

THE PROLIFERATION OF PLASMA CELLS FROM MOUSE BONE MARROW IN VITRO

I. THE ROLE OF THYMUS

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(Received for publication 3 November 1971)

Recent studies on the development of antibody-forming cells have implied cellular interaction among two or three cell types. Claman et al. (1) proposed synergistic interaction between thymus and bone marrow cells in hemolysin production in irradiated mice transplanted with either or both sorts of cells. Globerson and Auerbach (2) also found by using irradiated mice that thymus and bone marrow cells were inevitably necessary for the initiation of graft-vs.-host reaction in vitro. Mitchell and Miller (3, 4) showed that the thymus supplied the antigen-reactive cells, while lymphoid cells derived from bone marrow could differentiate to specific antibody-forming cells after interaction with antigen-reactive cells from thymus.

These results suggest that the in vitro reconstruction of an IgG-producing system must be attempted in order to obtain an accurate understanding of the pathways of antibody-forming reactions. There have been two reports (5, 6) concerning the formation of plasma cells, but these did not mention the role of thymic lymphocytes. As mentioned above, the important role of the thymus in immune responses was confirmed by using several techniques, such as neonatal thymectomy, irradiation, and cell transplantation (7-9). But it has not yet been determined whether the thymus has direct effects on the development of IgG-producing plasma cells. The following in vitro experiments suggest that bone marrow is one of the sources for plasmablasts, and that the thymus plays a very important role in the proliferation of antibody-forming plasma cells.

Materials and Methods

Mice and Cells.—A/Jax and C57BL/6 male and female 12-18-wk-old mice, weighing 18-25 g each, were used throughout the experiments. They were bred in our laboratory in air-conditioned animal rooms and fed Oriental mouse chow and water *ad libitum*. Bone marrow cells were harvested from femurs and tibias. Demascularized bones were crushed with a surgical needle holder in chilled and heparinized Hanks' balanced salt solution (Hanks' BSS)¹ (concentration of heparin, 5 units/ml). Residual large particles were removed by filtration through four sheets of gauze. Cells were sedimented by centrifugation at 600 g for 10 min and nucleated cells were counted and adjusted to a concentration of 3.5×10^6 /ml in the culture

¹Abbreviations used in this paper: Eagle's MEM, Eagle's minimal essential medium; FITC, fluorescein isothiocyanate; Hanks' BSS, Hanks' balanced salt solution; HGG, human gamma globulin; PBS, phosphate-buffered saline.

medium. Axillary and inguinal lymph nodes and thymuses were collected in cold Hanks' solution separately, decapsulated, filtered through four sheets of gauze, centrifuged, and resuspended in Eagle's minimal essential medium (MEM) at a concentration of 10^7 /ml.

Culture Medium.—The basal medium was consisted of eight parts of Eagle's MEM (enriched with a twofold concentration of vitamins and amino acids) and two parts of fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.).

Preparation of Cell Homogenates.—A suspension of lymph node cells or thymus cells, 98–99% of which were small lymphocytes, was sonicated for 90 sec at 10 kc with 10-sec intervals. Homogenates were centrifuged at 10,000 g for 30 min and supernatants were passed through a Millipore filter (type HA, pore size 450 m μ ; Millipore Corp., Bedford, Mass.) and stored at 4°C. The fibroblastic cells used as control cells derived from C57BL/6 mice kidneys (secondary culture) were treated as in above (i.e., nonimmunological cell homogenate originated in syngeneic mice). BAT-6 cells derived from hamster tumor caused by bovine adenovirus type 3 (10) were also treated for the preparation of xenogeneic nonimmunological cell homogenate.

Cell Culture Methods.—Cover glasses (9 × 12 mm) were fixed in Petri dish bottoms by heating with fetal calf serum. 7×10^6 nucleated bone marrow cells suspended in 2 ml of basal medium were seeded into the 35 × 15 mm glass Petri dishes and incubated in 5% CO₂, 95% air, and moisture-saturated incubator. After 24 hr, floating cells and clusters were removed and dishes were washed out with Hanks' BSS three times. Fresh medium containing human gamma globulin (HGG) at the concentration of 2.5 mg/dish was added to the bone marrow cultures of experimental groups. Thymus or lymph node cells or cell homogenates (equivalent to 3.3×10^6 cells) were added at this time. Thereafter at days 3, 5, 8, 12, 16, and 21, cover glasses were removed, dried, and fixed with ethanol solution (ethanol 95%, acetic acid 1%, distilled water 4%) for 5 min. After being passed through absolute ethanol and carbon tetrachloride, cover glasses were stored at 4°C for immunochemical staining. At each time of sampling, the total cell number per Petri dish was counted with a hemacytometer. 0.05–0.2 ml of a 10-fold concentration of Eagle's amino acids and vitamins was supplied every 4 days to each dish.

Preparation of Anti-Allotype Serum (BALB/c Anti-C57BL/6 Ig-1b Antisera).—C57BL/6 mice were sacrificed and their sera pooled. The IgG fraction was emulsified in complete Freund's adjuvant and injected into BALB/c mice, by the subcutaneous route and into the footpad, in the amount of 1 mg/mouse three times a week. After a booster injection into the tail vein, BALB/c anti-C57BL/6 Ig-1b antiserum was harvested (11).

Preparation of Rabbit Anti-Mouse IgG-Fc Antiserum.—The mouse IgG preparation was treated with papain and the Fc fragment was collected. This antigen was injected into rabbits, by the footpad and subcutaneous routes, in the amount of 1 mg/animal three times a week. After a booster injection via the auricular vein, rabbit anti-mouse Fc antiserum was harvested (12).

