IMMUNE RESPONSES IN VITRO

IV. Suppression of Primary γM, γG, and γA Plaque-Forming Cell Responses in Mouse Spleen Cell Cultures by Class-Specific Antibody to Mouse Immunoglobulins*

By CARL W. PIERCE, SUSAN M. SOLLIDAY, and RICHARD ASOFSKY

(From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, and the Laboratory of Microbiol Immunity, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014)

(Received for publication 2 November 1971)

Stimulation of precursors of antibody-forming cells to divide and differentiate into cells actively synthesizing and secreting antibody is initiated by interactions among precursor cells, antigen, and lymphocytes derived from the thymus (1–6). The clonal selection theory postulates that precursor cells are precommitted to respond to a limited range of antigenic specificities and that recognition of these specificities is mediated through surface antigen receptors (7–10).

Lymphocytes from several species have been shown by different techniques to have immunoglobulin molecules on their cell surfaces (11–28). Lymphocytes also have been shown to bind antigen specifically, presumably through these surface immunoglobulins (29–44). In the mouse, both the precursor cells and antigen-binding lymphocytes can be derived from bone marrow (41); these cells appear to be functional homologues of bursa-derived lymphocytes in birds (45). It is these nonthymus-derived lymphocytes which are the main source of lymphocytes with detectable surface immunoglobulin (25–27) which is largely of the γM class in normal animals (17, 35); but immunoglobulins of other classes have also been detected (11, 12, 25–27). The immunoglobulin on cell surfaces is thought to be representative of the antibody to be secreted later by that cell both in terms of its specificity and immunoglobulin class (29, 32, 38, 42, 44, 46–48).

Precursors of antibody-forming cells, then, are believed to have immunoglobulin receptors on their cell membranes which are antigen-specific and representative of the immunoglobulin class of the antibody to be later secreted by that cell. The combination of antigen with these specific antibody-like receptors and the collaboration of thymus-derived lymphocytes results in stimulation of the precursor cells into cells actively secreting antibody (46). One might predict that saturation of receptors of a given immunoglobulin class with a specific antiglobulin would suppress production of antibodies of only that immunoglobulin class after antigenic stimulation, if this "receptor = product" hypothesis is correct (48).

It has been shown that antibody to mouse κ-chain (49), to mouse immunoglobulin,

* This investigation was supported by US Public Health Service Research Grant AI-09897 from the National Institute of Allergy and Infectious Diseases.

to Fab of γ_{2a}, and to IgM inhibited primary γM responses in vitro under conditions which did not permit detection of antibody-forming cells of other immunoglobulin classes (42, 50). Antibody to mouse immunoglobulin in tissue culture similarly inhibited antibody production and the induction of tolerance to polymerized flagellin (51).

The present studies investigated the effects of specific goat anti-mouse globulins on primary immunoglobulin (Ig)[1] class-specific plaque-forming cell (PFC) responses in mouse spleen cell cultures. A recently developed technique was used which permits the detection of primary PFC responses of γG and γA, as well as γM Ig classes in vitro (52). The data show that anti-γ_1 or anti-γ_2 each suppressed both the γ_1 and γ_2, but not γM or γA PFC responses, anti-γA suppressed only the γA response, but antibody to μ-chain suppressed PFC responses in all Ig classes. The results are discussed in terms of the possible relationships between surface Ig receptors and the antibody ultimately secreted.

Materials and Methods

Animals.—C57BL/6, AKR, and C3H/He male mice, 2–4 months old (Jackson Laboratories, Bar Harbor, Maine), and BALB/c AnN male mice and New Zealand white male rabbits, 3–6 months old, from the NIH DRS Rodent and Rabbit Production Section, were maintained on the appropriate laboratory chow and water *ad libitum*. Goats were maintained on a standard diet at the NIH Ungulate Division.

Erythrocyte Antigens.—Horse (HRBC) and sheep (SRBC) erythrocytes in Alsever's solution (Grand Island Biological Co., Grand Island, N.Y.) were washed three times by centrifugation with 40 vol of sterile Hanks' balanced salt solution (HBSS) and resuspended in HBSS to 20×10^8 RBC/ml for use in the hemolytic plaque assay or 2.5×10^8 RBC/ml for use as antigen in cultures.

Preparation of Myeloma Proteins and Anti-Immunoglobulin Sera.—Myeloma proteins and light chains were purified from ascitic fluid or urine from BALB/c mice bearing appropriate ascitic plasma cell tumors by preparative electrophoresis on Geon/Pevikon resin blocks followed when needed by Sephadex G-200 filtration (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) (52–54). Fc fragments were prepared by a modification of the method of Porter (54). Antiglobulins used for development of γG and γA PFC in the hemolytic plaque assay were prepared in rabbits and purified as described previously (52, 53).

Antiglobulins for addition to mouse spleen cell cultures were raised in goats by repeated immunization with the immunogens listed in Table I incorporated in complete Freund's adjuvant (54). Anti-ferritin was raised in goats by repeated immunization with horse spleen ferritin (Pentex Biochemical, Kankakee, Ill.) incorporated in complete Freund's adjuvant. All antisera, myeloma protein, and light chain preparations 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/6 spleen cells per 10 ml serum. The antisera were rendered specific by appropriate absorption with purified light chain, myeloma protein, or germfree mouse serum as indicated in Table I. Monospecific antiglobulins coupled

[1] *Abbreviations used in this paper:* "γG" is used 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; Ig, immunoglobulin(s); LNC, lymph node cells; MΦ, macrophages; MEM, minimal essential medium; PFC, plaque-forming cell(s); RBC, red blood cell; SRBC, sheep red blood cells; -θLC, anti-θ-treated lymphoid cells.

to bromo-acetyl cellulose (55) did not bind ^{125}I-labeled light chains or myeloma proteins of Ig classes other than the one against which the antiglobulin was directed. Before addition to cultures, all reagents were sterilized by filtration through a prewashed 0.45 μ Millipore filter (Millipore Corporation, Bedford, Mass.)

Preparation of AKR Anti-θ C3H/He.—AKR mice were injected intraperitoneally with 25 × 10^6 C3H/He thymus cells (56–58) at 14-day intervals until the specific anti-θ titer against C3H/He thymus cells 7 days after the last immunization was greater than $\frac{1}{1000}$ using 50% cytotoxicity as the end point. Normal and immunized mice were then bled and the serum was decomplemented, absorbed with SRBC and HRBC, and sterilized as described above. The specificity of the anti-θ serum for only θ-bearing cells was demonstrated by cytotoxicity assays using either rabbit or guinea pig serum as complement, ^{51}Cr-labeled cells

TABLE I

Biological Reagents

Antiglobulin	Immunogen	Absorbants	Specificity
Anti-μ	MOPC-104E myeloma protein	λ-chain MOPC-104E urinary protein	μ
Anti-γ_1	MOPC-31c myeloma protein Fc piece	—	γ_1
Anti-γ_2	Adj PC-5 myeloma protein Fc piece	—	γ_2
Anti-γA	MPC-1 myeloma protein	Germfree serum	α
Anti-κ	Adj PC-9 urinary protein	—	κ
Anti-λ	MOPC-104E urinary protein	—	λ

Myeloma protein	Immunoglobulin class
MOPC-104E	γM
MPC-47, MOPC-70A, MOPC-31c	γ_1
LPC-1, Adj PC-5	γ_{2a}
MPC-11, MPC-37	γ_{2b}
SPC-1, MPC-40, MPC-1	γA
MOPC-104E urinary protein	λ
Adj PC-9 urinary protein	κ

from several lymphoid tissues (58), and anti-θ serum appropriately absorbed with lymphoid tissues or brain. Anti-allotype (59) and anti-LyC2 (60) activities were not detected.

Spleen Cell Cultures.—Single cell suspensions of spleen were prepared as described previously (52) and were incubated in 35 × 10 mm plastic culture dishes (Falcon 3005 [Falcon Plastics, Div. of Bioquest, Oxnard, Calif.]) at a density of 10 × 10^6 cells/1 ml in completely supplemented Eagle's minimal essential medium (MEM) (61), containing 10% fetal bovine serum (Reheis Chemical Co., Kankakee, Ill., lots E21505, E21806), and 50 units each/ml of penicillin and mycostatin and 50 μg/ml of streptomycin, with or without antigen, 3–7 × 10^6 RBC/culture; the incubations occurred in a water-saturated atmosphere of 7% O_2, 10% CO_2, and 83% N_2 at 37°C in an airtight box on a rocking platform oscillating at 3½ complete cycles/min. Media and supplements other than fetal bovine serum were obtained from Microbiological Associates, Inc., Bethesda, Md. Cultures were supplemented daily with 0.05 ml of a nutritional mixture and fetal bovine serum (52). Goat antiglobulins and anti-ferritin were diluted in HBSS and 0.05 ml were added to cultures as dictated by experimental protocol.

