IMMUNE RESPONSES IN VITRO

III. Development of Primary γM, γG, and γA Plaque-Forming Cell Responses in Mouse Spleen Cell Cultures Stimulated with Heterologous Erythrocytes*

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In vivo, after antigenic stimulation, mice develop antibody responses representing all five major immunoglobulin classes (1–5). Evaluation of serum antibody responses to heterologous erythrocytes at the cellular level is possible with the hemolytic plaque technique which facilitates the screening of large numbers of lymphoid cells for those cells releasing antibodies against erythrocyte antigens. The technique as described initially by Jerne et al. (6, 7) enumerated only cells releasing high hemolytic efficiency, γM antibodies (6–9). Modifications have permitted enumeration of those cells releasing antibodies of other immunoglobulin classes (10–12). By using heterologous anti-mouse globulins monospecific for each major immunoglobulin class, antibody-releasing cells from each class have been detected and the kinetics of their appearance after immunization have been described (1, 2, 5). γG[1] plaque-forming cells (PFC) usually are detected later and reach maximum numbers later than do γM PFC (1, 2, 5). γM PFC are thought to develop by rapid division of precursor cells restricted to production of antibody of the γM class (6, 13–17). The developmental pathway of γG PFC has still not been precisely defined. These cells may arise by proliferation of specific precursors restricted to production of antibodies of a single immunoglobulin class (2, 18, 19), or they may develop by conversion of cells from γM to a given class of γG antibody syn-

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[1] *Abbreviations used in this paper:* "γG" is used in this paper to refer to γ_1, γ_{2a}, and γ_{2b} immunoglobulins in aggregate; HBSS, Hanks' balanced salt solution lacking sodium bicarbonate; HRBC, horse red blood cells; MEM, minimal essential medium; PFC, plaque-forming cell(s); RBC, red blood cells; SRBC, sheep red blood cells.

thesis (20). γG PFC may also develop by recruitment of potential antibody-producing cells restricted to immunoglobulin class without cell division (1).

The cell suspension system for stimulation of antibody production to heterologous erythrocytes by murine spleen cells in vitro, as developed by Mishell and Dutton (21), is ideally suited for investigation of these possibilities. Here, critical variables such as antigen presentation and interactions among required cell types can be more precisely controlled and the essential events in the development of responses representing individual immunoglobulin classes analyzed. However, in previous studies with this culture system, numerically significant primary γG PFC responses were not detected (22).

The present studies were initiated to investigate whether this failure was the result of methodology and reagents or a more fundamental failure of the culture system to support development of primary γG and γA PFC responses. We now present a modification of the hemolytic plaque assay which has enabled us for the first time to directly enumerate PFC representing all the major immunoglobulin classes in cultures of mouse spleen cells stimulated in vitro with heterologous erythrocytes. The culture conditions found to support development of these responses and the kinetics of appearance of PFC representing each immunoglobulin class will be described. The analysis of the effects of anti-erythrocyte antibody on primary γM and γG PFC responses will illustrate some of the fundamental differences in these responses.

Materials and Methods

Animals.—C57BL/6N and BALB/cAnN male mice, 3–4 months old, and New Zealand white male rabbits, 3–6 months old, obtained from the NIH Rodent and Rabbit Production Section were maintained on the appropriate Purina chow and water *ad libitum*. Goats were maintained at the NIH Ungulate Division on a standard goat diet.

Erythrocyte Antigens and Anti-Erythrocyte Antisera.—Erythrocytes from one sheep (SRBC) and one horse (HRBC), maintained at the NIH Ungulate Division, were collected biweekly under sterile conditions into acid citrate-dextrose solution. Erythrocytes were washed by centrifugation three times with 40 volumes of sterile Hanks' balanced salt solution (HBSS) and resuspended in HBSS to 20×10^8 erythrocytes/ml for immunization or for use in the hemolytic plaque assay; erythrocytes for stimulation of cultures were resuspended in HBSS to 2.5×10^8 erythrocytes/ml.

Antisera against SRBC and HRBC were obtained from rabbits bled by cardiac puncture 10 days after the last of three weekly intravenous injections of 5 ml of a suspension containing 20×10^8 RBC/ml. Sera were separated, decomplemented by heat at 56°C for 30 min, sterilized by passage through a prewashed 0.22 μ Millipore filter, and stored at −20°C in 1 ml portions. Hemagglutinin titers were 1/6000 to 1/8000 against the immunizing erythrocyte and less than 1/10 against nonimmunizing erythrocytes. Antibody activity was resistant to treatment with 0.1 M 2-mercaptoethanol for 30 min at 37°C. Antibody concentration is expressed as the reciprocal of the titer in the culture dish. For example, a serum with a titer of 1/8000 was diluted 1/1000 and 0.05 ml was added to a 1 ml culture giving a concentration of 4×10^{-1}.

Preparation of Myeloma Proteins and Anti-Immunoglobulin Sera.—Purified myeloma proteins were prepared from ascitic fluid from BALB/cAnN mice bearing plasma cell tumors

adapted to the ascites form. Pools of urine from mice bearing myeloma tumor MOPC-104E were the source of λ-chain. Immunogens for preparation of polyvalent anti-γG and anti-γ2a + 2b were purified from pools of hyperimmune sera from several inbred strains of mice. Immunogens were purified by preparative electrophoresis in a Geon-Pevikon resin block (23) followed by gel filtration through Sephadex-G-200 (22, 23). Fc fragment was isolated from MOPC-31c myeloma protein after papain digestion for the preparation of anti-γ_1 sera (24, 25). Antiglobulins used for development of γG and γA PFC were prepared in rabbits using the immunogens prepared as described above and listed in Table I. Anti-mouse μ-chain antibody was prepared in goats.

TABLE I

Biological Reagents

Antiglobulin	Immunogen	Absorbed with*
Goat anti-μ	MOPC-104E myeloma protein	λ-chain (MOPC-104E urinary protein)
Rabbit anti-γM	" "	" "
Rabbit anti-γG (polyvalent)	Slow electrophoretic fraction from hyperimmune serum	No absorption
Rabbit anti-γ_{2a+2b}	" "	γ_1 (MOPC-70A myeloma protein)
Rabbit anti-γ_1	MOPC-31c myeloma protein Fc piece	γ_{2b} (MPC-37 myeloma protein)

Myeloma protein	Immunoglobulin class
MOPC-104E	γM
MPC-47, MOPC-70A, MOPC-31c	γ_1
LPC-1, RPC-5	γ_{2a}
MPC-11, MPC-37	γ_{2b}
SPC-1, MPC-40	γA

* Germfree serum was used to absorb antibodies to serum proteins other than immunoglobulins.

All separated rabbit and goat sera were decomplemented at 56°C for 30 min and absorbed twice with 1 ml each of washed, packed SRBC and HRBC/10 ml serum and once with 1 ml of washed, packed C57BL/6N spleen cells/10 ml serum. Initial specificity of the antiglobulins was tested by double diffusion in agar. Required absorptions to achieve monospecific antiglobulins were performed using the purified myeloma proteins and germfree serum as indicated in Table I. After absorption, monospecific antiglobulins had no detectable reaction with nonhomologous myeloma proteins by agar diffusion. Monospecific antiglobulins coupled to bromoacetyl-cellulose (26) did not bind ^{125}I-labeled light chains or myeloma proteins of immunoglobulin classes other than the one against which the antiglobulin was directed.