Preparation of Antiserum to Surface Antigen of Plasma Cell and Identification of Cultured Cells.—MOPC-31B myeloma cells were used as the source of plasma cell surface antigen. Rabbits were inoculated with 200×10^6 live cells subcutaneously and via the footpads once a month. Blood was harvested by heart puncture 10 days after the last inoculation. The pooled antiserum was absorbed with mouse liver powder, live thymus cells, mouse erythrocytes, and MOPC-104E myeloma cells which were producing IgM actively. The serum titers against MOPC-31B myeloma cells and normal plasma cells were examined repeatedly by cytotoxicity tests using super vital staining with Congo red and plaque formation-inhibition tests against sheep red blood cells.² This antiserum was used to identify plasma cells from cultured bone marrow. At the 17th day, cover glasses with the cultured cells were removed from Petri dish, washed five times with Hanks' BSS and immersed in anti-MOPC-31B antiserum (diluted

² Namba, Y. 1971. Private communication.

to 2% in phosphate-buffered saline (PBS)) for 20 min in 37°C incubator. After washing with Hanks' BSS five times, goat anti-rabbit IgG antiserum labeled with fluorescein isothiocyanate (FITC) was overlaid for 20 min. These samples enclosed in culture medium were observed under a fluorescein microscope.

Preparation of FITC-Labeled Antibody.—FITC (Baltimore Biological Laboratories, Cockeysville, Md.) and the gamma globulin fraction extracted from antiserum were mixed in pH 9.6 carbonate buffer at 1:100 of FITC/protein concentration. After stirring for 6 hr at 4°C, mixtures were filtered through Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) and fractionated by diethylaminoethyl (DEAE)-cellulose column, and the F/P ratio of each fraction was calculated. The fractions having a F/P value of 1 to 2 were used for staining (13). These preparations were adsorbed with rabbit or mouse liver powder before use to prevent nonspecific stainings.

Preparation of Enzyme-Labeled Antibody.—50 mg of IgG and of horseradish peroxidase (97–135 units/mg, Sigma Chemical Co., St. Louis, Mo.) were mixed in 2 ml of cold carbonate buffer, pH 10.0, 0.5 M, and then 0.25 ml of bifunctional agent FNPS (*p,p*-difluoro-*m,m*-dinitrodiiphenyl-sulfone; Tokyo Kasei Industrial Co., Tokyo) 0.5% in acetone was added. After being stirred for 6 hr at 4°C, the samples were dialyzed against 3 liters of PBS for 16 hr and centrifuged for 30 min at 10,000 *g* to remove large aggregates. Unconjugated enzyme was removed by sedimentation with 50% saturated ammonium sulfate and free IgG was separated by gel filtration through Biogel P-300. In order to eliminate nonspecific adsorption, the samples were adsorbed with liver powder of rabbit or mouse before use (14).

Enumeration of Antibody-Forming Cells.—At each sampling date, the number of cells which attached to the surface of the dishes were counted with a hemacytometer, after being treated with EDTA solution (0.5 mM ethylenediaminetetraacetate 2 Na in PBS). Cover glasses on which bone marrow cells were attaching were stained with the peroxidase-labeled anti-mouse IgG-Fc or anti-C57BL/6 Ig-1b allotype antiserum. The localization of antigen was visualized after the reaction with diaminobenzidine 0.05 M, and peroxide in tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.6, and nuclear staining with methyl green. The percentage value of antibody-forming cells was obtained by counting 3000 cells. Using both total cell numbers and percentage values, the number of IgG-forming cells per dish was calculated, and the kinetics of cellular development were followed until the 21st day after antigen stimulation.

Antibody Determination.—Cultured bone marrow cells were destroyed by ultrasonication after washing with an EDTA plus trypsin solution (0.5 mM EDTA and 0.2% trypsin, Difco Laboratories, Detroit, Mich.), and suspending in Tris-sucrose buffer (0.05 M Tris buffer, pH 7.6, 0.25 M sucrose, 0.005 M MgCl₂, 0.025 M KCl). They were centrifuged at 600 *g* for 10 min. The supernatant was treated with 0.5% sodium deoxycholate, and all samples were measured for their protein content by Lowry's method. They were then analyzed by the Ouchterony double-diffusion technique and immunoelectrophoresis in order to classify the produced globulins.

Cultured cells on cover glasses were examined by means of peroxidase-labeled antigen HGG staining to determine whether they were producing the specific immunoglobulin against the administered antigenic protein human gamma globulin. Not only qualitative but also quantitative analyses were prospected by counting positively stained cells with peroxidase-labeled anti-C57BL/6 IgG (Ig-1b) antibody or enzyme-labeled antigen staining.

Morphological Observations.—Living cells attached to cover glasses were examined and photographed under a phase-contrast microscope at the magnification × 1000. Fixed specimens were stained with methyl green-pyronin, Giemsa, FITC, or peroxidase-labeled antibody staining. Those specimens which were used for statistical analysis were usually prepared by the peroxidase-labeled antibody (rabbit anti-mouse IgG or IgG-Fc or BALB/c anti-C57BL/6 Ig-1b antiserum) stainings, which included a pretreatment with a 0.1% sodium azide solution

in PBS to prevent endogenous peroxidase activity. After thorough washing out sodium azide with PBS, antibody conjugation, peroxidase reactions, and nuclear staining with methyl green were performed.