Cells were harvested after 5 or 7 days' incubation and washed once by centrifugation before assay for hemolytic PFC.

Hemolytic Plaque Assay.—Ig class-specific PFC from spleen cell cultures were enumerated by a modification of the Jerne hemolytic plaque (62) described in detail elsewhere (52). γM PFC were enumerated after incubation of indicator erythrocytes and spleen cells in semisolid agarose in HBSS using guinea pig serum (Baltimore Biological Laboratories, Baltimore, Md.) as a complement source. Ig class-specific γG and γA PFC were developed with monospecific or polyvalent rabbit antiglobulins and complement in assay preparations to which sufficient goat anti-μ antibody had been added to inhibit development of all γM PFC.

Cells recovered from culture dishes were counted with a Coulter Counter Model B (Coulter Electronics, Inc., Hialeah, Fla.); PFC were expressed per 10^6 recovered cells or per culture. Data presented are from experiments with comparable cell recoveries in comparable experimental groups. In most graphs, data are expressed as per cent control response to allow comparison of data from three to five experiments with identical protocol. Statistical analyses are not presented on each graph since the coefficient of variability of experimental groups was always less than 15%.

^{51}Cr Cytotoxicity Assay.—Single cell suspensions of mouse spleen, thymus, bone marrow, or lymph node (50×10^6 cells/ml) in medium L-15 (Microbiological Associates, Inc.) with 10% fetal bovine serum were freed of RBC by treatment with tris(hydroxymethyl)aminomethane-buffered NH_4Cl and labeled with $Na_2\ ^{51}CrO_4$ as described by Raff and Wortis (58). For the assay, 0.1 ml of ^{51}Cr-labeled cells, (5×10^6/ml), 0.1 ml of antiserum at desired concentration or medium, and 0.1 ml of a noncytotoxic complement source at predetermined optimal dilution (lyophilized guinea pig serum [Baltimore Biological Laboratories] or fresh rabbit or mouse serum) or medium were incubated at 37°C for 1 hr in 10×75 mm glass tubes; 0.7 ml of L-15 was added and the cells were sedimented by centrifugation; 0.5 ml of the supernate was removed and counted in a Packard Autogamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Controls for spontaneous release, release due to complement alone as well as release from thrice-frozen and -thawed samples, and samples incubated with appropriate anti-H2 serum[2] were included.

RESULTS

Effect of Goat Antiglobulins on Primary Ig Class-Specific PFC Responses In Vitro.—Cultures of C57BL/6 spleen cells were stimulated with SRBC in the presence of goat antibodies against the major heavy chains of mouse immunoglobulins, κ- or λ-light chains or ferritin. Ig class-specific PFC responses were determined after 5 days of incubation and compared to responses in control cultures to which no goat serum was added (Fig. 1). High concentrations (50 μl, 1:10 dilution) of anti-κ, anti-λ (not shown in Fig. 1), or anti-ferritin had no effect on any Ig class-specific response; anti-γA suppressed only the γA PFC response, anti-γ_1 or anti-γ_2 each suppressed both the γ_1 and γ_2, but not γA or γM responses. Anti-μ suppressed all Ig class-specific PFC responses.

Since anti-μ produced the greatest suppression, and affected responses of all Ig classes, the specificity, mechanism, and site of action of this antiglobulin in various experimental situations was studied in detail. High concentrations (50

[2] Anti-H2 sera were obtained from Dr. Donald E. Kayhoe, Transplantation and Immunology Branch, Collaborative Research, National Institute of Allergy and Infectious Diseases.

μl, 1:10 and 1:25 dilutions) added at the initiation of the cultures profoundly suppressed the responses in all Ig classes (Fig. 2). However, at lower concentrations, for example 50 μl of the 1:1000 dilution, anti-μ consistently enhanced PFC responses in all Ig classes. The degree of suppression by high concentrations of anti-μ was about equal in all the Ig classes, but enhancement varied from about 30% for γA responses to 100% or more for γM and γG responses. In the experiments shown in Fig. 1 and in the remaining figures, responses of control cultures were within $\pm$0.3 to 0.5 log units of those indicated in Fig. 2.

Specificity of the Suppressive Activity of Anti-μ on PFC Responses In Vitro.— The specificity of the suppressing activity of the anti-μ was demonstrated in

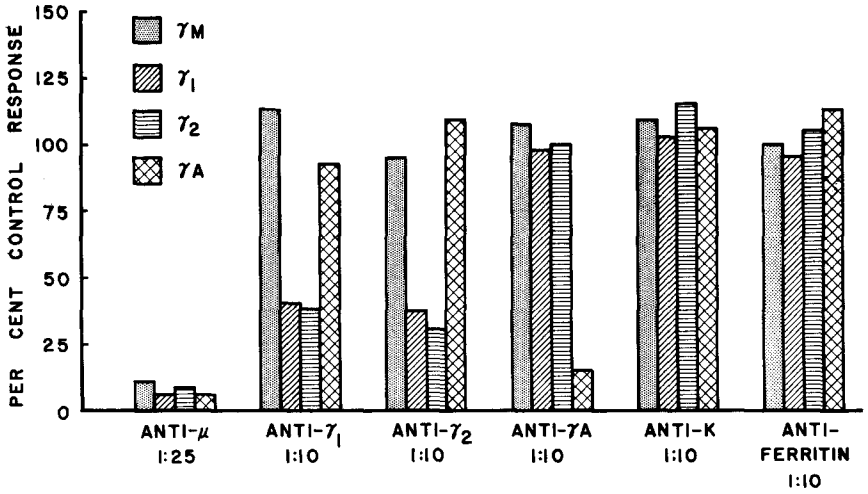

FIG. 1. Effect of goat antiglobulins on primary Ig class-specific PFC responses in vitro. Antiglobulins were added at initiation of cultures. Data are expressed as per cent of the response in each Ig class in cultures without any additives. Representative PFC responses per 10^6 cells are shown in Fig. 2.

two ways: first, absorption of anti-μ with γM myeloma protein insolubilized with ethylchloroformate (63) removed virtually all suppressive activity (Fig. 3). Increased concentrations of this absorbed preparation of anti-μ (50 μl, 1:5 dilution) also had no suppressive activity. Similar absorption with insolubilized γ_1 and γ_2 myeloma proteins had no measurable effect on the suppressive activity of the anti-μ. Second, when myeloma proteins were added together with anti-μ at the initiation of the cultures, only γM myeloma protein neutralized or partly neutralized the suppressive activity of anti-μ; γG or γA myeloma had no effect on the suppressive activity of anti-μ (Fig. 4). The degree of reversal of anti-μ–mediated suppression produced by 50 μg of γM myeloma protein was dependent on the amount of anti-μ added to the cultures. Addition of 50 μg of γM, γG, or γA myeloma protein to control cultures had no effect on Ig class-specific PFC responses.

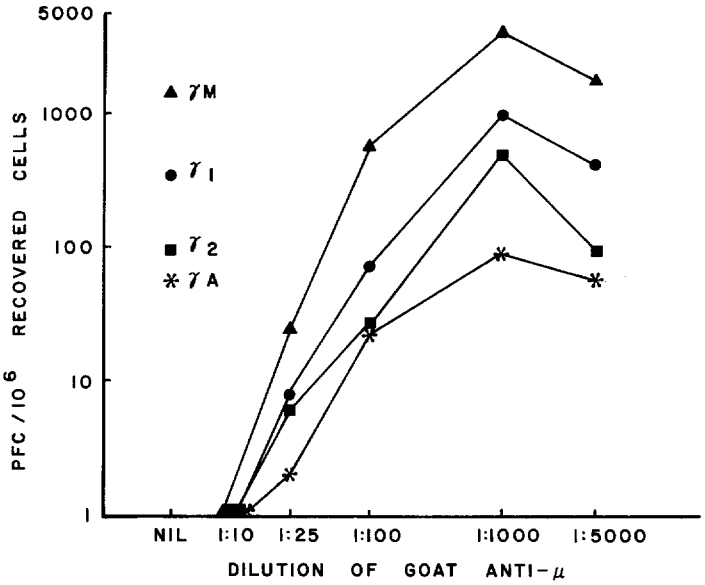

FIG. 2. Effect of various concentrations of goat anti-μ on Ig class-specific PFC responses in vitro. Data are from a representative experiment; PFC responses in all experiments were $\pm$ 0.3 to 0.5 log units of those indicated.