Spleen Cell Cultures.—Spleen cell suspensions were prepared under sterile conditions by gently teasing apart the spleens with forceps into cold HBSS; after residual tissue fragments had settled by gravity, the supernatant cell suspension was separated and centrifuged for 10 min at 600 *g* at 4°C. The sedimented cells were resuspended to the desired density, usually 1×10^7 cells/ml, in completely supplemented Eagle's minimal essential medium (MEM), monolayer type, with Hanks' salts (21, 22, 27), containing 10% fetal bovine serum (Reheis

Chemical Co., Kankakee, Ill., Lot E21505), and 50 units each/ml of penicillin and mycostatin, and 50 μg/ml of streptomycin. Tissue culture media and supplements except sera were obtained from Microbiological Associates, Inc., Bethesda, Md.

The spleen cells in 1 ml volumes were incubated with or without erythrocyte antigens, usually 10^6–10^7/culture, in a water-saturated atmosphere of 7% O_2, 10% CO_2, and 83% N_2 at 37°C in a large air-tight plastic box on a rocker platform oscillating at 3½ complete cycles/min. Experimental groups were composed of three culture dishes which were supplemented

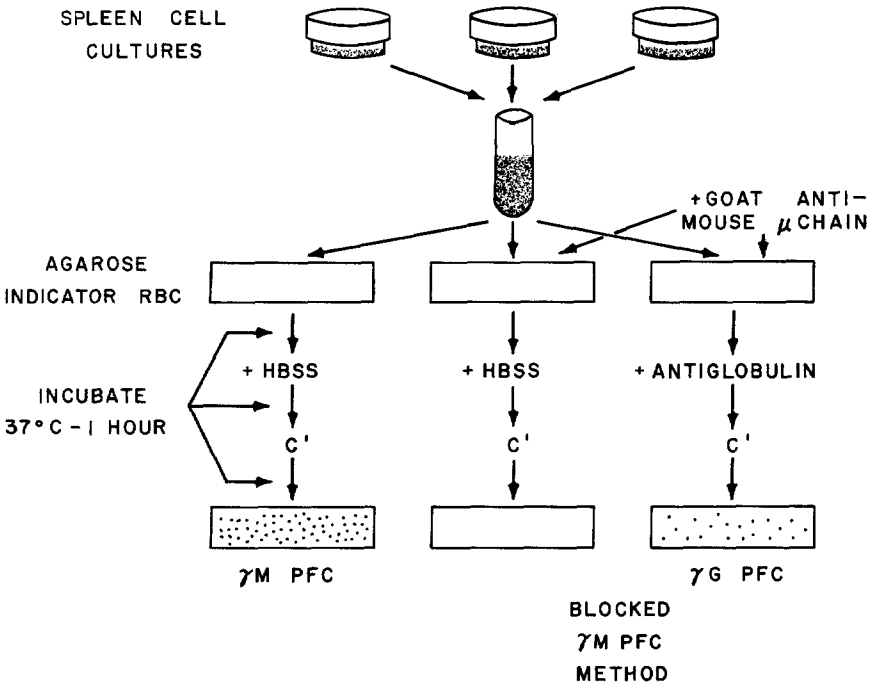

FIG. 1. Schematic representation of the assay procedure for direct (γM) PFC and indirect (γG and γA) PFC using the blocked γM PFC method.

daily with 0.05 ml fetal bovine serum and 0.05 ml of a nutritional mixture prepared by supplementing HBSS with 10 mg/ml dextrose and glutamine and amino acids in twice the concentration as in completely supplemented MEM (22, 27).

Hemolytic Plaque Assay.—Cells from each experimental group were harvested by aspiration, pooled and sedimented by centrifugation. The supernatant culture fluid was discarded and the sedimented cells were resuspended in HBSS at dilutions expected to give 30–300 plaques/slide. The number of PFC was determined by the Jerne hemolytic plaque technique (6, 7), modified for use with glass microscope slides (5, 21, 22) and illustrated in Fig. 1.

For enumeration of direct or γM PFC, agarose (L'Industrie Biologique Francaise S.A., Gennevilliers, France), 0.5% in tris(hydroxymethyl)aminomethane (Tris)-buffered Eagle's MEM without serum (Medium 327 from the NIH Media Unit), 0.40 ml; indicator erythrocytes, 7.5%, 0.05 ml; and the culture cell suspension, 0.1 ml, were mixed at 44°C in 10 × 75

mm glass tubes and poured onto microscope slides previously coated with 0.1% agarose in water. Duplicate slides, prepared from each group, were incubated for 1 hr at 37°C in a humid atmosphere; HBSS was flooded under the slides and incubation was continued for 1 hr. Guinea pig complement (Baltimore Biological Laboratories, Baltimore, Md.), diluted $\frac{1}{10}$ or $\frac{1}{20}$ in 0.85% NaCl buffered at pH 7.4 with 0.01 M phosphates and containing 5×10^{-4} M Mg^{++} and 1.5×10^{-4} M Ca^{++} (28) was added, and incubation was continued for another hour. PFC were counted at 7× magnification with indirect illumination.

Indirect PFC (γG and γA immunoglobulin classes) were enumerated by two new methods in addition to the conventional method for indirect PFC (29, 30). In the *blocked γM PFC method*, 0.05 ml of a predetermined optimal dilution of goat anti-mouse μ-chain antibody was added to the assay tubes containing agarose in Tris-MEM and indicator erythrocytes before the addition of the immune cell suspension (Fig. 1). The mixture was poured on agarose-coated microscope slides. After 1 hr incubation, a predetermined optimal concentration of polyvalent or monospecific rabbit anti-mouse globulin in HBSS was flooded under the slides; incubation was continued for 1 hr, the antiglobulin poured off, and the slides washed once gently with HBSS. Complement was then added and after 1 hr incubation, PFC were counted.

In the *sequential PFC development method*, assay slides containing indicator erythrocytes and immune cells in agarose in Tris-MEM were incubated as described for development of γM PFC. After these PFC were counted (count 1), antiglobulin at optimal dilution or HBSS was flooded under the slides and incubation was continued as described for development of γG PFC in the blocked γM PFC method. No goat anti-μ-antibody was ever added to these preparations. After incubation with complement, total plaques were counted (count 2).

Cells recovered from culture dishes were counted with a Coulter Counter, Model B, with a 100 μ aperture (Coulter Electronics Inc., Hialeah, Fla.). The γM PFC/10^6 recovered cells were calculated from slides developed only with complement. γG and γA PFC/10^6 recovered cells in the blocked γM PFC assay method were calculated directly from assay slides developed by antiglobulin and complement since the goat anti-μ-antibody in the agarose layer prevented development of any γM PFC. Control slides of indicator erythrocytes, goat anti-μ-antibody, and immune cells in agarose incubated only with complement insured the complete blocking of all γM PFC.

In the sequential PFC development method, counts 1 and 2 were converted to PFC/10^6 recovered cells. The net increase in PFC or indirect PFC/10^6 recovered cells was calculated by subtraction of PFC/10^6 cells (count 1) and additional γM PFC/10^6 cells developed by HBSS and complement from total PFC/10^6 cells (count 2). Data presented are from experiments with comparable cell recovery in all experimental groups and are representative of data obtained in at least three separate expreriments.