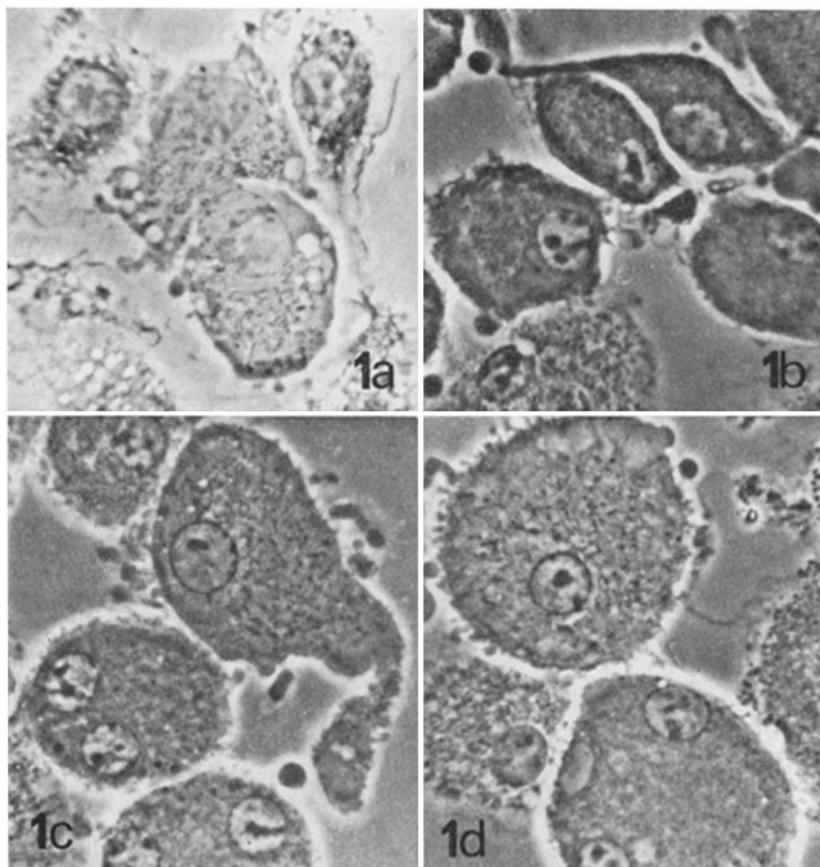


FIG. 1. In vitro proliferation of plasma cells derived from bone marrow was accelerated by the addition of thymic homogenate and HGG. Photographs by phase-contrast microscope. $\times 1000$. (a) Eccentric nucleus, enlarged endoplasmic reticulum (ER), well-developed Golgi apparatus showing "thread confused figures," varying thickness of nuclear membrane, and defined contour were characteristic. Cultured for 13 days after the addition of thymic homogenate and HGG. (b) Plasma cells having characteristic juxtannuclear Golgi apparatus. Cultured for 17 days. (c) Binucleated plasma cells and a foot-elongating cell. Cultured for 17 days. (d) Enlarged ER indicating the retention of synthesized globulin. Cultured for 21 days.

RESULTS

The Morphological Characteristics of Cultured Plasma Cells.—Among the 3-day specimens, a small proportion of long and slender bipolar pyroninophilic cells were recognized. Some cells possessed two nuclei, and all showed well-de-

fined contours. They were very weakly stained with peroxidase-labeled rabbit anti-mouse IgG antibody. Gradually, over a period of days, their bipoles became shorter, and the strength of pyroninophilicity, reactivity against peroxi-

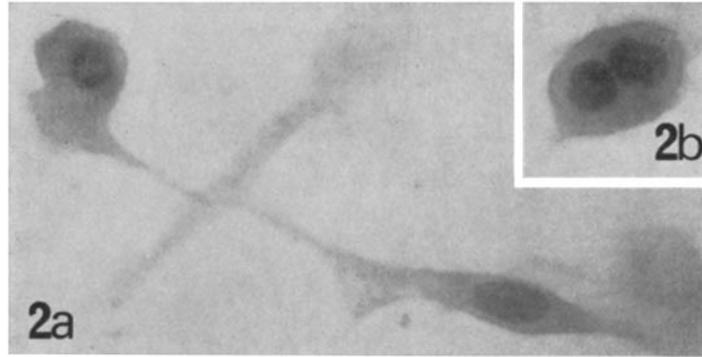


FIG. 2. (a) Pyroninophilic cells showing spindly or elongating cytoplasm with wheel axis-shaped nucleus. Methyl green-pyronin staining, at day 12. (b) Binucleated pyroninophilic cell showing wheel axis-shaped nucleus. Methyl green-pyronin staining, at day 17. $\times 1000$.

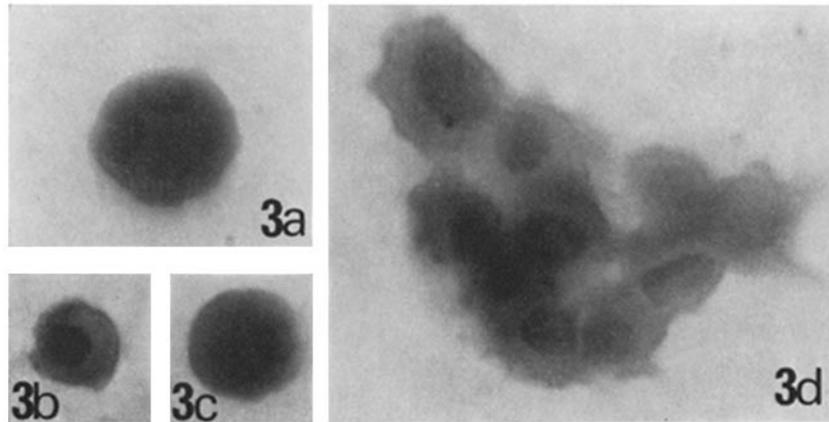


FIG. 3. Peroxidase-labeled antibody stainings of proliferating plasma cells in vitro, from bone marrow. BALB/c anti-C57BL/6 Ig-1b antiserum was used. Plasma cells were induced by the addition of thymic homogenate and HGG. Nuclear staining by methyl green. $\times 1000$. (a) Binucleated IgG-forming cell stained strongly brown. Cultured for 17 days. (b) Strongly brown-stained cell having the juxtannuclear light area, indicating well-developed Golgi apparatus, at day 17. (c) A cluster of IgG-forming cells, at day 21. (d) Round cell having eccentric nucleus, and strongly stained by the peroxidase-Ab procedure, at day 21.