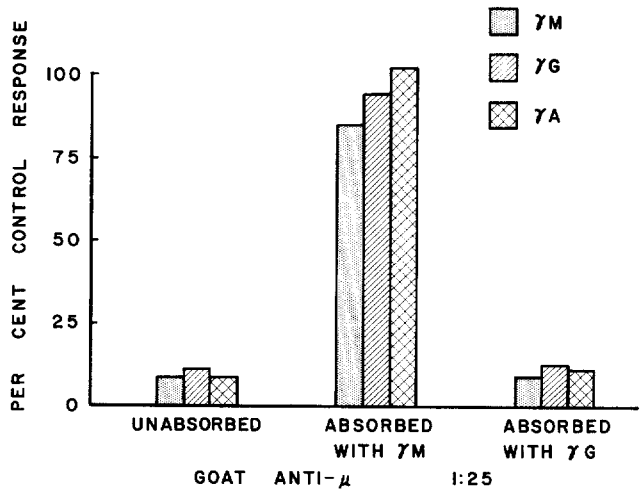

FIG. 3. Specificity of goat anti-μ for μ-chain determinants: effect of absorption of anti-μ with insolublized γM or γG myeloma proteins on suppressive activity.

None of the antiglobulins tested at any concentration had any cytotoxic activity for either ^{51}Cr-labeled thymus or spleen cells in the absence or presence of complement sources that killed virtually 100% of these cells in the presence of the appropriate anti-H2 antisera. Also, when spleen cells were labeled with

^{51}Cr and incubated in the culture system with SRBC with or without anti-μ, ^{51}Cr release into the culture supernate was not increased in cultures to which anti-μ had been added, but subsequent PFC responses in these cultures were suppressed.

Time Requirement for Excess Anti-μ for Suppression of PFC Responses In Vitro.—To determine whether an excess of anti-μ was required in the cultures throughout the 5 days' incubation for suppression of PFC responses, cells from stimulated cultures both with and without anti-μ were harvested at 24-hr intervals after initiation of the cultures, washed twice by centrifugation with 50 vol of HBSS, and returned to the original culture dishes in 1 ml of fresh medium. Cells adhering to the dishes were also washed with HBSS. Thereafter, the only anti-μ in the system was that which had remained bound to cells. When the

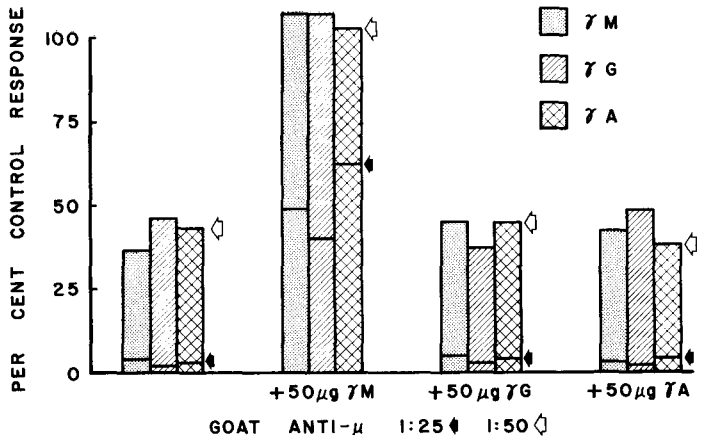

FIG. 4. Specificity of goat anti-μ for μ-chain determinants: effect of adding γM, γG, or γA myeloma proteins with anti-μ on suppressive activity. Data from experiments with 1:25 and 1:50 dilutions of anti-μ, as indicated by arrows, are shown.

excess anti-μ was removed at 48 hr or later, PFC responses were suppressed to the same degree as responses in cultures where excess anti-μ was not removed until immediately before PFC assay at 120 hr (Fig. 5). Addition of 50 μg of γM myeloma protein after removal of excess anti-μ at 48 hr had no effect on the 5-day PFC responses. However, when excess anti-μ was removed at 24 hr, suppression of the 5-day PFC responses was considerably less; responses in these cultures were about 50% of responses in control cultures. The suppressive activity was further reduced by addition of 50 μg of γM myeloma protein, but not by γG myeloma proteins after removal of excess anti-μ at 24 hr.

A similar experimental design was employed to determine if spleen cells, after 48 hr exposure to an excess of anti-μ, were totally unresponsive to antigenic stimulation. SRBC-stimulated cultures were incubated with or without anti-μ for 48 hr, excess anti-μ was removed by washing, and cultures were continued with no additions, with addition of 50 μg of γM or γG myeloma protein, or

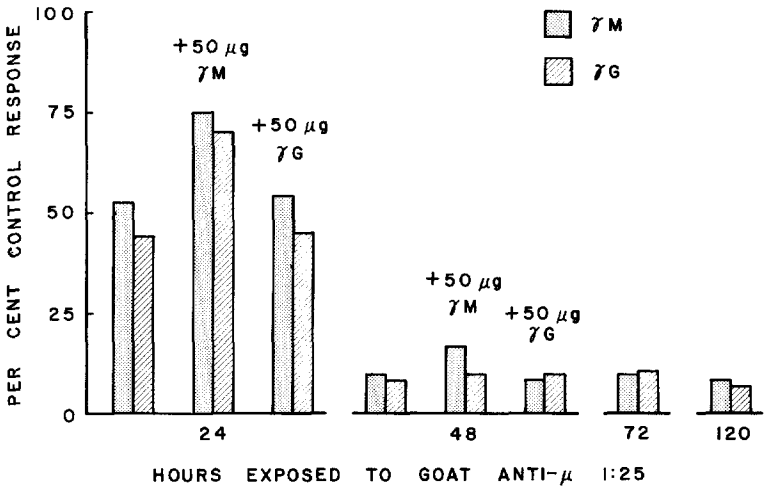

FIG. 5. Time requirement for excess goat anti-μ for suppression of PFC responses. Experimental details are in the text.

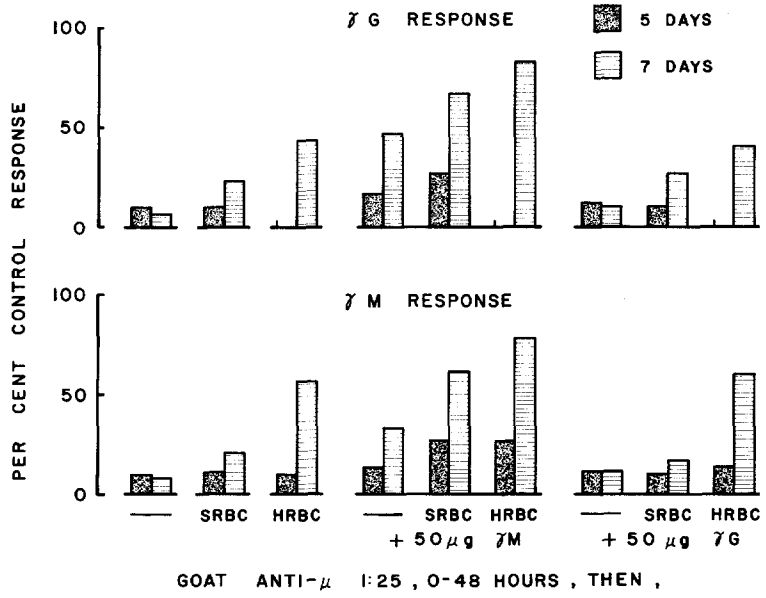

FIG. 6. Responses to new antigen after removal of excess goat anti-μ. Spleen cell cultures were stimulated with SRBC and incubated with anti-μ for 48 hr. After washing to remove non-cell-bound anti-μ, cells were returned to culture with no new antigen (—), or with SRBC or HRBC with or without γM or γG myeloma proteins as indicated. Day 5 and day 7 responses relative to identically treated cultures which were not incubated with anti-μ are displayed. Cultures incubated with HRBC were assayed against these RBC; all other cultures were assayed against SRBC.

with 3–7 × 10^6 HRBC or SRBC with or without γM or γG myeloma protein. γM and γG PFC were enumerated on days 5 and 7.