RESULTS

Development of the Blocked γM PFC Assay Method.—Previous studies in our laboratory had not detected significant primary γG PFC responses in spleen cell cultures of BALB/c AnN mice using conventional methods of facilitation with several polyvalent antiglobulins (22). These polyvalent antiglobulins are known to inhibit γM PFC development; this inhibition must be corrected for in calculation of γG PFC developed by such antiglobulins (2, 29, 30). In the present studies, we found that the inhibition of γM PFC development by polyvalent antiglobulins in cultures containing only γM PFC varied considerably from experiment to experiment and among mouse strains. The mono-

specific antiglobulins also variably inhibited γM PFC development, but to a far lesser degree. After taking all these variables into account, consistent primary γG PFC responses were recently detected in our culture system using the conventional assay procedures and the antiglobulin reagents described herein (31). However, the result obtained with this method showed considerable variability; therefore, a more reliable method for detection of indirect PFC was developed which does not require application of correction factors to the data.

Anti-γM antibody is known to inhibit development of γM PFC (2, 29, 30). We found that goat anti-μ-chain antisera prepared against MOPC-104E and absorbed with λ-light chains reproducibly inhibited development of all γM PFC when incorporated into the assay reagent mixture at final dilutions of $\frac{1}{2000}$–$\frac{1}{3600}$ depending on the antiserum. This inhibition was almost completely reversed by the addition of MOPC-104E myeloma protein at a concentration of 100 μg/ml; other myeloma proteins (γ_1, γ_{2a}, γ_{2b}, and γA) produced at most 10% reversal at 100 μg/ml. Unrelated proteins, such as bovine serum albumin, had no effect on the blocking of γM PFC development even at 1 mg/ml.

The incubation of assay slides containing the anti-μ-antibody and immune cells from 5-day cultures with polyvalent or monospecific antiglobulins and complement reproducibly resulted in the development of significant numbers of plaques. The number of γG or γA PFC enumerated by the blocked γM PFC method was comparable to the number detected by the sequential PFC development method (Table II); both these methods compared favorably with the conventional assay method for detection of γG or γA PFC. In addition, the number of indirect PFC detected by developing PFC with anti-γ_1 and anti-γ_{2a+2b} together was similar to the sum of the number of γ_1 and γ_{2a+2b} PFC detected separately (Table II).

Cultures of spleen cells from BALB/cAnN mice developed lower γG PFC responses to both SRBC and HRBC at all ages tested than did cultures of spleen cells from C57BL/6N mice; therefore, C57BL/6N mice were used for the remainder of the experiments reported.

Specificity of Plaques Developed by Antiglobulins in the Blocked γM PFC Assay Method.—The specificity of antiglobulins for development of γG and γA plaques was demonstrated by absorption of antiglobulins with myeloma proteins and a competitive inhibition method.

Monospecific and polyvalent antiglobulins were absorbed with myeloma proteins as indicated in Table III. Absorption was accomplished by adding sufficient homologous myeloma proteins to precipitate the antibody completely; samples of antiserum with equal amounts of other myeloma proteins added served as controls. The number of plaques developed by these antiglobulins after absorption and removal of precipitates by centrifugation was

compared with the number developed by untreated antiglobulin at the same dilution and the per cent of inhibition of plaque development was determined.

The developing activity of the monospecific antiglobulins (anti-γ_1, anti-γA, and anti-γ_{2a+2b}) was removed only by absorption with the homologous myeloma protein(s). The activity of the polyvalent anti-γG was markedly decreased after absorption with myeloma proteins of the γG class; about 20%

TABLE II

Comparison of the Blocked γM PFC, Sequential PFC Development, and Conventional Methods for Enumeration of γG and γA PFC

Antiglobulins used to develop plaques	PFC/10^6 recovered cells		
	Blocked γM PFC method		Conventional method
	Exp. 1	Exp. 2	Exp. 2
Anti-γ_1	606	377	356
Anti-γ_{2a+2b}	303	309	259
Anti-γ_1 + anti-γ_{2a+2b}	947 (909)*	656 (686)	578 (601)
Anti-γA	306	271	234

	Sequential PFC Development Method			
	Total increase in PFC/10^6 cells (Count 2—count 1)		Net increase (γG or γA PFC) PFC/10^6 cells‡	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
HBSS	70	33	—	—
Anti-γ_1	698	397	628	364
Anti-γ_{2a+2b}	407	339	337	306
Anti-γA	400	337	330	304

Immune cells were from 5-day stimulated cultures of C57BL/6N spleen cells; γM PFC/10^6 recovered cells were Exp. 1, 2235; Exp. 2, 1358.

* Numbers in parentheses are the sums of PFC/10^6 cells from the separate assays with anti-γ_1 and anti-γ_{2a+2b}.

‡ Number of additional γM PFC appearing after incubation with HBSS and complement have been subtracted from the total increase in PFC/10^6 cells to obtain the net increase due to PFC developed by antiglobulin and complement.

reduction in activity was also detected after absorption with γM or γA myeloma proteins (Table III). Results using both the blocked γM PFC and sequential PFC development methods were comparable (Table III). However, a word of caution must be raised with regard to the blocked γM PFC method when antiglobulins to which myeloma proteins have been added either for the purpose of absorption or competitive inhibition of activity are used in this assay method. γM myeloma proteins, or reagents contaminated with γM protein not detectable by other methods, may cause some reversal of the blocking of γM PFC development by the anti-μ-serum. This reversal of blocking of γM PFC development was highly reproducible for any antiglobulin

to which myeloma proteins had been added. γ_1, γ_{2a+2b}, and γA myeloma proteins caused less than 10% reversal at concentrations of 100 μg/ml. γM myeloma protein, however, produced almost 95% reversal at 100 μg/ml, but less than 10% reversal at 10 μg/ml. Therefore, the degree of reversal for each antiglobulin reagent to which myeloma proteins have been added must be determined on an immune cell suspension containing only γM PFC (spleen cells

TABLE III

Inhibition of γG- and γA-Plaque Development by Absorption of Antiglobulins with Myeloma Proteins

Antiglobulin	Myeloma protein absorbed with	Average per cent inhibition of plaque development*	
		Blocked γM PFC method	Sequential PFC development method
Anti-γ_1	γ_1	72	81
	γ_{2a+2b}	4	2
	γM	2	1
	γA	5	3
Anti-γ_{2a+2b}	γ_1	8	6
	γ_{2a+2b}	81	89
	γM	0	0
	γA	0	0
Anti-γA	$\gamma_1 + \gamma_{2a+2b} + \gamma$M	0	0
	γA	97	95
Anti-γG (Polyvalent)	$\gamma_1 + \gamma_{2a+2b}$	93	90
	γM	20	26
	γA	17	13

* The values represent averages from at least four separate experiments with different C57BL/6N immune cell suspensions from 5-day stimulated cultures using the absorbed antiglobulins at the same dilutions as the control, unabsorbed antiglobulins. The method used for absorption is presented in the text.

from a 3 day stimulated culture) on the same day that the reagent is used in assays on cell suspensions containing γG or γA PFC. Only in this manner can an accurate evaluation of the effects of proteins on the activity of antiglobulin reagents in the blocked γM PFC method be made. This correction has been applied to the data obtained with the blocked γM PFC method in Table III.