dase-labeled anti-mouse IgG antibody, and brightness with FITC-labeled anti-mouse IgG-Fc antibody staining, became stronger. The typical plasma cells became distinctly recognizable by day 12. They demonstrated broad pyroninophilic cytoplasm, eccentric nuclei, juxtannuclear light areas, and so-called wheel

axis-shaped nuclei. They were stained with FITC or peroxidase-labeled anti-mouse IgG antibody fairly strongly, Golgi apparati were observed as "thread confused figure" at the juxtannuclear area under a phase-contrast microscope (Fig. 1 *a*). However, many pyroninophilic cells still showed spindly or rhombic shapes, and had long elliptic nuclei and sometimes juxtannuclear light areas (Fig.

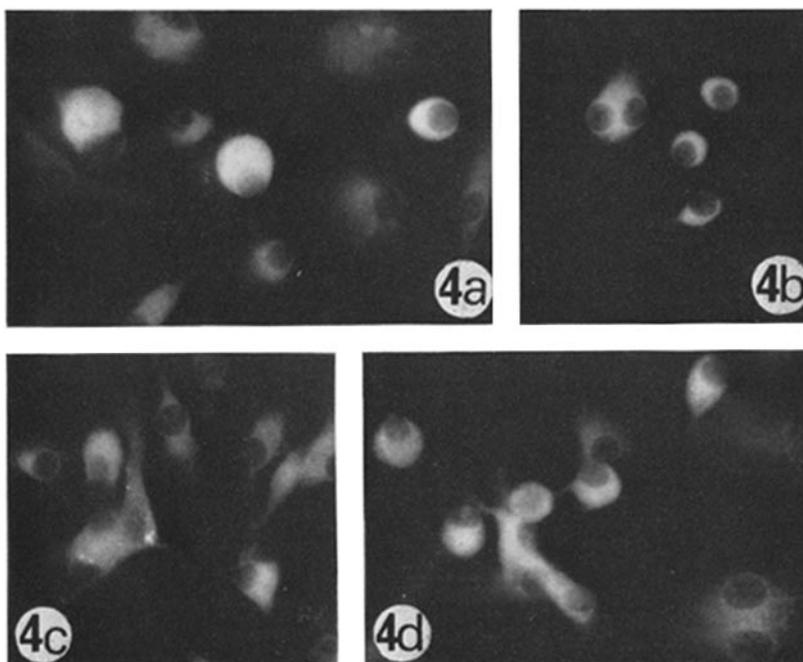


FIG. 4. Fluorescein antibody-stainable cells whose proliferation from bone marrow were accelerated by the administration of thymic homogenate and HGG. Anti-mouse IgG-Fc antiserum labeled with FITC was used. Cultured for 17 days. $\times 400$. (*a*) The round and eccentric nucleus was characteristic, and most cells were oval or round, but a few still demonstrated indefinite forms. (*b*) Binucleated cell with entire cytoplasm stained. (*c*) At the juxtannuclear region of Golgi area was not stained. Binucleated and spindly cells were also stainable. (*d*) Many positive cells joined by intercellular bridges of cytoplasm. A large cell in the lower right was not classified as a plasma cell although it was binucleated.

2 *a*). They were also weakly but positively stained with FITC and peroxidase-labeled anti-mouse IgG antibody. These cells were examined further to study the morphogenesis from plasmablasts to mature IgG-producing cells. At day 16 or 17, shapes of almost all IgG-producing cells had the figure of the so-called "Marsharko's type" (Fig. 4*a*). A few remained spindly. Sometimes mature plasma cells abutted on other sorts of cells, or made a cell cluster. Some cells observed under the phase-contrast microscope appeared like typical matured plasma cells. Golgi apparati were found in compact order, and nuclear membranes were

thick and irregular (Figs. 1 *b* and 4 *c*). Binucleated cells strongly positive for IgG were observed. They also had pyroninophilic cytoplasm and juxtannuclear light areas (Figs. 1 *c*, 2 *b*, 3 *a*, and 4 *b*).

From the 21-day specimens, the effect of thymic agent and the development of plasma cells were seen clearly. In the group to which no thymic agent was added, pyroninophilic cells appeared at different stages of development, forming a parade around the large cells. Some were spindly, some rhombic, some of

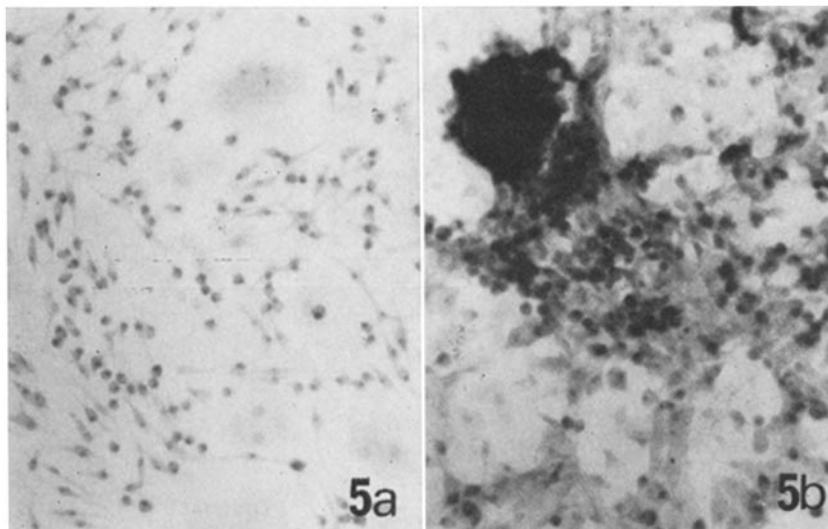


FIG. 5. (a) Bone marrow cells cultured without the addition of thymic cells or their homogenate, at different stages of development. Their figures were spindly, rhombic, oval, or roundish, but fairly pyroninophilic. Cultured for 21 days. Methyl green-pyronin staining. $\times 100$. (b) The development of plasma cells has been stimulated by the addition of thymic homogenate and HGG, making a cluster with other cells. Cultured for 21 days. Methyl green-pyronin staining. $\times 100$.

