcholine which is in agreement with Claflin's and Davie's demonstration (25) of poor hapten inhibition with anti-idiotypic to TEPC 15 raised within murine strains. However, phosphorylcholine on a carrier did significantly inhibit and may reflect steric hinderance or the increased affinity of a hapten-carrier conjugate for the myeloma protein due to multivalent presentation of determinants.

The finding by Lieberman et al. (24) of natural antibody bearing the TEPC 15 idio type in BALB/c mice enabled us to test the specificity of the anti-idiotypic serum for serum from nonimmune, bacterial-immunized, and germfree BALB/c mice and C57BL/6 mice, which lack the natural TEPC 15 idio type antibody. As

TABLE IV  
*Specificity of Anti-TEPC 15 Idiotypic Antibody: Hapten and Serum Inhibition\**

Inhibitors	cpm‡	Inhibition %
None	1,955	—
PC, $5 \times 10^{-3}$ M	1,896	3
PC-Hy, $2 \times 10^{-4}$ M	745	62
BALB/cJ, nonimmune serum	885	55
BALB/cAnN, nonimmune serum	1,035	47
BALB/c, immune (R36A)§	548	72
BALB/c, germfree serum	1,935	1
C57BL/6, nonimmune serum	1,877	4

\* Inhibition is recorded as percent inhibition of control binding of  $^{125}$ I-labeled TEPC 15 to a 1:8,000 dilution of anti-TEPC 15 serum. Inhibition was tested with 0.1  $\mu$ l of pooled serum from nonimmune and immune mice.

‡ Nonspecific binding has been subtracted.

§ The serum from mice immunized with *Pneumococcus* and bled 1 wk later was tested.

shown in Table IV, sera from nonimmune and bacterial-immunized BALB/c mice can inhibit idio type binding to anti-idio type, whereas sera from germfree BALB/c and C57BL/6 are ineffective. The amount of protein containing the TEPC 15 idio type in normal BALB/c serum was 52  $\mu$ g/ml, which correlates with previous observations (24).

Having established the specificity of the anti-TEPC 15 serum, the extent of inhibition with unlabeled idio type was determined to quantitate inhibition by culture fluids. In order to maximize inhibition with nanogram quantities of inhibitor, a series of twofold dilutions of the anti-idio type serum were made to determine the equivalence point between labeled idio type and anti-idio type. At lower dilutions of serum, the anti-idio type would be in excess, and therefore bind

both labeled and unlabeled idiootype. If the labeled idiootype was in slight excess however, it would be more easily displaced from binding to anti-idiootype by unlabeled inhibitor. The equivalence point was defined as the last dilution that bound 2 ng of labeled idiootype maximally before the counts rapidly decreased due to diminished anti-idiootype. With this particular serum, a 1:8,000 dilution was used in the assay, which was a dilution somewhat higher than the equivalence point of 1:5,000, thus insuring conditions of idiootype excess.

Fig. 1 demonstrates the sensitivity of the radioimmunoassay to inhibition by nanogram quantities of unlabeled TEPC 15 idiootype. A similar, but not identical, phosphorylcholine-binding protein, MOPC 603, was ineffective as an inhibitor as were W3129 protein ( $\alpha, \kappa$ ), with specificity for  $\alpha$ -1,6 dextran, and MOPC 104E