Competitive inhibition of antiglobulin activity with myeloma proteins also demonstrated specificity. Myeloma proteins at a final concentration of 100–0.1 μg/ml were incubated with optimal dilutions of the antiglobulins at 37°C for 1 hr with periodic agitation. The activity of these preparations in developing indirect plaques was compared with untreated antiglobulins and the per cent of

inhibition of indirect PFC development for each myeloma protein at each concentration was calculated. Significant inhibition of activity of any antiglobulin was achieved only with the homologous myeloma protein(s) (Table IV). Homologous myeloma proteins inhibited facilitation almost completely at concentrations of 10 μg/ml. By contrast, other myeloma proteins produced at most 20% inhibition at concentrations of 100 μg/ml. Corrections were applied

TABLE IV

Competitive Inhibition of γG- and γA-Plaque Development by Myeloma Proteins

Antiglobulin	Myeloma protein	Average per cent inhibition at concentration of myeloma protein (μg/ml)*			
		100	10	1	0.1
Anti-γ_1	γ_1	96 (97)‡	87	57	39
	γ_{2a+2b}	6 (3)	4	2	1
	γM	8 (7)	3	3	0
	γA	7 (3)	6	0	0
Anti-γ_{2a+2b}	γ_{2a+2b}	100 (100)	98	89	67
	γ_1	20 (17)	0	0	0
	γM	21 (23)	9	3	2
	γA	13 (17)	11	2	0
Anti-γA	γA	100 (98)	97	94	78
	γ_1	0 (2)	2	3	3
	γ_{2a+2b}	2 (2)	1	4	2
	γM	5 (2)	2	3	3
Anti-γG	$\gamma_1 + \gamma_{2a+2b}$	100 (98)	98	100	85
	γM	11 (7)	3	3	1
	γA	7 (3)	2	1	1

* The values represent the averages from at least four separate experiments with different C57BL/6N immune cell suspensions from 5-day stimulated cultures at each myeloma protein concentration. Details of the procedure and calculations are in the text.

‡ Values in parentheses are averages from three separate experiments using the sequential PFC-development method.

for reversal of blocking of γM PFC in all experiments at each concentration of myeloma protein. This was especially important when γM myeloma protein was the added protein; the data with the 100 μg/ml concentration of this myeloma protein were at best an approximation since blocking of γM PFC was completely reversed at this concentration. Confirmation of all data in the competitive inhibition test with the 100 μg/ml concentrations was obtained using the sequential PFC development method with immune cell concentrations that gave accurately countable numbers of PFC per slide (Table IV).

The obvious control of substituting normal rabbit serum, decomplemented

and absorbed with SRBC and mouse spleen cells, at the same dilution as the rabbit antiglobulins, should be adequate. However, several "normal rabbit sera" developed indirect PFC. Some of these sera consistently developed as many or more PFC than did optimal dilutions of anti-γG, anti-γ_1, or anti-γ_{2a+2b}. This developing activity was not competitively inhibited by 100 μg/ml of any myeloma protein. Furthermore, the addition of γ_1 or γ_{2a+2b} myeloma proteins in amounts exceeding that required to absorb activity from specific antiglobulins did not significantly reduce activity in the normal rabbit sera. Only absorption with antigen-antibody complexes significantly reduced the developing capacity of these sera; activity of the monospecific and polyvalent anti-γG sera were unaffected by absorption with the same antigen-antibody complexes. Data similar to those obtained with the blocked γM PFC method were also obtained with the sequential PFC development method.

It is uncertain whether this facilitating activity of some normal rabbit sera is due to a rheumatoid-like factor (32, 33) or some other artifact. This observation has merited a more detailed separate study.[2] It is clear, however, that antiglobulins with nonspecific facilitating activity as found in normal rabbit sera and some unabsorbed polyvalent anti-γG sera must be sought out and eliminated from routine use. Monospecific and absorbed polyvalent antiglobulins probably lack this abnormal reactivity since during their purification the immune complexes formed in the reaction mixture removed the serum components mediating the nonspecific facilitating activity.

Culture Variables Affecting γM, γG, and γA PFC Responses.—As indicated above, C57BL/6N mice consistently developed higher PFC responses than BALB/cAnN mice; responsiveness among other inbred mouse strains has not yet been fully investigated. No difference was found in the γM, γG, and γA PFC responses of C57BL/6N males and females. SRBC and HRBC both stimulated adequate γM, γG, and γA PFC responses in cultures of C57BL/6N spleen cells. Since adequate numbers of PFC representing all the immunoglobulin classes were detected with the culture conditions described above, modifications of these conditions were not made.

Optimal spleen cell concentration for development of maximal 5-day γM, γG, and γA PFC responses was consistently between 15 and 8.5 $\times$ 10^6 spleen cells/culture dish. In subsequent experiments, the spleen cell concentration was adjusted as closely as possible to 10 $\times$ 10^6 cells/culture. The plot of the log of the γM or γG PFC response per dish against the log of the number of spleen cells initially cultured as the spleen cell concentration was varied from 25 to 0.5 $\times$ 10^6 cells/dish produced a regression line with a slope approximately 2.5 over the linear portion of the plot. Earlier, a similar analysis of the γM response alone produced a regression line with a similar slope; it was suggested

[2] Pierce, C. W., B. Benacerraf, and R. Asofsky. Development of mouse indirect hemolytic plaques by normal rabbit sera. Manuscript in preparation.

that interactions among three cell types were required for development of that response (34).

Optimal antigen dose for stimulation of maximum γM PFC responses was about 1×10^7 red blood cells (RBC)/culture; however, maximal γG PFC responses developed consistently in cultures stimulated with 10^6 RBC/culture (Table V). Optimum γA responses were also obtained with 10^6 RBC (data

TABLE V

Effect of Mouse Age and Antigen Dose on 5-Day Primary γM and γG PFC Responses In Vitro

Culture Variables: 1.2 × 10^7 spleen cells/culture		PFC/10^6 recovered cells			
		Exp. 1		Exp. 2	
Mouse age	SRBC dose	γM	γG	γM	γG
months					
2	10^8	786	149	942	243
	10^7	749	147	939	318
	10^6	690	340	835	395
	10^5	609	221	260	162
	0	48	6	29	0
4	10^8	1346	427	1122	347
	10^7	1201	481	1474	351
	10^6	1066	528	1008	475
	10^5	495	275	474	458
	0	50	3	171	5
6	10^8	832	177	1466	273
	10^7	837	191	1946	249
	10^6	665	235	1059	296
	10^5	309	138	640	130
	0	29	7	60	8
9	10^8	715	220	661	101
	10^7	889	271	859	153
	10^6	686	350	688	373
	10^5	401	121	290	97
	0	111	2	30	4

not shown). RBC used within 2 wk after collection stimulated consistently reproducible responses in all immunoglobulin classes; RBC used after 14 days storage stimulated lower and more varied responses and were more fragile in the assay system. Subsequent experiments used RBC less than 14 days old at $3–7 \times 10^6$ RBC/culture. Maximal γM and γG responses developed in cultures of spleens from mice 4–6 months old; however, responses in spleen cell cultures from mice as young as 2 months or as old as 9 months were adequate for the study of cellular events in the development of either γM or γG responses (Table

V). Responses in unstimulated cultures were not consistently increased in elderly mice. The optimal RBC doses for stimulation of maximal γM and γG responses did not vary with mouse age.