indefinite shape with short processes, and some roundish with typical plasma cell shapes (Fig. 5 *a*). On the other hand, in the group to which thymic inducer was administered, cell clusters consisted of round or oval cells which were strongly pyroninophilic (Fig. 5 *b*). Such pyroninophilic round cells were also seen all over the cover glasses. These cells or clusters were shown by the FITC and peroxidase-labeled anti-mouse IgG antibody stainings to be actively producing IgG (Figs. 3 *c*, 3 *d*, 4 *d*). They were also observed under the phase-contrast microscope (Fig. 1 *d*).

The allotype of produced IgG was inquired by using BALB/c anti-C57BL/6 IgG (Ig-1b) anti-allotype antisera. Plasma cells induced by the addition of allogeneic mouse thymus cells or their homogenate (A/Jax) were revealed to be synthesizing "recipient bone marrow type" IgG. Namely plasma cells origi-

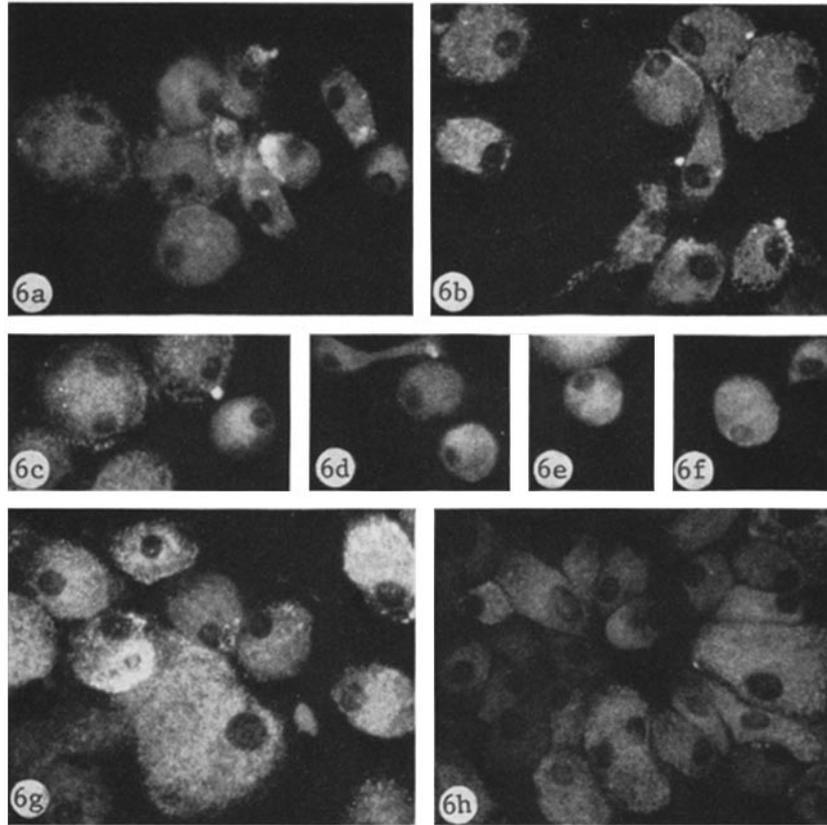


FIG. 6. Surface antigen of cultured plasma cells stained by indirect fluorescein antibody technique. Rabbit antiserum to live myeloma MOPC-31B, and goat anti-rabbit IgG antiserum labeled with FITC, were used. Cells were cultured for 17 days after antigenic stimuli. $\times 400$. (a) A positively stained cell. (b) A cell stained at its marginal zone. (c) A stained binucleated cell with a ring-like form, and a cell with an eccentric nucleus having negative area at under the nucleus. (d-f) Cells demonstrating the "mosaic figures" of positively stained regions on the cell surface. (g) A cell at the upper right was positively stained on a broad area of the surface, but a small region near the nucleus remained negative. Mosaic patterned and ring-like cells are shown. (h) Control staining with normal rabbit serum and goat anti-rabbit IgG antiserum labeled with FITC gave negative results. Even typical plasma cells having juxtannuclear dark area, or two nuclei, or eccentric nuclei were not stained with these procedures.

nating in A/Jax bone marrow could not be stained with FITC-labeled anti-allotype Ig-1b antiserum, however these cells deriving in C57BL/6 bone marrow and being induced by allogeneic thymus cells or their homogenate were always stained with that allotype antiserum (Fig. 3).