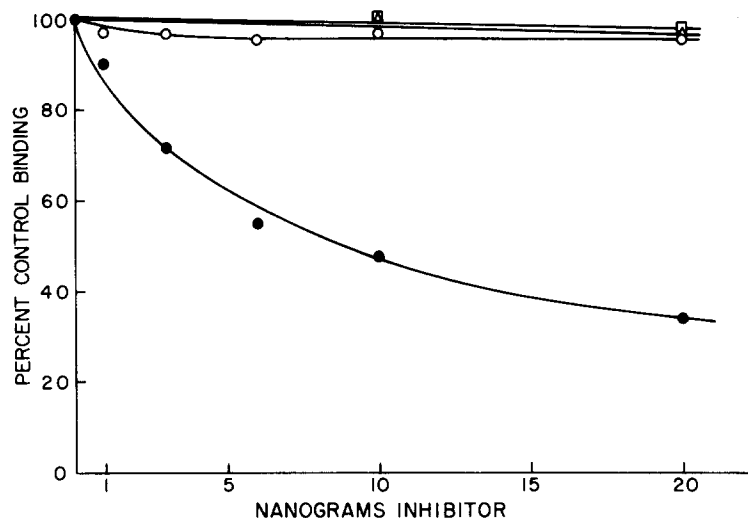


FIG. 1. Specific inhibition of iodinated TEPC 15 binding to anti-TEPC 15 serum. Closed circles indicate the extent of inhibition with unlabeled TEPC 15; open circles, MOPC 603; squares, W3129; and triangles, MOPC 104E. Percent control binding = (cpm bound in the presence of inhibitor/cpm bound without inhibitor)  $\times$  100. Each point denotes the average of four determinations. All proteins used for inhibition except MOPC 104E were reduced and alkylated before purification. Concentrations of the undiluted proteins were calculated by optical density, using an extinction coefficient of 1.40/mg/ml, 1-cm light path.

protein ( $\mu, \lambda$ ) binding  $\alpha$ -1,3 dextran. This assay was used to quantitate antibody of the TEPC 15 idiootype produced by culture fluids of stimulated fragments in the in vitro focus system. Fluids that contained antiphosphorylcholine antibody detected by the radioimmunoassay using PC-BSA-BAC and  $^{125}\text{I}$ -labeled anti-Fab were then tested in the anti-idiootype assay for the presence or absence of the TEPC 15 idiootype.

*The Immunoglobulin Class and Idiootype of Monoclonal Anti-PC Antibodies.* The frequency of B cells specific for the PC determinant is presented in another publication.<sup>2</sup> All monoclonal antibodies obtained from unimmunized conventionally reared BALB/c mice were analyzed for their immunoglobulin class and idiootype. The data from these analyses is shown in Table V. It can be



TABLE V  
*In Vitro Clonal Response to Phosphorylcholine*

Anti-PC antibody‡	% Total number of clones analyzed*	
	Idiotype positive§	Idiotype negative
IgM only	15.0	5.0
IgG1 only	21.2	2.5
IgM and IgG1	38.8	13.8
Other	2.5	1.3
Total	77.5	22.6

\* The products of 80 clones from three donor mice were analyzed for idiotype and heavy-chain class. Fragments contained unimmunized spleen cell precursors in a Hy-primed environment and were stimulated in vitro with PPC-TGG-Hy.

‡ Duplicate 20- $\mu$ l culture fluid samples were added to PC-BSA-BAC, and labeled anti-IgM or anti-IgG1 antibody was added. A clone was determined to be positive if the counts in the radioimmunoassay were significantly above background.

§ Duplicate 20- $\mu$ l samples of culture fluid collected 10 or 12 days after stimulation were tested for inhibition of  $^{125}$ I-labeled TEPC 15 binding to anti-TEPC 15 serum-coated test tubes.

|| The product of these clones was detected with labeled anti-IgA.

seen that most, but not all, of the clones responding to PC possess the TEPC 15 idiotype. One of the donor mice had as much as 50% non-TEPC 15 idiotype-producing foci. Both IgM and IgG1 classes are represented in the immune response; preliminary examination with  $^{125}$ I-labeled anti-IgA also revealed idiotype-positive and -negative clones bearing the  $\alpha$ -heavy chain. Monoclonal antibodies were further tested for binding specificity by their ability to bind to strain R36A *Pneumococcus*. Antibody with and without the TEPC 15 idiotype bound to bacteria used as an immunoadsorbent and was detected with  $^{125}$ I-labeled antimouse Fab.

Fig. 2 plots on a weight basis the amounts of monoclonal antibodies detected by the radioimmunoassay for Fab vs. idiotype. Given an uncertainty of as much as 10% for each method, the data fit remarkably well to the theoretical curve constructed with purified TEPC 15 myeloma protein.