Kinetics of Primary γM, γG, and γA PFC Responses In Vitro.—The PFC responses of cells representing major immunoglobulin classes were determined daily in cultures of spleen cells incubated with or without SRBC. After a 1

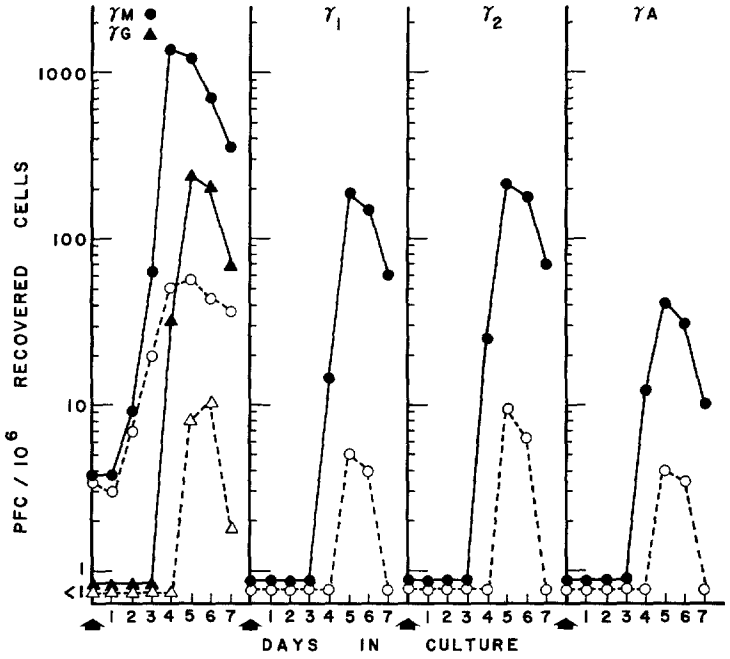

FIG. 2. Kinetics of appearance of γM, γG (polyvalent), and immunoglobulin class-specific γ_1, γ_{2a+2b}, and γA PFC in cultures of mouse spleen cells incubated with (closed figures) or without (open figures) SRBC. Daily cell recoveries in stimulated and unstimulated cultures were comparable. Data is from a representative experiment.

day lag period, the number of γM PFC increased exponentially from background levels, about three PFC/10^6 spleen cells, to about 1000 PFC/10^6 cells on day 4 in stimulated cultures. On day 5, only a slight decrease from this maximum was detected, but thereafter γM PFC decreased steadily (Fig. 2). An increase in γM PFC against SRBC was detected also in unstimulated cultures; this response reached a maximum on days 4–5 and was always less than 10% of the response in stimulated cultures. Similar background responses have been regularly detected by other investigators and are most likely due to antigens cross-reactive with SRBC antigens present in fetal bovine serum (21).

PFC responses of cells representing γG and γA immunoglobulin classes pre-

sented a definite contrast. No significant increase from background levels of 0.1–0.3 PFC/10^6 spleen cells was detected during the first 3 days of culture. Thereafter, a rapid exponential increase to maximum numbers on days 5 or 6 was observed for γ_1, γ_{2a+2b}, and γA PFC responses in stimulated cultures. After day 6, all responses decreased quite sharply; all attempts to date to prolong these responses beyond day 8 have failed. It is significant to note that γ_1, γ_{2a+2b}, and γA PFC increase in numbers in parallel. γ_1 and γ_{2a+2b} PFC/10^6 cells were about equal in numbers, whereas γA PFC were always somewhat lower. The PFC responses developed by polyvalent anti-γG were representative of the γ_1 and γ_{2a+2b} responses; this antiglobulin reagent is free of nonspecific facilitating activity and has been used in subsequent experiments to conserve valuable monospecific antiglobulins. γG and γA PFC also increased in unstimulated cultures, reaching numbers no greater than 10% of the responses in stimulated cultures. These background responses usually reached maximums on day 6 and declined very sharply by day 7.

At no time during the 7 day incubation of stimulated cultures were γM PFC of low hemolytic efficiency detected despite use of assay conditions and rabbit anti-γM antiglobulin known to develop indirect γM PFC against SRBC in vivo (5) and in other immune systems (35, 36). In the remainder of this communication we will present data comparing the effects of anti-erythrocyte antibody on the γM and γG PFC responses to evaluate the role of antigen in the induction of antibody production.

Suppression of Primary γM and γG PFC Responses by Anti-SRBC Antibody. —Previous studies had indicated that antibody suppresses the γM PFC response in vitro by neutralizing the effective antigenic stimulus at the macrophage-dependent phase of the response (37). A rabbit antiserum against SRBC was chosen for suppression of immune responses in the present experiments since mouse antibodies produced "false hemolytic plaques" in the assay of indirect PFC.

To investigate the effect of antibody concentration on suppression of the γM and γG PFC responses, spleen cells were incubated with 3×10^6 SRBC and 3×10^6 HRBC, and 0.05 ml of 10-fold dilutions of rabbit anti-SRBC with an initial hemagglutinin titer of $\frac{1}{8000}$. Antibody concentration (Fig. 3) was the reciprocal of the titer in the culture dish. High concentrations of antibody, 4×10^0 and 4×10^{-1}, caused marked suppression of both γM and γG PFC responses to SRBC, but had no effect on PFC responses to HRBC. As the concentration of antibody was decreased, the degree of suppression decreased. At any given antibody concentration, the degree of suppression of γG PFC was always greater than the suppression of γM PFC. Antibody did not suppress the development of background γM and γG PFC (Fig. 3); this has been verified for all concentrations of antibody used in the present experiments. Similar suppression of γM and γG PFC to HRBC has been obtained with rabbit anti-

HRBC; in these experiments the responses to SRBC were unaffected. The observation that responses to HRBC were unaffected by anti-SRBC showed that suppression of the responses to SRBC was specific and not due to toxicity of the antibody for the cultures.

As was shown previously for γM responses alone (37), suppressive activity of the anti-SRBC was also demonstrated with antigen-antibody complexes prepared with large amounts of antibody. Both γM and γG PFC responses to a

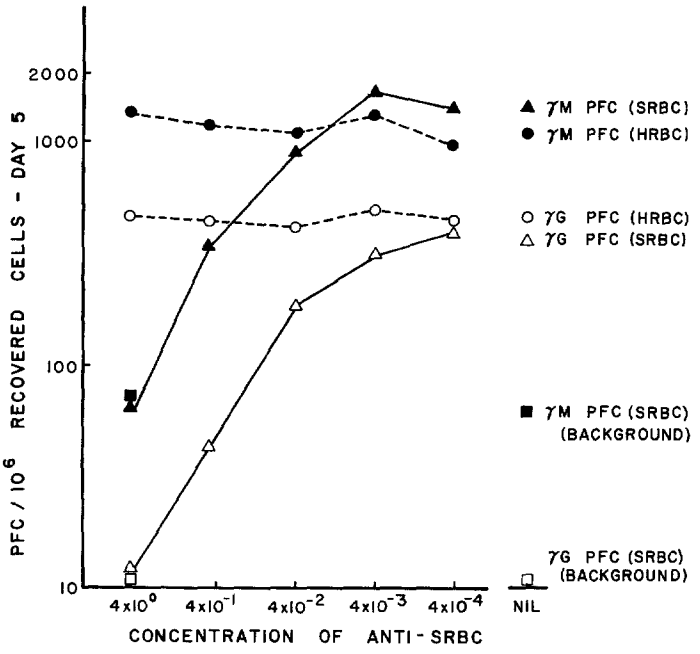

FIG. 3. Effect of concentration of anti-SRBC on the suppression of primary γM and γG PFC responses to SRBC. Cell recoveries in experimental groups were comparable; data is from a representative experiment.

complex containing 10^7 SRBC prepared with an antibody concentration of 4×10^{-1} were suppressed to almost the same degree as responses in cultures to which antigen and antibody were added separately. Suppressive activity was removed from the rabbit anti-SRBC during preparation of the complexes as demonstrated by reduced suppressive capacity of the supernate from the complexes. HRBC did not remove suppressive activity from the anti-SRBC when substituted for SRBC in the preparation of complexes.