Surface Antigen of Cultured Plasma Cells.—About 12% of the bone marrow cells cultured in the presence of thymic cell homogenate and HGG, were posi-

tively stained with rabbit anti-MOPC-31B myeloma antiserum at day 17. Most of these cells showed mosaic figures in fluorescein-stainable areas (Figs. 6 *a*, 6 *c-g*). Some of them were stained at their peripheral zones showing a ring-shaped contour (Fig. 6 *b*). Binucleated cells were also positively stained as above (Fig. 6 *c*). The cytoplasm of many of these cells appeared yellowish except at the fluorescein-stained areas. The yellowish cytoplasm might be due to rich ribosomal RNA, and might indicate active synthesis of protein at the ribosomes. They were not stained by Congo red (super vital staining) indicating that they

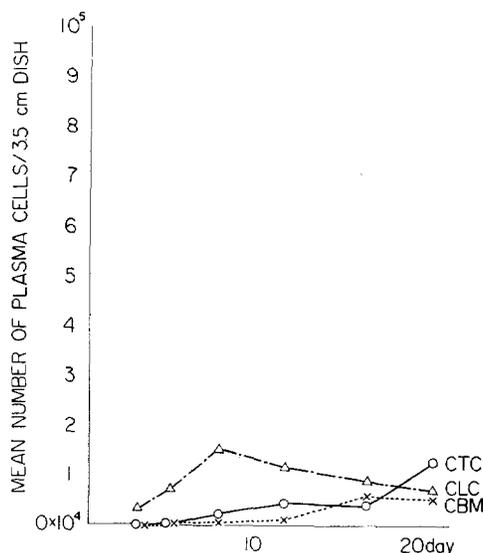


FIG. 7. Kinetics of proliferation of plasma cells. When C57BL/6 bone marrow cells were cultured in basal medium without HGG and other cells, only slight proliferation was observed (X---X, CBM). When syngeneic thymic lymphocytes were added to C57BL/6 bone marrow cultures, some proliferation of plasma cells was obtained (O—O, CTC). Similar results were obtained by syngeneic lymph node lymphocytes (Δ --- Δ , CLC).

were alive and not in a degenerative stage. When bone marrow cultures were treated with normal rabbit serum, even typical plasma cells did not show positive fluorescein (Fig. 6 *h*).

Kinetics of Plasma Cells Induced from Bone Marrow In Vitro.—The kinetics of the proliferation of plasma cells were investigated. The results mentioned below are a summary of at least three experiments performed repeatedly under the same conditions. Each subgroup in one experiment consisted of three dishes at every sampling time. Therefore, each point representing a number of plasma cells was averaged from at least nine dishes.

The effects of thymic lymphocytes or lymph node lymphocytes derived from syngeneic C57BL/6 mice: When bone marrow cells were cultured in basal medium

in the absence of other cells and proteins, very little proliferation of plasma cells was observed, the maximum number being 6500/dish at day 17. Percentage values of IgG-possessing cells were 0.5 at minimum and 3.3 at maximum. When thymic lymphocytes or lymph node lymphocytes were added to the above system, there was no significant proliferation of plasma cells. The maximum number in each case was 13,300 (3.6%) at day 21 after the administration of thymic cells, and 16,100 (14.0%) at day 8 with lymph node cells (Fig. 7). When HGG

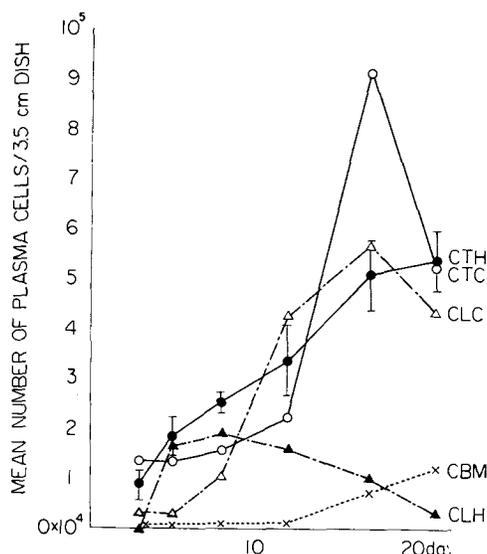


FIG. 8. Abundant proliferation of plasma cells was observed after addition of syngeneic thymic lymphocytes and HGG to C57BL/6 bone marrow cultures (O—O, CTC). Syngeneic lymph node lymphocytes (Δ---Δ, CLC) and thymic cell homogenate (●—●, CTH) were fairly effective in stimulating plasma cell proliferation. A homogenate of syngeneic lymph node lymphocytes (▲---▲, CLH) had little effect on plasma cell proliferation. When bone marrow cells were cultured in the medium containing HGG and without other cells, plasma cells increased only very slightly (×---×, CBM).

was added to bone marrow cultures in the absence of another kind of cell, a slight increase of plasma cell number was observed and the maximum was 12,200 at day 21 (4.7%). However, a remarkable proliferation of plasma cells was obtained by means of addition of syngeneic thymic lymphocytes to bone marrow cultures in basal plus HGG medium. Although from day 3 to 12, the number of plasma cells did not increase, at day 3 it was higher than any other groups. IgG-possessing cells were found to be 13,400 (6.7%) at day 3, and 18,200 (9.8%) at day 12, and the maximum number was recorded at day 17 91,500 (20.8%). Thereafter plasma cells attached to the dishes decreased to 52,800 at day 21, but percentage value increased slightly to 24.0%.

Lymph node lymphocytes also proved to have the capacity to induce plasma

cells in basal plus HGG medium, but the peak of plasma cell proliferation was lower than that obtained by the administration of thymic lymphocytes. The maximum number of plasma cells was found to be 57,000 (19.0%) at day 17. The efficient difference between thymic and lymph node lymphocytes was statistically confirmed, $P < 0.001$ (X^2 test), at day 17 (Fig. 8).