After removal of excess anti-μ at 48 hr, cultures with no additions had no increase in PFC responses relative to controls when incubation was prolonged to 7 days. However, addition of 50 μg of γM myeloma protein at 48 hr, although it produced no dramatic effect by 5 days, did result in considerably increased responses in 7-day cultures (Fig. 6). Addition of fresh SRBC alone at 48 hr produced only marginal increases in 5- and 7-day responses; however, addition of γM myeloma with the SRBC resulted in markedly increased responses by day 7. A primary response to a second antigen, HRBC, initiated when the excess

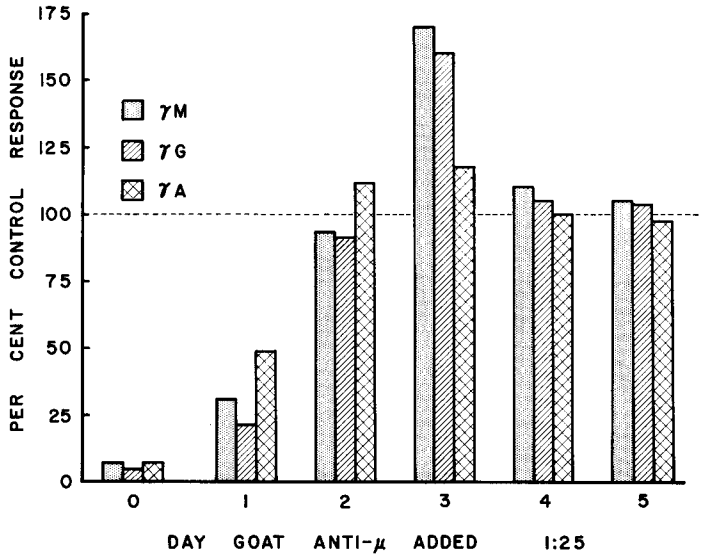

FIG. 7. Effect on PFC responses in vitro of goat anti-μ added after initiation of cultures.

anti-μ was removed at 48 hr was about 50% of the response in control cultures after 5 days' incubation with HRBC (7 days total); addition of γM myeloma protein with the HRBC further reduced the suppressive activity of the anti-μ and responses in these cultures were about 75% of control responses. γG myeloma protein had no effect alone and no additive effect when added with SRBC or HRBC. In experiments where the order of addition of HRBC and SRBC to the cultures was reversed, the results were similar.

Effect of Anti-μ Added after Initiation of Spleen Cell Culture on PFC Responses In Vitro.—Anti-μ may be blocking antigen receptors on precursor cells and thus preventing antigenic stimulation. If this were true, once stimulation of the precursors had been achieved, anti-μ should have no effect. The suppressive effect of anti-μ was most pronounced when it was added at the initation of the cultures (Fig. 7). Less suppression resulted when anti-μ was added at 24 hr with

dispersion of cell clusters in the cultures. No significant effect was observed when anti-μ was added at 48 hr. Paradoxically, an amount of anti-μ which was suppressive when added at the initiation of the cultures (50 μl, 1:25 dilution), significantly enhanced γM, γG, but not γA PFC responses when added to cultures at 72 hr. Anti-μ added at 96 hr or 30 min before asay at 120 hr had no significant effect.

Effect of Antigen Dose on Suppressive Activity of Anti-μ on PFC Responses In Vitro.—The effects of several dilutions of anti-μ on γM and γG PFC responses in cultures stimulated with 10^7, 10^5, or 10^3 SRBC were compared. Antigen did not effectively compete with anti-μ for antigen receptors (Fig. 8). The degree

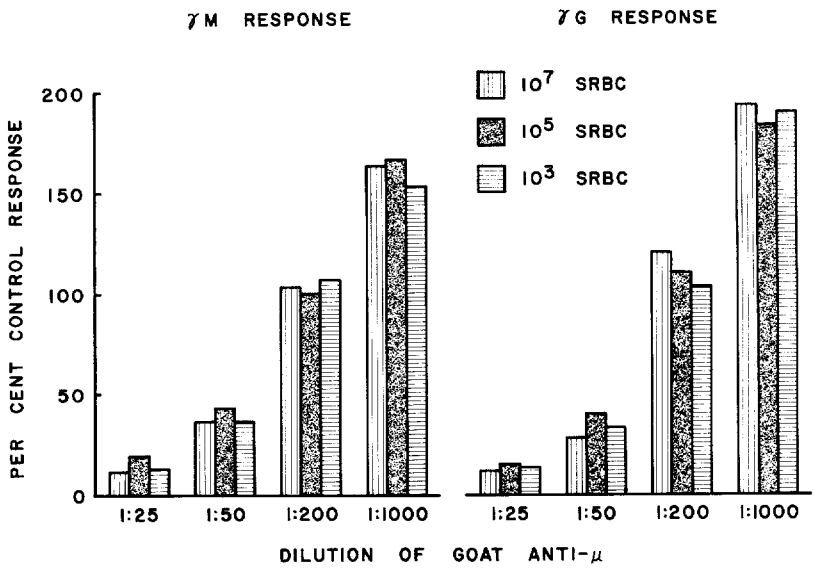

FIG. 8. Effect of antigen dose on suppressive activity of goat anti-μ on PFC responses in vitro.

of suppression by each dilution of anti-μ was almost identical at each SRBC dose for γM and γG responses.

Duration of Exposure to Anti-μ Required to Achieve Suppression of PFC Responses In Vitro.—Before the investigation of the site of action of anti-μ, the duration of exposure of spleen cells to anti-μ in cultures without antigen necessary to achieve suppression of a subsequent PFC response was determined. Spleen cells were incubated with 50 μl of a 1:10 dilution of anti-μ for various intervals, excess anti-μ was removed by washing and cultures were then stimulated with SRBC; PFC responses were assayed 5 days after addition of antigen. Exposure of spleen cells to anti-μ for 24 hr was necesary to achieve reproducible suppression of both γM a nd γG responses (Fig. 9). γM myeloma protein par-

tially reversed the suppression when added with the antigen. Shorter exposure to anti-μ did not result in significant and reproducible suppression.

Site of Action of Anti-μ in Suppression of PFC Responses In Vitro.—Interactions among three functionally distinct type of cells, macrophages, thymus-derived lymphocytes, and precursor lymphocytes, are required for development of PFC responses in vitro[3] (5, 6, 64–72).

Macrophages (MΦ) were separated from spleen cell suspensions by adherence to the plastic culture dishes (72, 73) and freed of loosely adhering lymphoid cells by washing with HBSS. The nonadherent lymphoid cells from spleen (10×10^6/ml) were treated with AKR anti-θ C3H (titer 1:1256, final dilution 1:20) and guinea pig complement (Baltimore Biological Laboratories, final dilution

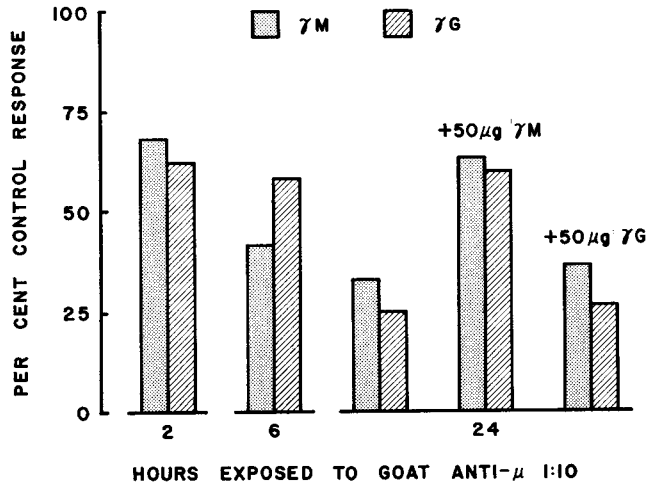

FIG. 9. Duration of exposure to goat anti-μ required to achieve suppression of subsequently stimulated cultures.