Previous studies indicated that increasing the antigen dose overcame the suppressive effects of antibody, further suggesting that the mechanism by which antibody suppressed immune responses was by neutralization of the effective antigenic stimulus (37, 38).

At a dose of 10^5 SRBC, γM and γG PFC responses were suppressed to background levels by anti-SRBC (Fig. 4 *a*). As the SRBC dose was increased, the degree of suppression of both γM and γG responses decreased; at a dose of 10^8 SRBC, suppression of both responses was completely abrogated (Fig. 4 *a*, 4 *b*). Further, it is significant that at all SRBC doses the degree of suppression of the γG response again was always greater than the suppression of the γM response (Fig. 4 *b*).

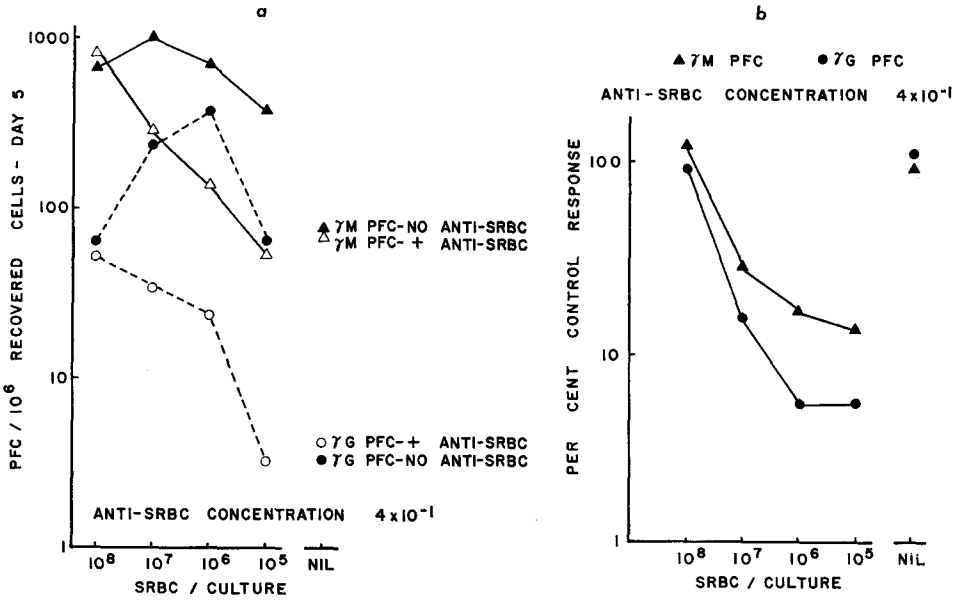

FIG. 4. Abrogation of anti-SRBC-mediated suppression of primary γM and γG PFC responses to SRBC by increased SRBC concentration. (*a*) Raw data from a representative experiment illustrating the SRBC dose-PFC response relationship for primary γM and γG responses and the effect of anti-SRBC on these relationships. (*b*) Data are presented as per cent of control responses (responses in cultures to which no antibody was added) that developed in antibody-treated cultures at each SRBC concentration. These data clearly depict the greater sensitivity of the γG response to antibody at each SRBC dose. Data are pooled from three experiments.

The greater susceptibility of γG responses to the suppressive effects of antibody was further demonstrated in experiments where antibody was added at 24-hr intervals after initiation of the cultures (Fig. 5). γM PFC responses were almost completely suppressed by antibody added at the initiation of the cultures or 24 hr later. However, antibody added at 48 hr or later had little or no suppressive effect. In contrast, considerable suppression of γG PFC responses was obtained when antibody was added during the first 48 hr of incubation. However, at 72 hr and thereafter little or no suppression of γG responses was obtained by the addition of anti-SRBC.

Spleen cells exposed to an anti-SRBC concentration of 4×10^0 in the absence of SRBC for 24 hr and then washed repeatedly before the addition of 3×10^6 SRBC and HRBC had suppressed γM and γG PFC responses to SRBC, but not to HRBC. γG responses were more affected than γM responses and the degree of suppression of both responses was less than if the 4×10^0 concentration of anti-SRBC was present throughout the incubation period. Further, if an additional 10^7 SRBC were added 6 hr after the initial SRBC, the suppres-

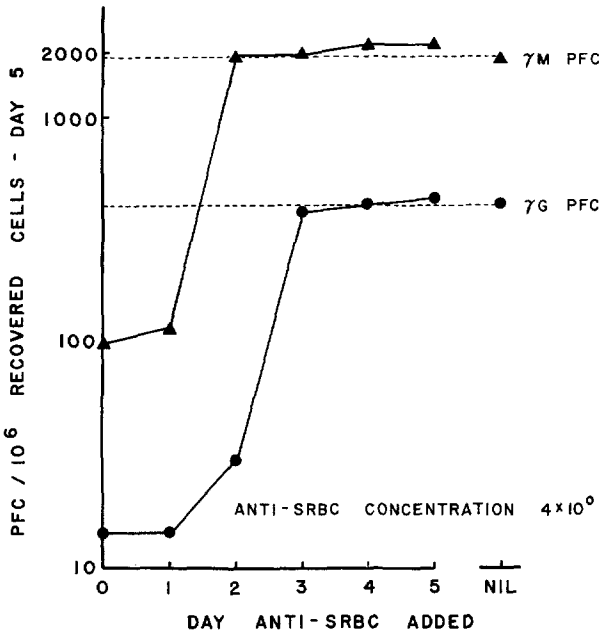

FIG. 5. Suppression of primary γM and γG PFC responses to SRBC by anti-SRBC added at 24-hr intervals after initiation of cultures.

sion of both γM and γG PFC responses was abrogated. These data indicated that antibody may also act directly on one of the interacting cell types required for generation of γM and γG PFC responses.

Mouse spleen cells can be conveniently separated into adherent (mostly macrophages) and nonadherent (mostly lymphocytes) populations (39–41); both populations are required for generation of γM and γG PFC responses in vitro[3] (42). Experiments identical in design and methodology to those reported previously (37) have confirmed our original observations for γM response and extended the observations to the γG PFC responses. Incubation of separated adherent cells with anti-SRBC antibody caused suppression of the γM and

[3] Pierce, C. W., and B. M. Johnson. Immune responses in vitro. IV. Cell interactions in the development of primary γM and γG PFC responses in vitro. Manuscript in preparation.

γG PFC responses to SRBC, but not to HRBC in cultures reconstituted with untreated nonadherent cells. Incubation of nonadherent cells, which contain antibody-forming cell precursors (40), with antibody did not produce suppression of either the γM or γG PFC responses. As in other experiments with anti-SRBC, the degree of suppression of the γG responses was greater than the degree of suppression of the γM response. These data confirm our original findings that antibody suppresses the immune response by neutralizing the effective antigenic stimulus at the level of the macrophage.

DISCUSSION

We have described the development of γ_1, γ_{2a+2b}, and γA as well as γM PFC responses in cultures of mouse spleen cells stimulated in vitro with heterologous erythrocytes. A new procedure for detecting indirect (γG and γA) PFC makes analysis of these immunoglobulin class-specific responses possible. This new method makes use of the fact that development of direct (γM) PFC can be completely inhibited by incorporation of appropriate amounts of a suitable anti-IgM specific antibody into the agarose-indicator erythrocyte-assay mixture. The technique offers several advantages over the conventional methods (1, 2, 5, 10, 11, 29, 30) for assaying indirect PFC. First, the indirect PFC can be directly enumerated from the assay slides. In conventional procedures a correction must be made for the frequently observed suppressive effect of facilitating antiglobulins on development of γM PFC. Second, responses studied with this method are more reproducible than with conventional procedures. Finally, the new procedure is rapid; it is certainly less cumbersome than the sequential PFC development method which requires two periods of incubation and two separate PFC counts. The number of indirect PFC determined by this latter procedure and the number determined by the new blocked γM PFC method were in very close agreement. The sequential PFC development method was extremely useful, however, in confirming the results of the specificity controls.