The effect of syngeneic cell homogenate on the proliferation of plasma cells:

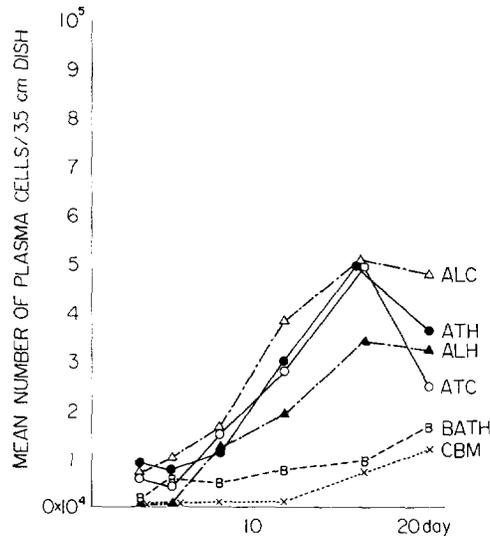


FIG. 9. The effect of allogeneic (A/Jax) cells and their homogenates on the proliferation of plasma cells from C57BL/6 bone marrow cultures. A/Jax thymic lymphocytes (○—○, ATC), lymph node lymphocytes (△—△, ALC), and homogenate of thymic lymphocytes (●—●, ATH) had the same effect on the proliferation of plasma cells. Homogenate of A/Jax lymph node lymphocytes induced a rather low rate of proliferation of plasma cells, but they were superior to syngeneic C57BL/6 lymph node lymphocyte homogenate (▲—▲, ALH) (cf. Fig. 8). Nonimmunological hamster tumor cells had very little effect on plasma cells (B—B, BATH).

When a cell homogenate of thymic lymphocytes was added to bone marrow cultures with HGG, the formation of plasma cells was stimulated to the same degree as by intact lymph node lymphocytes derived from syngeneic mice. Formation was relatively high, the plasma cell count being 8500 (7.1%). The number gradually increased to 17,900 (10.5%) at day 5, 25,400 (12.1%) at day 8, 34,000 (12.3%) at day 12, 51,000 (11.6%) at day 17, and 54,500 (15.6%) at day 21. This curve will be used as a standard for the following results (Fig. 8). The homogenate of syngeneic lymph node lymphocytes was not as effective in stimulating the proliferation of plasma cells as was the thymic homogenate. A peak of 19,000 (10.0%) was seen at day 8, and thereafter a gradual decrease was observed until a minimum of 3800 (1.6%) at day 21 (Fig. 8).

The effect of allogeneic (A/Jax) thymic or lymph node lymphocytes and their homogenates on the proliferation of plasma cells: When C57BL/6 bone marrow cultures were incubated with A/Jax thymic or lymph node lymphocytes or a homogenate of A/Jax thymic cells, a maximum number of about 49,500 plasma cells was obtained on day 17, the percentage values being 14.5, 16.5, and 13.8%, respectively. However, when the bone marrow cultures were incubated with lymph node cell homogenate, the maximum number of plasma cells was only 34,600 (10.8%) at day 17, and the efficient difference for all groups was $P < 0.001$ (Fig. 9).

The effects of homogenates of nonimmunological cells on the formation of plasma cells: A homogenate of hamster tumor cells induced by bovine adenovirus type 3 (Bat-6) was administered to bone marrow cultures which originated from

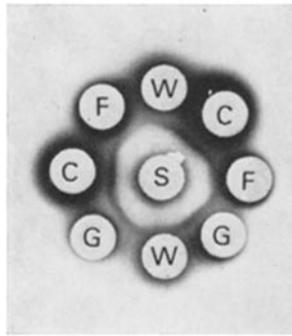


FIG. 10. Ouchterony double-diffusion analysis for the classification of synthesized globulin. Only one precipitate line was seen for each of the following: rabbit anti-mouse whole serum (*W*), rabbit-anti-mouse IgG (*G*), rabbit anti-mouse IgG-Fc (*C*), and rabbit anti-mouse IgG:Fab (*F*). These precipitate lines fused in a reaction of identity.

C57BL/6 mice, and proved to be ineffective, the plasma cell number being 19,000 (8.7%) at day 21. And a homogenate of syngeneic mouse kidney fibroblastoid cells (secondary culture) did not induce a great increase of plasma cells, the maximum number being 12,200 (5.3%) at day 21. On the other hand, the homogenate of thymic cells administered to fibroblastoid cells derived from syngeneic mouse kidney (secondary culture), was found to have no ability to induce fibroblasts to differentiate to plasma cells.

Results of Immunochemical Analyses.—The homogenate of cultured bone marrow cells was examined by immunochemical assays for detection of immunoglobulin. By means of Ouchterony double-diffusion method in agar, the immunoglobulin in the cultured cells were grossly classified. The reactive antisera against homogenates were (*a*) rabbit anti-mouse IgG-Fab antiserum, (*b*) rabbit anti-mouse IgG antiserum, (*c*) rabbit anti-mouse IgG-Fc antiserum, and (*d*) rabbit anti-mouse whole serum (Fig. 10). These precipitate lines fused in a reaction of identity. However, immunoelectrophoresis showed that the antibody

produced in vitro was IgG. Rabbit anti-mouse IgG antiserum, anti-mouse whole serum, anti-mouse IgG-Fc, an anti-mouse IgG-Fab antisera each demonstrated only one line of precipitate.

Antibody Reactivity of Produced IgG against the Administered Antigenic Protein (Human Gamma Globulin).—Immunoglobulin-G contained in proliferating plasma cells was examined in its antibody specificity by peroxidase-labeled antigen HGG staining. Among the 17-day specimens, IgG containing cells were counted to be 51,000 (11.6%) per dish, when bone marrow cells were cultured with thymic cell homogenate and HGG. Among them, 34,700 (7.9%) plasma cells were stained with peroxidase-labeled antigen HGG. Namely 68% of plasma cells were proved to be forming the specific antibody IgG against antigenic protein HGG (Table I). The specificity of this antigenic staining was certified by a blocking test using unlabeled HGG. Successful stainings could be obtained in many cases at the dilution of enzyme-labeled HGG about 30–100 $\mu\text{g}/\text{ml}$.