1:10) to remove θ-positive, thymus-derived cells (57, 58, 65). The lymphoid cells, antiserum, and complement were incubated at 37°C for 60 min; cells were washed twice by centrifugation with 25 vol of HBSS. None of the cells remaining could be killed by anti-θ and complement; this population contains the precursors of antibody-forming cells and will be referred to as -θLC (anti-θ-treated lymphoid cells). Axillary and inguinal lymph nodes were the source of thymus-derived cells and are referred to as LNC; 75–85% of these cells were θ-positive. None of these partially purified cell populations when incubated alone or in combinations of any two cell types at cell densities indicated in Table II developed γM or γG PFC responses greater than those detected in unstimulated

[3] Pierce, C. W., and B. M. Johnson. Immune responses in vitro. VI. Cell interactions in the development of primary γM and γG plaque-forming cell responses in vitro. Manuscript in preparation.

whole spleen cell cultures. Further details of preparation of these cell populations and their interactions in development of γM and γG PFC responses in vitro will be presented elsewhere.[3] Cell populations were incubated with or without anti-μ (50 μl, 1:10 dilution) for 24 hr and washed by centrifugation; cultures were reconstituted to 1 ml final volume as follows: MΦ 1–2 $\times$ 10^5/dish; lymph node cells (LNC) 1.0–1.5 $\times$ 10^6/dish; -θLC 8.0–8.5 $\times$ 10^6/dish, and 1 $\times$ 10^7 SRBC.

Cultures in which all three cell populations had been exposed to anti-μ or to which anti-μ (50 μl, 1:25 dilution) was added at reconstitution had suppressed

TABLE II

Cellular Requirements for Development of Primary γM and γG PFC Responses In Vitro

Culture variables		PFC/10^6 recovered cells—day 5					
		γM			γG		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
10×10^6 spleen cells	+ SRBC	840	1137	1417	360	420	463
1×10^5 MΦ	+ SRBC	23	21	13	3	0	3
2×10^6 LNC	+ SRBC	10	13	29	0	0	7
8×10^6 $-\theta$LC	+ SRBC	168	177	181	31	39	23
1×10^5 MΦ + 2×10^6 LNC	+ SRBC	13	17	13	1	2	0
1×10^5 MΦ + 8×10^6 $-\theta$LC	+ SRBC	151	183	140	33	50	21
2×10^6 LNC + 8×10^6 $-\theta$LC	+ SRBC	169	152	189	38	53	29
1×10^5 MΦ + 2×10^6 LNC + 8×10^6 $-\theta$LC	+ SRBC	977	1376	1110	387	516	444
1×10^5 MΦ + 2×10^6 LNC + 8×10^6 $-\theta$LC	—	133	141	179	30	41	30
10×10^6 spleen cells	—	147	163	155	27	49	37

Cell populations are prepared as described in the text. MΦ = macrophages; LNC = lymph node cells; $-\theta$LC = splenic lymphoid cells treated with anti-θ and complement; SRBC = 3–7 $\times$ 10^6 sheep erythrocytes.

responses (Fig. 10). However, cultures in which only MΦ or LNC or both of these cell types had been exposed to anti-μ were not suppressed. Cultures with anti-μ–treated -θLC or precursors had suppressed responses which were partially restored by addition of γM, but not γG myeloma protein at the time of reconstitution.

DISCUSSION

These experiments demonstrate that goat anti-mouse μ-chain antibody almost completely suppresses primary PFC responses of γM, γG, and γA Ig classes in mouse spleen cell cultures, whereas anti-γ_1 or anti-γ_2 suppress both γ_1 and γ_2, but not γM or γA PFC responses, and anti-γA suppresses only γA

responses. The degree of suppression by anti-μ is dependent on the amount of anti-μ added to the culture, and the time at which it is added. The anti-μ chain serum was highly specific for μ-chain determinants: Suppressive activity was eliminated by absorption with insolubilized γM myeloma protein, but not with similarly treated γG myeloma proteins; further, this suppressive activity was inhibited competitively by γM myeloma protein in solution, but not by γG or γA proteins. The activity of anti-μ was not the result of a cytotoxic effect;

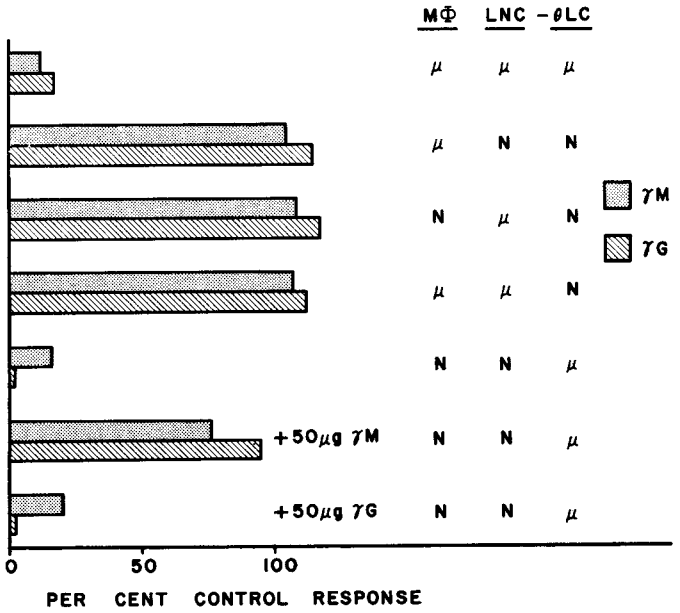

FIG. 10. Cellular site of action of goat anti-μ in suppression of PFC responses. Macrophages (MΦ), lymph node cells, as a source of thymus-derived cells (LNC), and anti-θ–treated spleen lymphoid cells as a source of precursors of antibody-forming cells (-θLC) were prepared as described in the text, and incubated with (μ) or without (N) anti-μ for 24 hr. Cultures were then reconstituted with 1–2 $\times$ 10^5 MΦ/dish, 1.0–1.5 $\times$ 10^6 LNC/dish, and 8.0–8.5 $\times$ 10^6 -θLC/dish and 10^7 SRBC. Cell populations alone or in any combination of two gave no response.

anti-μ was not cytotoxic alone or in the presence of a variety of complement sources, as judged by ^{51}Cr release from labeled spleen or thymus cells.

More compelling evidence for lack of cytotoxicity was obtained in studies of spleen cell function after anti-μ was removed from the culture system. Spleen cells incubated in, then separated from medium containing anti-μ after 48 hr responded to new antigen, SRBC, or HRBC, if the culture period was prolonged to 7 days. Addition of γM myeloma protein together with the new antigen improved the response, indicating that anti-μ bound to the cells could be enticed from the cell surfaces by excess γM protein in solution. This phenomenon

was also observed in experiments assayed after 5 days' incubation with antigen in which the suppression produced by incubation of spleen cells with anti-μ for 24 hr was partially reversed by addition of γM myeloma protein after removal of excess anti-μ. The experiments in which anti-μ was added at 24 hr-intervals after initiation of the cultures also demonstrated lack of nonspecific cytotoxicity of this reagent, but more importantly provided strong evidence that stimulation of precursor cells by antigen is completed by 48 hr. At this time or later, anti-μ no longer had a suppressive effect, suggesting that the event(s) with which it interfered when added earlier in the response was completed. In fact, when the normally suppressive amount of anti-μ was added at 72 hr after initiation of the cultures, γM and γG responses were significantly enhanced. This enhancement may be due to neutralization of an early, regulatory γM antibody, which normally modulates responses, as has been suggested previously (52), or to a mitogenic effect of the anti-μ on stimulated precursor cells with surface μ-chain determinants which results in an expansion of this clone of cells. These possibilities are currently being investigated.

Another observation deserves comment at this juncture: Effective suppression of PFC responses requires incubation of spleen cells with anti-μ for 24 hr or longer. Brief exposures, which should have been sufficient for anti-μ to combine with μ-chain determinants in receptors, resulted in only marginal suppression. The mechanism of suppression by anti-μ appears to be more complex than simply the saturation of Ig receptors and prevention of antigenic stimulation. Perhaps a time-dependent phenomenon, such as endocytosis of an anti-Ig–cell surface Ig complex (74, 75), which results in removal of Ig from the cell surface plays a part in the suppression. Whatever the explanation, these Ig receptors probably can no longer be considered as static membrane structures, but must be considered in terms of their kinetics and mobility in the cell membrane.

Unlike soluble γM protein, increased numbers of erythrocytes added to the cultures proved incapable of overcoming the suppressive effect of anti-μ. It is possible that the anti-μ had a greater affinity for the receptor than did the antigen. It is also conceivable that it is impossible to raise the effective antigen dose sufficiently to overcome the effects of anti-μ. In this context, it is worth noting that the dose-response curve for SRBC in mouse spleen cell cultures is quite flat; an increase in the dose of antigen from 10^8 to 10^7 RBC/culture produces only a 10- to 20-fold increase in PFC responses (52, 73).