Absorption and competitive inhibition controls established the specificity of both blocking (anti-μ) and facilitating (anti-γG, anti-γ_1, anti-γ_{2a+2b}, and anti-γA) reagents. In general, homologous myeloma proteins were more than 10 times as efficient in blocking a specific antiglobulin activity than were nonhomologous myeloma proteins. The small amount of blocking obtained with heterologous myeloma proteins can be readily explained by the inevitable small contamination of purified myeloma proteins with normal immunoglobulins. The absolute requirement for such specificity controls was demonstrated by the observation that certain normal rabbit sera developed indirect PFC in all assay procedures employed. The facilitating activity of these sera could neither be blocked nor absorbed with myeloma proteins, but was removed only by immune complexes as has been discussed above.

A PFC response specific for each major class of mouse immunoglobulin

developed in vitro. Although the antiglobulin specific for γ_{2a+2b} did not distinguish between these individual immunoglobulins, it has been shown elsewhere (5) that this antiglobulin is only partially blocked by either class of myeloma protein alone. Reagents specific for the γ_3 immunoglobulin class were not available during this study (43).

In general, tissue culture conditions which supported adequate γM responses also supported γG and γA responses in vitro. Concentrations of spleen cells and ages of mice from which the cells were obtained that produced optimum γM responses also produced optimum γG responses. As has been observed for γM responses (21, 22), a low level of γG response was seen in unstimulated cultures. Finally, both γM and γG PFC responses were specifically suppressed by inclusion in the cultures of appropriate amounts of specific anti-erythrocyte antibody. It was shown that this inhibition was the result of neutralization of the effective antigenic stimulus at the macrophage-dependent phase of the response. This has been established previously in this laboratory (37) and suggested by others (38) only for γM responses in vitro, and is consistent with results of the suppressive effects of antibody in vivo (44–46).

A detailed study of the kinetics of appearance of γG and γA PFC showed that γ_1, γ_2, and γA PFC appeared 1–2 days later than did γM PFC and reached a peak 1 day later. The γ_1 and γ_{2a+2b} responses were somewhat greater than γA responses, but all responses showed identical kinetics. These differences in rates of appearance of γM and γG PFC are similar to those observed after immunization in vivo (1, 2, 5).

A greater sensitivity of the γG PFC response to antibody was revealed in two ways. First, any given dose of antibody had a proportionally greater effect on the γG than on the γM response. Second, the γG response could be inhibited by antibody added up to 48 hr after stimulation; in the case of the γM response, antibody had to be added before 24 hr after stimulation to achieve complete suppression. It has been shown that in the development of the γM PFC response, the first 24 hr is the interval during which critical interactions take place among the various types of lymphoid cells, macrophages, and antigen (41). The finding that γG responses are susceptible to suppressive antibody for 48 hr suggests that these responses may require more prolonged interactions among macrophages, antigen, and lymphoid cells for their development (42). The investigations of these cell interactions in the development of the primary γG response will be described in a later communication.[3]

A small but consistent difference was seen in the present experiments in the erythrocyte doses which stimulated maximum γM and γG responses. Doses of antigen which produced optimum γM responses were of an order of magnitude higher than those that produced optimum γG responses. With higher erythrocyte doses, γG responses were suboptimal. At no erythrocyte dose was the γG response equal to the γM response. Indeed, γM responses equal to optimal γG responses were usually obtained with low doses of antigen (see Table V). These

findings may be related to the greater observed sensitivity of the γG PFC responses to anti-erythrocyte antibody added to the cultures as discussed previously. It is known that γM antibody may have an enhancing effect on immune response in vivo (46). It must be remembered, however, that γM antibody in vivo is almost all confined to the circulating blood, and in the mouse is catabolized at a very rapid rate (47). In vitro, a greater accumulation of γM antibody in intimate contact with reactive and differentiating cells is to be expected, and it may, in these circumstances, play an important role in the development and regulation of γG PFC responses.

SUMMARY

We have demonstrated for the first time that mouse spleen cells stimulated in vitro with heterologous erythrocytes developed immunoglobulin class-specific γM, γ_1, γ_{2a+2b}, and γA plaque-forming cell (PFC) responses. A modification of the hemolytic plaque technique, the addition of goat anti-mouse μ-chain antibody to the assay preparation, specifically prevented development of all γM PFC and enabled accurate and reproducible enumeration of immunoglobulin class-specific PFC after treatment with appropriate monospecific antiglobulins and complement. Culture conditions, with regard to medium, atmosphere, agitation, and spleen cell densities, were similar to those previously shown to support only γM PFC responses. Evaluation of the kinetics of appearance of PFC showed that γM PFC reached maximum numbers on days 4–5; the magnitude of this response was 3–10 times greater than γ_1, γ_{2a+2b}, or γA PFC which reached maximum numbers on days 5–6. Optimal erythrocyte antigen dose for γM PFC responses was 10^7/culture, whereas a dose of 10^6 erythrocytes/culture consistently stimulated optimal γ_1, γ_{2a+2b}, or γA PFC responses. Investigations of the effects of anti-erythrocyte antibody on γM and γG PFC responses indicated that antibody suppressed these responses by neutralizing the effective antigenic stimulus at the macrophage-dependent phase of the response. At the same antibody concentration, γG PFC responses were more effectively suppressed than γM PFC responses. Further, γG responses could be almost completely suppressed by antibody as long as 48 hr after initiation of cultures, whereas γM PFC responses could only be completely suppressed during the first 24 hr. These results were discusssed in terms of the role of antigen in the stimulation γM and γG antibody.

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BIBLIOGRAPHY

1. Sell, S., A. B. Park, and A. A. Nordin. 1970. Immunoglobulin classes of antibody-forming cells in mice. I. Localized hemolysis-in-agar plaque-forming cells belonging to five immunoglobulin classes. *J. Immunol.* **104**:483.
2. Wortis, H. H., D. W. Dresser, and H. R. Anderson. 1969. Antibody production

studied by means of the localized haemolysis in gel (LHG) assay. III. Mouse cells producing five different classes of antibody. *Immunology.* **17:**93.