*The Immunoglobulin Class and Idiotype of Serum Anti-PC Antibodies.* Because of the unexpected finding of in vitro phosphorylcholine-responding clones of the IgG1 class, a careful study of serum anti-PC antibody was made to detect IgG. Mice primed with Hy 8 wk previously were immunized with PPC-TGG-Hy and bled at regular intervals. Serum obtained 21 days after immunization was applied to a Sephadex G-200 column and the fractions tested for anti-PC activity. Specific antibody bearing  $\mu$ - and  $\gamma$ 1-determinants was observed in the first and second peaks, respectively, with some trailing of IgM antibody into the IgG region, which may have been due to monomer IgM. A stepwise elution of serum from DEAE-cellulose was made in order to obtain an IgG fraction of antibody devoid of IgM.

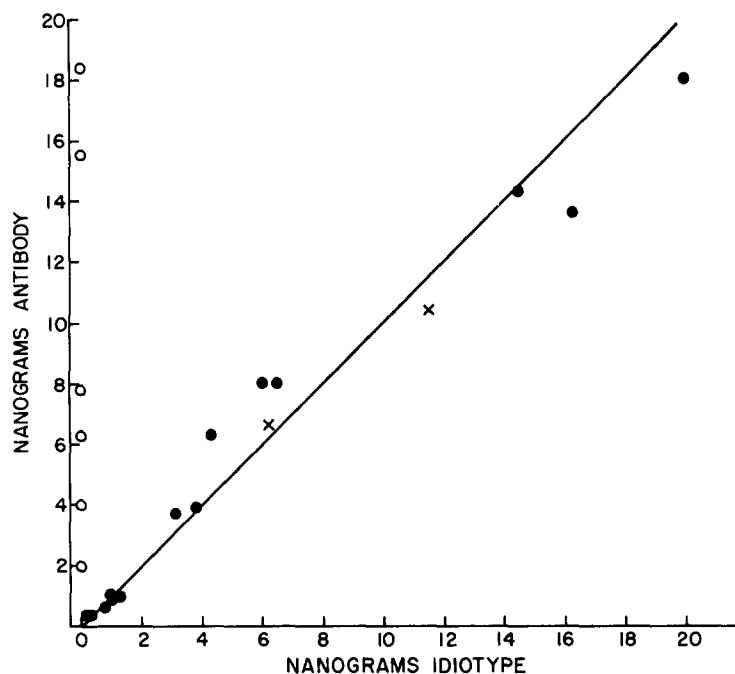


FIG. 2. Comparison of the amount of idiotype as detected by anti-Fab and anti-idiotype. Samples from positive culture fluids and TEPC 15 ascites were tested for antibody in the radioimmunoassay using PC-BSA-BAC and  $^{125}\text{I}$ -labeled anti-Fab, and in the anti-idiotype inhibition assay. Standard curves for both assays were derived using known amounts of purified reduced and alkylated TEPC 15. Closed circles signify the amount of idiotype positive antibody in  $20\ \mu\text{l}$  of culture fluid; open circles indicate culture fluid from idiotype negative clones; crosses represent two dilutions of a 45%  $(\text{NH}_4)_2\text{SO}_4$  preparation of TEPC 15 ascites fluid. It should be noted that the quantity of monoclonal antibodies present in positive culture fluids is highly variable regardless of the idiotype.

Both G-200 and DEAE-cellulose-eluted fractions were examined for heavy-chain specificity and idiotype. As seen in Table VI, the first collection of protein from G-200 chromatography had IgM antibody but not IgG1 and was positive for the idiotype. Fraction 2 from DEAE-cellulose contained IgG1 antibody and also possessed the TEPC 15 idiotype.

## Discussion

Previous investigations of the immune response to phosphorylcholine in BALB/c mice have shown the response to be essentially monoclonal and of the IgM class (2, 7, 8). This report has combined the use of two extremely sensitive radioimmunoassays, maximization of carrier recognition, and the stimulation of isolated B cells in fragment cultures to more carefully examine the potential for anti-PC responsiveness inherent in the B-cell repertoire of BALB/c mice. The results indicate that while B cells expressing the TEPC 15 idiotype predominate in the B-cell population responsive to PC, B cells of other idiotypes can respond to stimulation with PC on an appropriate carrier. In addition, when carrier help