The data indicate that the suppressive effect of anti-μ is mediated through the precursor lymphocytes. These cells have surface Ig molecules (25–27) which in the mouse are largely of the γM class (17, 35). Thus, the combination of anti-μ with μ-chain determinants in γM receptors for SRBC and the effective saturation of or interference with the function of these receptors would prevent stimulation of these cells by antigen and suppress subsequent antibody production. The data do not preclude the existence of μ-chain determinants on thymus-derived lymphocytes as has been reported (42); they only indicate that the

suppression of PFC responses by anti-μ is not mediated through μ-chain determinants in receptors in thymus-derived cells.

The class of the surface Ig receptor on the precursor cell has been thought to represent the Ig class of the antibody that will be produced by that cell after antigenic stimulation (29, 32, 38, 42, 44, 46–48): The prediction that saturation of Ig class-specific receptors on precursor cells by antiglobulin would result in suppression of the antibody response in only that Ig class was not verified, especially in the case of anti-μ. The prediction was strictly correct only for anti-γA which suppressed only γA responses. Although anti-γ_1 or anti-γ_2 did not suppress γA or γM responses, either reagent suppressed both γ_1 and γ_2 responses. This cross inhibition may be due to cross-reacting antibodies in the reagents that went undetected in conventional assay methods. However, the possibility that the cells capable of synthesizing γ_1 or γ_2 antibodies have both γ_1 and γ_2 receptors present on the same cell must be considered (25).

Mouse spleen lymphocytes have γ- and α-determinants in immunoglobulin receptors on their surfaces (11, 12, 25–27). This might account for the ability of antiglobulins to suppress responses in these classes without affecting γM responses; it also lends support to the concept that sometimes the surface Ig is of the same class as the antibody later to be secreted. However, cells with γ- or α-determinants may have arisen by a previous encounter with a closely related antigenic determinant and may reflect remnants of a memory cell pool remaining in the "experimentally virgin beast."

The lack of effect of anti-κ in the present experiments is most likely due to the quality of this reagent, since other investigators using anti-κ without complement have successfully inhibited PFC responses in vitro (49). Experiments in which anti-IgG was used to inhibit γM PFC responses were undoubtedly successful due to antibody activity to common Ig determinants in the polyvalent reagents (42, 50, 51). Similarly, failure of anti-Fc reagents to suppress γM PFC responses is probably related to the fact that the anti-Fc was directed against γ_{2a} Fc determinants and not γM Fc. Effects on γ_{2a} class-specific responses were not reported (50).

There are several possible explanations for the observation that anti-μ suppresses γG and γA as well as γM PFC responses; all are related to the Ig receptors on virgin precursor cells since the demonstrated site of action of the anti-μ is on this cell population and not on thymus-derived lymphocytes.

Precursor cells may have many receptors in close proximity on their surfaces, some with μ-determinants, others with γ- or α-determinants. Blocking the function of the receptor with the μ-chain determinant may, due to steric hindrance, also prevent antigen binding to receptors with γ- and α-determinants and subsequent stimulation of these cells. This explanation is unlikely since anti-γ_1, -γ_2, or -γA reagents did not cause suppression of all Ig class-specific responses, which would be expected, by analogy, if steric factors alone were important in suppression by anti-μ. However, such steric factors may help explain suppression of

both γ_1 and γ_2 PFC responses with either anti-γ_1 or anti-γ_2 reagents if γ_1 and γ_2 receptors are represented on the same cell surfaces in close proximity.

γG and γA PFC might arise by direct conversion from cells secreting γM antibody earlier in the immune response. Inhibition of γM PFC development by anti-μ would preclude γG or γA PFC development. Studies in which conversion from γM to γG antibody secretion were reported indicate that this is a limited event of such low frequency that numbers of observed γG PFC cannot all have arisen by direct conversion (76–78).

Antibody-forming cell precursors may be precommitted to synthesis of γM, γG, or γA antibody and bear surface receptors of only that Ig class. Stimulation of precursors with γM receptors might be a necessary and integral step in the stimulation and development of precursors with γG or γA receptors into PFC. This implies, however, some necessary interaction of antigen-stimulated γM precursors with γG or γA precursors for complete stimulation of these latter precursor cells into antibody-secreting cells. The data presented here do not exclude this possibility. However, such a model complicates the cell interaction scheme currently in vogue for development of humoral antibody responses to a four-cell model and restricts humoral immune responses to always having γM antibody production or at least stimulation of γM antibody-producing cell precursors as a necessary condition for stimulation of γG or γA precursor cells. Data presented in the accompanying paper suggest that such a model is unlikely (79).

Virgin precursor cells with γM receptors may not be precommitted to synthesis of antibody of a given Ig class and could conceivably differentiate to synthesize γM, γ_1, γ_2, or γA antibody, depending on as yet unknown factors operative at the time of stimulation of the precursor cell by antigen. Suppression of all Ig class-specific responses by anti-μ is readily explained in this model. However, some indirect evidence may be interpreted to support the concept that precursor cells are precommitted to synthesis of antibodies of only one Ig class (28, 46, 48, 80, 81); analysis of surface receptors in these cells is incomplete, but γM has been shown to be the predominating surface Ig on spleen lymphocytes (17, 25, 35). Thus a precursor cell with a γM receptor might have differentiated without antigenic stimulation, but under other selective pressures, into a cell capable of, for example, only γ_1 antibody synthesis. In this model, too, supression of responses in all Ig classes by anti-μ is easily explained. Recent studies in germfree mice treated from birth with goat anti-μ demonstrated that these mice had fewer lymphocytes with surface Ig of any class, depressed Ig synthesis in vitro in all classes and no humoral antibody response to ferritin (82). The data were interpreted to indicate that anti-μ directly suppressed development of precursor lymphocytes and that cells ultimately committed to synthesis of γ_1, γ_2, or γA Ig were derived from cells which expressed γM determinants at an earlier stage of differentiation. However, suppression of γG or γA responses with anti-γ_1, anti-γ_2, or anti-γA reagents is not readily explained in these models.

It is conceivable that precursor cells precommitted to γ_1, γ_2, or γA antibody synthesis already have receptors of that Ig class in addition to γM receptors, or develop receptors of that Ig class after antigenic stimulation. Stimulation of precursors with both γM and γG or γA receptors might require antigen binding to both receptors. Or, where γG or γA receptors appear after antigenic stimulation via the γM receptor, antigen binding to the second receptor may be necessary for actual antibody synthesis and secretion; failure to bind antigen by the second receptor may be the mechanism of generation of memory cells. Anti-μ by reaction with the γM receptor common to all precursors could suppress responses in all Ig classes, whereas other antiglobulins would effect γA or γG, but not γM responses. Although determinants of two heavy chain classes have not been directly demonstrated on lymphocyte membranes, recent studies suggest this might be possible (25, 28, 83). Thus, during the development of γG or γA antibody-producing cells in a primary response, it appears that for some interval the precursors of these cells must display the Ig class later to be secreted as an Ig receptor in addition to a γM receptor. After immunization, however, clones of precursor cells appear whose γG responses in vitro are not markedly suppressed by anti-μ, but are suppressed by anti-γ_1 or anti-γ_2. Immunization appears to generate changes in the class of Ig receptors on certain precursor cell populations; the kinetics of these cell populations suggest that they represent memory cell pools of γ_1 and γ_2 antibody-producing cell precursors. Subsequent antigenic stimulation of these cells may be mediated primarily through receptors of γ_1 or γ_2 Ig classes and not through receptors of the γM class (79).

Whether cells bearing γM receptors are precommitted or not to synthesis of other Ig classes, and whether or not receptors of two Ig classes are present on some virgin precursor cells, the finding that anti-μ suppresses development of PFC of all Ig classes raises certain obvious questions. First, are γM receptors restricted to precursor cells present before stimulation with antigen; second, does immunization produce changes in the precursor cell population with respect to its susceptibility to suppressive antiglobulins specific for different Ig classes; and third, are such changes, if present, permanent? The accompanying paper describes an investigation of these questions (79).

SUMMARY

The suppressive effects of monospecific goat anti-mouse globulins on primary immunoglobulin class-specific plaque-forming cell responses in mouse spleen cell cultures were investigated. Anti-μ suppressed responses in all immunoglobulin classes, whereas anti-γ_1 and anti-γ_2 suppressed the γ_1 and γ_2 responses but not γM or γA responses, and anti-γA suppressed only γA responses. The mechanism of action of the anti-μ was studied in detail because of its suppression of responses in all immunoglobulin classes.