3. Barth, W. F., C. L. McLaughlin, and J. L. Fahey. 1965. The immunoglobulins of mice. VI. Response to immunization. *J. Immunol.* **95:**781.
4. Warner, N. L., N. M. Vaz, and Z. Ovary. 1968. Immunoglobulin classes in antibody responses in mice. I. Analysis by biological properties. *Immunology.* **14:**725.
5. Plotz, P. H., N. Talal, and R. Asofsky. 1968. Assignment of direct and facilitated hemolytic plaques in mice to specific immunoglobulin classes. *J. Immunol.* **100:** 744.
6. Jerne, N. K., A. A. Nordin, and C. Henry. 1963. The agar plaque technique for recognizing antibody producing cells. *In* Cell Bound Antibodies. B. Amos and H. Koprowski, editors. The Wistar Institute Press, Philadelphia, Pa. 109.
7. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody producing cells. *Science (Washington).* **140:**405.
8. Ingraham, J. J., and A. Bussard. 1964. Application of a localized hemolysin reaction for specific detection of individual antibody-producing cells. *J. Exp. Med.* **119:**667.
9. Humphrey, J. H., and R. R. Dourmashkin. 1965. Electron microscope studies of immune cell lysis. *In* Complement. G. E. W. Wolstenholme and J. Knight, editors. J & A Churchill Ltd., London. 175.
10. Šterzl, J., and I. Riha. 1965. Detection of cells producing 7S antibodies by the plaque technique. *Nature (London).* **208:**858.
11. Dresser, D. W., and H. H. Wortis. 1965. Use of an antiglobulin serum to detect cells producing antibody of low haemolytic efficiency. *Nature (London).* **208:**859.
12. Weiler, E., E. W. Melletz, and E. Breuninger-Peck. 1965. Facilitation of immune hemolysis by an interaction between red cell sensitizing antibody and γ globulin allotype antibody. *Proc. Nat. Acad. Sci. U.S.A.* **54:**310.
13. Dutton, R. W., and R. I. Mishell. 1967. Cell populations and cell proliferation in the in vitro response of normal mouse spleen to heterologous erythrocytes. Analysis by the hot pulse technique. *J. Exp. Med.* **126:**443.
14. Nordin, A. A., H. Cosenza, and S. Sell. 1970. Immunoglobulin classes of antibody forming cells in mice. IV. The incorporation of tritiated thymidine into IgM and γ_1 plaque forming cells during the primary immune response. *J. Immunol.* **105:** 154.
15. Koros, A. M. C., J. M. Mazur, and M. J. Mowery. 1968. Radioautographic studies of plaque-forming cells. I. Antigen-stimulated proliferation of plaque-forming cells. *J. Exp. Med.* **128:**235.
16. Kennedy, J. C., J. E. Till, L. Siminovitch, and E. A. McCulloch. 1965. Radiosensitivity of the immune response to sheep red cells in the mouse as measured by the hemolytic plaque method. *J. Immunol.* **94:**715.
17. Rowley, D. A., F. W. Fitch, D. E. Mosier, S. Solliday, L. W. Coppleson, and B. W. Brown. 1968. The rate of division of antibody-forming cells during the early primary immune response. *J. Exp. Med.* **127:**983.
18. Cudkowicz, G., G. M. Shearer, and R. L. Priore. 1969. Cellular differentiation of the immune system of mice. V. Class differentiation in marrow precursors of plaque-forming cells. *J. Exp. Med.* **130:**481.

19. Miller, H. C., and G. Cudkowicz. 1971. Density gradient separation of marrow cells restricted for antibody class. *Science (Washington).* **171:**913.
20. Nossal, G. J. V., A. Szenberg, G. L. Ada, and C. M. Austin. 1964. Single cell studies on 19S antibody production. *J. Exp. Med.* **119:**485.
21. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126:**423.
22. Pierce, C. W. 1969. Immune responses in vitro. I. Cellular requirements for the immune response by nonprimed and primed spleen cells in vitro. *J. Exp. Med.* **130:**345.
23. Fahey, J. L., and C. McLaughlin. 1963. Preparation of antisera specific for 6.65 γ globulins, β2A-globulins, γ_1 macroglobulins, and for the type I and II common γ globulin determinants. *J. Immunol.* **91:**484.
24. Porter, R. R. 1959. The hydrolysis of rabbit γ globulins and antibodies with crystalline papain. *Biochem. J.* **73:**119.
25. Sell, S. 1967. Studies on rabbit lymphocytes in vitro. V. The induction of blast transformation with sheep antisera to rabbit IgG subunits. *J. Exp. Med.* **125:** 289.
26. Mann, D., H. Granger, and J. L. Fahey. 1969. Use of insoluble antibody for quantitative determination of small amounts of immunoglobulin. *J. Immunol.* **102:** 618.
27. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Washington).* **130:**432.
28. Hubner, K. F., and N. Gengozian. 1969. Critical variables of the Jerne plaque technique as applied to rodent antibody forming systems responding to heterologous red cell antigens. *J. Immunol.* **102:**155.
29. Wortis, H. H., R. B. Taylor, and D. W. Dresser. 1966. Antibody production studied by means of the LHG Assay. I. The splenic response of CBA mice to sheep erythrocytes. *Immunology.* **11:**603.
30. Wortis, H. H., R. B. Taylor, and D. W. Dresser. 1968. Antibody production studied by means of the localized haemolysis in gel (LHG) Assay. II. Assay procedure. *Immunology.* **14:**69.
31. Pierce, C. W., and B. M. Johnson. 1970. The development of primary γG plaque forming cell responses *in vitro*. *Fed. Proc.* **29:**571.
32. Abruzzo, J. L., and C. L. Christian: 1961. The induction of a rheumatoid factor-like substance in rabbits. *J. Exp. Med.* **114:**791.
33. Gell, P. G. H., and A. S. Kelus. 1967. Anti-antibodies. *Advan. Immunol.* **6:**461.
34. Mosier, D. E. and L. W. Coppelson. 1968. A three cell interaction required for the induction of a primary immune response in vitro. *Proc. Nat. Acad. Sci. U.S.A.* **61:**542.
35. Plotz, P. H., H. Colten, and N. Talal. 1968. Mouse macroglobulin antibody to sheep erythrocytes: a non-complement-fixing type. *J. Immunol.* **100:**752.
36. Baker, P. J., and P. W. Stashak. 1969. Quantitative and qualitative studies on the primary antibody response to pneumococcal polysaccharides at the cellular level. *J. Immunol.* **103:**1342.
37. Pierce, C. W. 1969. Immune responses in vitro. II. Suppression of the immune response in vitro by specific antibody. *J. Exp. Med.* **130:**365.

38. Lang, W., S. Nase, and K. Rajewsky. 1969. Inhibition of the immune response *in vitro* to sheep red blood cells by passive antibody. *Nature* (*London*). **223:**949.
39. Mosier, D. E. 1967. A requirement for two cell types for antibody formation *in vitro*. *Science* (*Washington*). **158:**1575.
40. Hartmann, K., R. W. Dutton, M. M. McCarthy, and R. I. Mishell. 1971. Cell components in the immune response. II. Cell attachment separation of immune cells. *Cell. Immunol.* **1:**182.
41. Pierce, C. W., and B. Benacerraf. 1969. Immune response *in vitro:* independence of "activated" lymphoid cells. *Science* (*Washington*). **166:**1002.
42. Pierce, C. W., and B. M. Johnson. 1971. Cell interactions in primary immune responses *in vitro*. *Fed. Proc.* **31:**526.
43. Grey, H. M., J. W. Hirst, and M. Cohn. 1971. A new mouse immunoglobulin: IgG3. *J. Exp. Med.* **133:**289.
44. Cerottini, J. C., P. J. McConahey, and F. J. Dixon. 1969. The immunosuppressive effect of passively administered antibody IgG fragments. *J. Immunol.* **102:**1008.
45. Uhr, J. W., and G. Möller. 1968. Regulatory effect of antibody on the immune response. *Advan. Immunol.* **8:**81.
46. Henry, C., and N. K. Jerne. 1968. Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. *J. Exp. Med.* **128:**133.
47. Fahey, J. L., and S. Sell. 1965. Immunoglobulins of mice. V. The metabolic (catabolic) properties of five immunoglobulin classes. *J. Exp. Med.* **122:**41.