TABLE VI  
*Analysis of Serum Antibody for Heavy-Chain Class and Idiotype\**

Antibody	cpm detected by:‡			Comparative concentration with TEPC 15 standard§	
	Anti-Fab	Anti- $\mu$	Anti- $\gamma$ 1	ng anti-body	ng idiotype
Fraction 1 from G-200 Sepharose	880	1,070	0	3.4	4.1
Fraction 2 from DEAE-cellulose¶	896	0	2,184	3.8	3.0

\* Hy-primed mice were immunized with PPC-TGG-Hy and bled 21 days later. The pooled serum was fractionated by G-200 and DEAE-cellulose chromatography, and analyzed for heavy-chain class by the radioimmunoassay and for idiotype content by inhibition of labeled TEPC 15 binding to anti-TEPC 15 antisera.

‡ Counts representing nonspecific binding to the immunoadsorbent were subtracted.

§ The values were obtained from a standard curve constructed for both assays using a known quantity of purified TEPC 15 protein.

|| Fraction 1 was the first peak to elute from G-200 Sephadex chromatographic separation of whole antiserum.

¶ Antiserum was applied to a DEAE-cellulose column, and fraction 2 was eluted with 0.02 M  $\text{PO}_4$ -0.05 M NaCl buffer, pH 8.0.

is maximized, antibody with the TEPC 15 idiotype and anti-PC antibody of other idiotypes which is not of the IgM class is synthesized.

The radioimmunoassay used for the detection of anti-PC antibody is an adaptation of a radioimmunoassay previously used to detect a variety of antihapten and antiprotein antibodies (18). The use of PC-BSA-BAC as an immunoadsorbent and either  $^{125}\text{I}$ -labeled anti-Fab, anti- $\mu$ , anti- $\gamma$ 1, or anti- $\alpha$  antibody as a detecting reagent has permitted accurate quantitation of as little as 1 ng of anti-PC antibody. The specificity of the assay was clearly demonstrated by a lack of reactivity of serum from germfree BALB/c and C57BL/6 mice and inhibition of antibody binding by phosphorylcholine.

The radioimmunoassay used for the quantitation of the TEPC 15 idiotype is a modification of that of Carson and Weigert (22). With this method, as little as 1 ng of immunoglobulin of the TEPC 15 idiotype can be quantitated. The specificity of this assay is demonstrated by the fact that 20 ng of a different anti-PC-binding myeloma protein, MOPC 603, does not inhibit to the extent of 1 ng of TEPC 15 protein (Fig. 1). The small amount of inhibition observed with high quantities of MOPC 603 protein appears to be nonspecific since different antigen-binding myeloma proteins, i.e. MOPC 104E and W3129, inhibit to the same degree. The validity of both of these radioimmunoassays as accurate methods for the quantitation of specific antibody is seen in Fig. 2, which demonstrates that these two independent radioimmunoassays give excellent quantitative correlation on a weight basis for the TEPC 15 myeloma protein as well as several monoclonal antibodies of this idiotype.

The availability of these radioimmunoassays permitted not only a careful analysis of anti-PC in the serum of nonimmune and immunized mice, but also allowed the analysis of antibodies produced by the clonal progeny of isolated precursor cells in fragment cultures. This system utilizes the transfer of syngeneic donor spleen cells in limiting cell doses into lethally irradiated carrier-primed recipients. The homing efficiency and stimulability of the cells reaching the recipient spleen has been previously determined to be 4% of the injected cells (reference 26 and footnote 3). Diced fragments of the recipient spleen are then stimulated in the wells of microtiter plates. Each fragment statistically contains zero to one B cells for a specific antigen and an excess of primed T cells (9); therefore, each precursor cell is stimulated individually, isolated from the influence of other precursors for that antigen. The monoclonality of the antibody derived from such cultures has previously been demonstrated by the linear dose dependence of the response (9), and the homogeneity of this antibody is established by the criteria of isoelectric focusing (27), homogeneous binding activity (27), as well as regain of this activity after heavy- and light-chain recombination (28). Fig. 2 in fact provides additional evidence for the monoclonality of each positive fragment. If a focus contained two different precursors for PC (one with the TEPC 15 idiotype and the other without), the amount of antibody from the sample would not correlate with the idiotype data obtained from the same clone.