The anti-μ was specific for μ-chain determinants; its activity was dose dependent, but was not mediated by killing cells with surface μ-chain deter-

minants. Free γM but not γG myeloma proteins in solution effectively competed with μ-bearing cells for the anti-μ. An excess of anti-μ was necessary in the cultures for 48 hr to insure complete suppression of 5-day responses. However, after removal of excess anti-μ at 48 hr, responses could be stimulated by newly added antigen in cultures where incubation was prolonged to 7 days. Anti-μ was most effective when added at the initiation of cultures and had no suppressive effect when added at 48 hr. Excess antigen did not effectively compete with anti-μ for antigen receptors. Precursors of antibody-forming cells were shown to be the cell population where the suppressive activity of anti-μ was mediated.

The experiments suggest that anti-μ combines with μ-chain determinants in antigen-specific receptors on the surfaces of antibody-forming cell precursors, prevents effective stimulation by antigen and subsequent antibody production. To explain suppression of responses in all Ig classes by anti-μ, several models were proposed. It is not possible to determine from the data whether stimulation of precursor cells with γG or γA receptors requires concommitant stimulation of separate cells with only γM receptors, or whether cells bearing γM receptors are precommitted to or differentiate into cells capable of synthesis of other Ig classes, or whether receptors of γM and another Ig class are present on some virgin precursors or the second Ig receptor appears after antigenic stimulation.

We thank Dr. Baruj Benacerraf for encouragment and advice during the course of these experiments.

REFERENCES

1. Claman, H. N., and E. A. Chaperon. 1969. Immunologic complementation between thymus and marrow cells—a model for the two-cell theory of immunocompetence. *Transplant. Rev.* **1**:92.
2. Taylor, R. B. 1969. Cellular cooperation in antibody response of mice to two serum albumins: specific function of thymus cells. *Transplant. Rev.* **1**:114.
3. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen-reactive cells. *Transplant. Rev.* **1**:3.
4. Davies, A. J. S. 1969. The thymus and the cellular basis of immunity. *Transplant. Rev.* **1**:43.
5. Hartmann, K.-U. 1970. Induction of a hemolysin response in vitro. Interaction of cells of bone marrow origin and thymic origin. *J. Exp. Med.* **132**:1267.
6. Hartmann, K.-U. 1971. Induction of a hemolysin response in vitro. II. Influence of the thymus-derived cells during the development of antibody-producing cells. *J. Exp. Med.* **133**:1325.
7. Burnet, F. M. 1959. *In* The Clonal Selection Theory of Acquired Immunity. Vanderbilt University Press, Nashville, Tenn. 209.
8. Siskind, G. W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Advan. Immunol.* **10**:1.
9. Mitchison, N. A. 1967. Antigen recognition responsible for the induction in vitro of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32**:431.

10. Bretscher, P. A., and M. Cohn. 1968. Minimal model for the mechanism of antibody induction and paralysis by antigen. *Nature (London)*. **220:**444.
11. Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue. 1970. Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.* **133:**156.
12. Raff, M. C., M. Steinberg, and R. B. Taylor. 1970. Immunoglobulin determinants on the surface of mouse lymphoid cells. *Nature (London)*. **225:**553.
13. Pernis, B. L., L. Forni, and L. Amante. 1970. Immunoglobulin spots on the surface of rabbit lymphocytes. *J. Exp. Med.* **132:**1001.
14. Sell, S., and P. G. H. Gell. 1965. Studies on rabbit lymphocytes in vitro. I. Stimulation of blast transformation with antiallotype serum. *J. Exp. Med.* **122:**423.
15. Coombs, R. R. A., B. W. Guerner, C. A. Janeway, A. B. Wilson, P. G. H. Gell, and A. S. Kelus. 1970. Immunoglobulin determinants on the lymphocytes of normal rabbits. I. Demonstration by the mixed antiglobulin reaction of determinants recognized by anti-γ, anti-μ, anti-Fab and anti-allotype sera, anti-As4 and anti-As6. *Immunology*. **18:**447.
16. Baur, S., E. Vitetta, C. J. Sherr, I. Schenkein, and J. W. Uhr. 1971. Isolation of H and L chains of immunoglobulin from surfaces of lymphoid cells. *J. Immunol.* **106:**1133.
17. Vitetta, E. S., S. Baur, and J. W. Uhr. 1971. Cell surface immunoglobulin. II. Isolation and characterization of immunoglobulin from mouse spleen lymphocytes. *J. Exp. Med.* **134:**242.
18. Daguillard, F., and M. Richter. 1969. Cells involved in the immune response. XII. The differing responses of normal rabbit lymphoid cells to phytohemagglutinin, goat anti-rabbit immunoglobulin antiserum and allogeneic and xenogeneic lymphocytes. *J. Exp. Med.* **130:**1187.
19. Oppenheim, J. J., G. N. Rogentine, and W. D. Terry. 1969. Transformation of human lymphocytes by monkey antisera to human immunoglobulins. *Immunology*. **16:**123.
20. Klein, E., T. Eskeland, M. Inoue, R. Strom, and B. Johansson. 1970. Surface immunoglobulin-moieties on lymphoid cells. *Exp. Cell Res.* **62:**133.
21. Bert, G., A. L. Massaro, D. L. Di Cassano, and M. Maja. 1969. Electrophoretic study of immunoglobulins and immunoglobulin subunits on surface of human peripheral blood lymphocytes. *Immunology*. **17:**1.
22. Davie, J. M., W. E. Paul, R. G. Mage, and M. B. Goldman. 1971. Membrane associated immunoglobulin of rabbit peripheral blood lymphocytes: allelic exclusion at the b locus. *Proc. Nat. Acad. Sci. U.S.A.* **68:**430.
23. Johansson, B., and E. Klein. 1970. Cell surface localized IgM-kappa immunoglobulin reactivity in a case of chronic lymphatic leukemia. *Clin. Exp. Immunol.* **6:**421.
24. Ivanyi, J., E. Skamene, and A. Kurisu. 1970. Stimulation of chicken lymphocytes *in vitro* by anti-immunoglobulin serum. *Folia Biol. (Praha)*. **16:**34.
25. Bankhurst, A. D., and N. L. Warner. 1971. Surface immunoglobulins on mouse lymphoid cells. *J. Immunol.* **107:**368.
26. Raff, M. C. 1970. Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence. *Immunology*. **19:**637.
27. Unanue, E. R., H. M. Grey, E. Rabellino, P. Campbell, and J. Schmidtke. 1971.

Immunoglobulins on the surface of lymphocytes. II. The bone marrow as the main source of lymphocytes with detectable surface-bond immunoglobulin. *J. Exp. Med.* **133:**1188.