In addition to permitting the stimulation of each B cell separately from other relevant B cells, the transfer to a carrier-primed environment maximizes precursor cell stimulation. One of us has previously shown that there is an obligatory dependence for the stimulation of primary precursor cells in splenic foci for recognition of the homologous carrier used to immunize the recipients (9). Furthermore, recognition of Hy is maximized in this study by use of a tripeptide spacer. The diazotization process generally used to couple haptens may alter determinants on a carrier such that T cells may not recognize it (29), but such alterations apparently do not occur when the spacing group is introduced between PC and the carrier. This notion is experimentally confirmed by the poor stimulation achieved after injection of normal spleen cells into Hy-primed recipients followed by stimulation with PC-Hy in splenic fragment cultures (P. Gearhart, unpublished results).

An analysis of the monoclonal antibodies produced in fragment culture revealed that while a majority of clones produced antibody of the TEPC 15 idiotype, 2-50% of clones derived from different donors produced anti-PC antibody of other idiotypes. This finding is in contrast to those of previous investigators (7, 8). Preliminary analysis by isoelectric focusing (17) indicates that the non-TEPC 15 idiotype monoclonal antibodies are of several distinct clonotypes (N. Sigal, unpublished results). Evidence that these non-TEPC 15 antibodies are indeed the products of anti-PC specific B cells is obtained from the fact that (a) the clones were stimulated by the PC haptenic determinant, (b) the

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<sup>3</sup>Pickard, A., and N. Klinman. Relationship between antigen-binding cells and precursors to antibody-forming cell clones before and after immunization. Manuscript submitted for publication.

radioimmunoassay is carried out on PC-BSA-BAC which should carry no determinants of the antigen other than PC, and (c) these antibodies can also be shown to bind to R36A pneumococcus.

Several factors may be responsible for our ability to generate anti-PC antibody-producing clones other than those of the TEPC 15 idiotype. First, the splenic focus technique isolates hapten-specific B cells, allowing full expression of each specificity. It is possible that the TEPC 15 clone exhibits a clonal dominance in vivo much like that described previously for a transferred DNP-specific clone (30). Secondly, it is possible that only the TEPC 15 clone can be expressed when stimulation is carried out in the absence of maximal carrier-specific help. Thus stimulation by hapten on a modified carrier or the R36A bacterium, which has been described as carrier independent (31), may only be able to stimulate clones of the TEPC 15 idiotype. The latter explanation is supported by the finding of Lee et al. (8) who reported that the immune response to PC is totally suppressed by anti-TEPC 15 antibody, thus indicating the inability of these investigators to obtain anti-PC antibodies of other idiotypes even in the absence of a response of the TEPC 15 clone. It should be noted, however, that careful analysis of isoelectric focusing of serum anti-PC antibody produced in response to prolonged immunization with R36A bacteria in our laboratory indicates that the serum antibody does not all react with anti-TEPC 15 antibody.<sup>2</sup> Such an analysis of serum anti-PC antibody has not been carried out previously so that the presence of non-TEPC 15 anti-PC antibodies may have been obscured (24). Furthermore, Claflin et al. (7) have described incomplete inhibition of plaque-forming cells specific for PC with anti-idiotypic serum against the TEPC 15 determinant.

Analysis of the monoclonal anti-PC antibody produced in fragment culture also revealed that a majority of monoclonal antibodies contained the IgG1 class. This was true regardless of whether or not the clone was of the TEPC 15 idiotype. Again, this finding is in contrast to previous reports which indicated that the anti-PC response was solely of the IgM class (2, 7, 8) and may result from the fact that carrier-specific enhancement is magnified in the fragment culture system; IgG production has been reported to be at least partially dependent on T-cell help (32). It should be noted that a majority of the monoclonal antibodies analyzed contain both IgM and IgG1. This is consistent with a previous report from this laboratory showing double class production of anti-DNP-specific antibody by single clones (33). A subsequent publication will demonstrate in detail the monoclonality of the IgM and IgG antibodies in these culture fluids. In agreement with others (7), we have found that the majority of antibody in the serum of nonimmune conventionally reared BALB/c mice and mice immunized with R36A *Pneumococcus* is of the IgM class. However, we have confirmed our in vitro findings of both IgM and IgG production in the presence of maximal carrier recognition by demonstrating IgM and IgG anti-PC antibody in the serum of mice immunized with PPC-TGG-Hy after preliminary priming with Hy. Indeed, antibody of the TEPC 15 idiotype can be demonstrated in both the IgM and IgG fraction of this serum, again emphasizing the obligate carrier dependence of IgG production in the primary immune response. Some IgG1 could be detected by our methods in the serum of nonimmune and bacterial-immunized mice, which may

reflect the sensitivity of the radioimmunoassay. The inability of other investigators (2, 7, 8) to observe indirect plaque-forming cells from *in vitro* stimulated cells may result from the lack of appropriate T-cell help or insensitivity of the plaque assay to this antibody. Nevertheless it is clear that B cells specific for PC, given the proper T-cell help and immunogenic antigen, can make IgG1 either *in vivo* or *in vitro*.

The ability to correlate the production of anti-PC antibody of both the IgM and IgG1 classes with detection of the TEPC 15 idiotype allows us to make more definitive statements about the IgM and IgG1 produced. The purified IgM and DEAE-cellulose-eluted IgG anti-PC fractions from the serum of immune mice both contained the TEPC 15 idiotype. This demonstrates that regardless of the class of antibody, the idiotype of the variable region is apparently conserved, a conclusion also reached by Cosenza and Köhler (3) who demonstrated the TEPC 15 idiotype on IgA and IgM. Confirming this point is the data from Fig. 2 which indicates that on a weight basis, each of the tested monoclonal antibodies (some of which are IgM and some IgG1) binds the anti-idiotype antibody as well as the standard TEPC 15 myeloma protein does. If only one heavy-chain class of monoclonal anti-PC antibody contained the idiotype, the linear relationship would not exist.

The combination of the splenic foci technique with the anti-idiotypic analysis of culture fluids allows a high degree of resolution in studying the response of a clone of a known idiotype to various parameters. The modulation of the immune response by T-independent or -dependent antigens as well as antigen dose requirements for stimulating primary and secondary B cells can now be probed at the level of single cell precursors for a given antibody product. In this regard an interesting finding is demonstrated in Fig. 2 where it can be seen that B cells of a single clonotype can not only give rise to antibody of differing immunoglobulin classes but also can be stimulated to produce clones of differing size under apparently identical conditions of stimulation. By such analyses, it should now be possible to analyze the natural history of a defined precursor cell clone.

## Summary

Immune responsiveness to phosphorylcholine (PC) in BALB/c mice has been characterized by combining (a) usage of highly sensitive radioimmunoassays for quantitation of antibody, heavy-chain class, and idiotype on a weight basis; (b) isolation of PC-specific B cells in fragment cultures; and (c) stimulation in a carrier-primed environment with the PC hapten coupled to carrier through a tripeptide spacer in order to maximize carrier recognition. The specificity and accuracy of the radioimmunoassays have been verified by specific inhibition, lack of nonspecific binding, and excellent concordance of values for monoclonal antibody concentration obtained independently for Fab and idiotype content. The latter evidence also serves as strong confirmation of the monoclonality of *in vitro* monofocal responses as well as the preservation of the idiotype on antibodies of differing immunoglobulin classes. The results indicate that while B cells expressing the TEPC 15 idiotype predominate, other idiotypes may be represented by 2-50% of PC-specific precursors, and monoclonal antibodies even

of the TEPC 15 idiotype are produced in both the IgM and IgG1 immunoglobulin classes. These findings are confirmed by the analysis of serum antibodies produced in carrier-primed mice immunized with hapten coupled through a tripeptide spacer, thus re-emphasizing the enhancement of primary responsiveness, particularly IgG1 production, by maximizing carrier recognition. The finding of idiotype diversity in the PC response, as well as diversity of expression in terms of quantity and immunoglobulin class of antibody synthesized by the clonal progeny of B cells within the TEPC 15 clonotype, emphasize the heterogeneity of the B-cell population both in terms of the specificity repertoire and the physiological state of cells even within a single clonotype.

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