28. Sell, S. 1970. Development of restrictions in the expression of immunoglobulin specificities by lymphoid cells. *Transplant. Rev.* **5:**22.
29. Paul, W. E. 1970. Functional specificity of antigen-binding receptors of lymphocytes. *Transplant. Rev.* **5:**130.
30. Davie, J. M., and W. E. Paul. 1971. Receptors on immunocompetent cells. II. Specificity and nature of receptors on dinitrophenylated guinea pig albumin-^{125}I–binding lymphocytes of normal guinea pigs. *J. Exp. Med.* **134:**495.
31. Davie, J. M., A. S. Rosenthal, and W. E. Paul. 1971. Receptors on immunocompetent cells. III. Specificity and nature of receptors on dinitrophenylated guinea pig albumin-^{125}I–binding lymphocytes of immunized guinea pigs. *J. Exp. Med.* **134:**517.
32. Wigzell, H., and O. Mäkelä. 1970. Separation of normal and immune lymphoid cells by antigen-coated columns. Antigen-binding characteristics of membrane antibodies as analyzed by hapten-protein antigens. *J. Exp. Med.* **132:**110.
33. Ada, G. L., and P. Byrt. 1969. Specific inactivation of antigen-reactive cells with ^{125}I-labelled antigen. *Nature (London).* **222:**1291.
34. Byrt, P., and G. L. Ada. 1969. An *in vitro* reaction between labelled flagellin or haemocyanin and lymphocyte-like cells from normal animals. *Immunology.* **17:** 503.
35. Warner, N. L., P. Byrt, and G. L. Ada. 1970. Blocking of the lymphocyte antigen receptor site with anti-immunoglobulin sera *in vitro*. *Nature (London).* **226:**942.
36. Naor, D., and D. Sulitzeanu. 1967. Binding of radioiodinated bovine serum albumin to mouse spleen cells. *Nature (London).* **214:**687.
37. Mason, S., and N. L. Warner. 1970. The immunoglobulin nature of the antigen recognition site on cells mediating transplantation immunity and delayed hypersensitivity. *Immunology.* **104:**762.
38. Wigzell, H., and B. Andersson. 1969. Cell separation on antigen-coated columns. Elimination of high rate antibody-forming cells and immunological memory cells. *J. Exp. Med.* **129:**23.
39. McConnell, I., A. Munro, B. W. Gurne, and R. R. Coombs. 1969. Studies on actively allergized cells. I. The cyto-dynamics and morphology of rosette forming lymphoid cells in mice and inhibition of rosette formation with antibody to mouse immunoglobulins. *Int. Arch. Allergy Appl. Immunol.* **35:**209.
40. Truffa-Bachi, P., and L. Wofsy. 1970. Specific separation of cells on affinity columns. *Proc. Nat. Acad. Sci. U.S.A.* **66:**685.
41. Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye. 1971. Specific inactivation of thymus-derived (T) and non-thymus derived (B) lymphocytes by ^{125}I-labelled antigen. *Nature (New Biol.) (London).* **231:**104.
42. Greaves, M. F. 1970. Biological effects of anti-immunoglobulins: evidence for immunoglobulin receptors on 'T' and 'B' lymphocytes. *Transplant. Rev.* **5:**45.
43. Ada, G. L. 1970. Antigen binding cells in tolerance and immunity. *Transplant. Rev.* **5:**105.
44. Wigzell, H. 1970. Specific fractionation of immunocompetent cells. *Transplant. Rev.* **5:**76.

45. Cooper, M. D., R. D. A. Peterson, M. A. South, and R. A. Good. 1966. The functions of the thymus system and the bursa system in the chicken. *J. Exp. Med.* **123:**75.
46. Mäkelä, O. 1970. Analogies between lymphocyte receptors and the resulting humoral antibodies. *Transplant. Rev.* **5:**3.
47. Walters, C. S., and H. Wigzell. 1970. Demonstration of heavy and light chain antigenic determinants on the cell-bound receptor for antigen. Similarities between membrane-attached and humoral antibodies produced by the same cell. *J. Exp. Med.* **132:**1233.
48. Mäkelä, O., and A. M. Cross. 1970. The diversity and specialization of immunocytes. *Progr. Allergy.* **14:**145.
49. Lesley, J., and R. W. Dutton. 1970. Antigen receptor molecules: inhibition by antiserum against kappa light chains. *Science (Washington).* **169:**487.
50. Hartmann, K.-U., S. Reeg, and C. Mehner. 1971. The induction of haemolysin producing cells *in vitro*: inhibition by antiglobulin antisera. *Immunology.* **20:**29.
51. Feldmann, M., and E. Diener. 1971. Reversible blocking effect of anti-mouse immunoglobulin serum on the induction of immunity and tolerance. *Nature (New Biol.) (London).* **231:**183.
52. Pierce, C. W., B. M. Johnson, H. E. Gershon, and R. Asofsky. 1971. 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. *J. Exp. Med.* **134:**395.
53. 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.
54. Biozzi, G., R. Asofsky, R. Lieberman, C. Stiffel, D. Mouton, and B. Benacerraf. 1970. Serum concentrations and allotypes of immunoglobulins in two lines of mice genetically selected for "high" and "low" antibody synthesis. *J. Exp. Med.* **132:**752.
55. 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.
56. Reif, A. E., and J. M. V. Allen. 1966. Mouse thymic isoantigens. *Nature (London).* **209:**521.
57. Raff, M. C. 1969. Theta isoantigen as a marker of thymus derived lymphocytes in mice. *Nature (London).* **224:**378.
58. Raff, M. C., and H. H. Wortis. 1970. Thymus dependence of θ bearing cells in the peripheral lymphoid tissues of mice. *Immunology.* **18:**931.
59. Baird, S., J. Santa, and I. Weissman. 1971. Anti-theta antiserum may contain anti-allotype contamination. *Nature (New Biol.) (London).* **232:**56.
60. Boyse, E. A., K. Itakura, E. Stockert, C. A. Iritani, and M. Miuri. 1971. Ly C: a third locus specifying alloantigens expressed only on thymocytes and lymphocytes. *Transplantation.* **11:**351.
61. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Washington).* **130:**432.
62. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody producing cells. *Science (Washington).* **140:**405.

63. Avrameas, S., and T. Ternyck. 1967. Biologically active water insoluble protein polymers. I. Their use for isolation of antigens and antibodies. *J. Biol. Chem.* **242:**1651.

64. Pierce, C. W., and B. M. Johnson. 1971. Cell interactions in primary immune responses *in vitro*. *Fed. Proc.* **31:**526.

65. Chan, E. L., R. I. Mishell, and G. F. Mitchell. 1970. Cell interaction in an immune response *in vitro*: requirement for theta-carrying cells. *Science* (*Washington*). **170:**1215.

66. Doria, G., M. Martinozzi, G. Agarossi, and S. Di Pietro. 1970. *In vitro* primary response resulting from interaction between bone marrow-derived and thymus cells. *Experientia* (*Basel*). **26:**410.

67. Haskill, J. S., P. Byrt, and J. Marbrook. 1970. In vitro and in vivo studies of the immune response to sheep erythrocytes using partially purified cell preparations. *J. Exp. Med.* **131:**57.

68. Hirst, J. A., and R. W. Dutton. 1970. Cell components in the immune response. Neonatal thymectomy: restoration in culture. *Cell. Immunol.* **1:**190.

69. Mosier, D. E., F. W. Fitch, D. A. Rowley, and A. J. S. Davies. 1970. Cellular deficit in thymectomized mice. *Nature* (*London*). **225:**276.

70. Munro, A., and P. Hunter. 1970. In vitro reconstitution of the immune response of thymus-deprived mice to sheep red blood cells. *Nature* (*London*). **225:**277.

71. Talmage, D. W., J. Radovich, and H. Hemmingsen. 1970. Cell interaction in antibody synthesis. *Advan. Immunol.* **12:**271.

72. Mosier, D. W. 1967. A requirement for two cell types for antibody formation in vitro. *Science* (*Washington*). **758:**1573.

73. 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.

74. Unanue, E. R., W. D. Perkins, and M. J. Karnovsky. 1972. Endocytosis by lymphocytes of complexes of anti-Ig with membrane-bound Ig. *J. Immunol.* In press.

75. Pernis, B., M. Ferrarini, L. Forni, and L. Amante. 1971. Immunoglobulins on lymphocyte membranes. *In* Progress in Immunology. B. Amos, editor. Academic Press, Inc., New York. 95.

76. Nossal, G. J. V., N. L. Warner, and H. Lewis. 1971. Incidence of cells simultaneously secreting IgM and IgG antibody to sheep erythrocytes. *Cell. Immunol.* **2:**41.

77. Nordin, A. A., H. Cosenza, and S. Sell. 1970. Immunoglobulin class of antibody-forming cells in mice. II. Class restriction of plaque-forming cells demonstrated by replica plating. *J. Immunol.* **104:**495.

78. Merchant, B., and Z. Brahmi. 1970. Duplicate plating of immune cells products: analysis of globulin class secretion by single cells. *Science* (*Washington*). **167:** 69.

79. Pierce, C. W., S. M. Solliday, and R. Asofsky. 1972. Immune responses in vitro. V. Suppression of γM, γG and, γA plaque-forming cell responses in cultures of primed mouse spleen cells by class-specific antibody to mouse immunoglobulins. *J. Exp. Med.* **135:**698.

80. Miller, H. C., and G. Cudkowicz. 1971. Density gradient separation of marrow cells restricted for antibody class. *Science* (*Washington*). **171:**913.

81. 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.

82. Lawton, A. R., R. Asofsky, M. B. Hylton, and M. D. Cooper. 1972. Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to μ-chain. *J. Exp. Med.* **135:**277.

83. Greaves, M. F. 1971. The expression of immunoglobulin determinants on the surface of antigen-binding lymphoid cells in mice. I. An analysis of light and heavy chain restrictions on individual cells. *Eur. J. Immunol.* **1:**186.