TABLE I
*Specific Antibody-Forming Cells against Administered Antigenic Protein HGG Were Counted by Means of Peroxidase-Labeled Antigen Staining among the 17-Day Specimens**

Stainings	Percentage	Cell No. per dish
		440,000
Rabbit anti-mouse IgG antiserum	11.6	51,000
Antigenic protein HGG	7.9	34,700
Specific antibody-forming cells in plasma cells		68.1%

* These cells were induced by the addition of thymic cell homogenate and HGG to C57BL/6 bone marrow cultures.

These optimal concentrations of antigen might indicate the staining to be due to specific immunological reactions.

DISCUSSION

It was reported in this paper that a proliferating system of plasma cells was established in vitro by mixing bone marrow cells and the homogenate of thymic lymphocytes or lymph node lymphocytes. Although the effects of the thymic lymphocyte and lymph node lymphocyte differed to some extent, it was demonstrated that both organs had significant ability to accelerate the development of plasma cells in vitro. Furthermore, bone marrow was clearly shown, with several immunological techniques, to be one source of plasma cells. These proliferating plasma cells proved to be producing specific reactive IgG against administered antigenic protein.

The reason for the superiority of thymic lymphocytes over lymph node lymphocytes in plasmopoietic activity can be explained as follows. Although the basic functions of thymic lymphocytes and lymph node lymphocytes may

differ, the thymic lymphocytes may migrate into peripheral lymph nodes to give certain informational stimuli to plasmablasts. These thymic immigrants may be called "thymus-dependent lymphocytes" (15) or "thymus-derived lymphocytes." Since thymic lymphocytes can be found in peripheral lymph nodes, it may be that peripheral lymph node cells have the activity to promote the proliferation of plasma cells. So-called "lymph node lymphocytes" may also have the function of IgM synthesis as a consequence of antigenic stimulations. Although the functional mechanisms of thymic cell homogenate cannot be discussed here, it has been clearly shown that a direct cell-to-cell contact between plasmablasts and thymic cells is not necessary for the maturation of immunologically competent cells.

The variation in inducing activity for plasma cells with different donor strains, may be due to a difference in the population of thymic lymphocytes in thymus and peripheral lymph nodes. The proportion of thymic lymphocytes and lymph node lymphocytes, or the migration speed of thymic lymphocytes

TABLE II
*Differences in the Thymus and Lymph Node Weights in A/Jax and C57BL/6 Mice**

Mouse	Average body weight	Thymus	Lymph node
	g	mg/20 g b.w.	mg/20 g b.w.
A/Jax	24.0	17.5	2.14
C57BL/6	22.7	25.4	1.98

* The average weight was obtained from 12 mice of each strain.

may affect the cell population in the thymus or lymph nodes. In adult A/Jax mice, thymuses were observed to be smaller than those of C57BL/6 mice. But A/Jax axillary and inguinal lymph nodes were larger than those of C57BL/6 mice. In adult A/Jax peripheral lymph nodes, thymus-dependent lymphocytes may propagate faster than in C57BL/6. For this reason, the atrophy of A/Jax thymus may be more severe than in C57BL/6 (Table II).

The immune responses against the proteins in culture media (deriving from fetal bovine serum) were to be negligible in C57BL/6 mice. However, bone marrow cells harvested from A/Jax mice were affected by serum proteins, complicating the analyses of immune responses in vitro. Therefore, recipient bone marrow cells were harvested primarily from C57BL/6 mice.

The primary immune responses in vitro system were discussed only about the 17-day specimens. Further investigations about this problem will be reported in the serial papers by using plural soluble and insoluble antigens.

SUMMARY

An in vitro proliferative system for immunoglobulin-G-forming plasma cells from the bone marrow of mice was established by the addition of antigenic pro-

tein and thymic cells or their homogenate to bone marrow cultures. The promoting activity of the thymus on plasma cells was independent from mouse strain, but it differed in strength with the variations of donor strains. Synthesis of immunoglobulin-G in proliferating plasma cells and its antibody reactivity against the administered antigen were demonstrated by immunocytological analyses.

BIBLIOGRAPHY

1. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Thymus-marrow cell combinations. Synergism of antibody production. *Proc. Soc. Exp. Biol. Med.* **122**:1167.
2. Globerson, A., and R. Auerbach. 1967. Reactivation in vitro of immunocompetence in irradiated mouse spleen. *J. Exp. Med.* **126**:223.
3. Miller, J. F. A. P., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:801.
4. Mitchell, G. F., and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128**:821.
5. Ioachim, H. L. 1965. Continuous formation of plasma cells in long term cultures of spleen. *Exp. Cell Res.* **38**:249.
6. Teodorescu, M., and J. Brucher. 1969. Cell differentiation and pseudodifferentiation in in vitro cultures of spleen. *Exp. Cell Res.* **55**:410.
7. Metcalf, D. 1960. The effect of thymectomy on the lymphoid tissues of the mouse. *Brit. J. Haematol.* **6**:324.
8. Osoba, D. 1968. The regulatory role of the thymus in immunogenesis. *In Regulation of the Antibody Response*. B. Cinader, editor. Charles C Thomas, Publisher, Springfield, Ill. 232.
9. Martinez, C., A. P. Dalmaso, and R. A. Good. 1964. Homotransplantation of normal and neoplastic tissue in thymectomized mice. *In The Thymus in Immunology*. R. A. Good and A. E. Gabrielsen, editors. Hoeber, New York. 465.
10. Nakamura, Y., and Y. Kanda-Inoue. 1969. A new cell line (BAT-6) of hamster tumor induced by Bovine Adenovirus type 3. *Ann. Rep. Inst. Virus Res. Kyoto Univ.* **13**:114.
11. Herzenberg, L. A., N. L. Warner, and L. A. Herzenberg. 1965. Immunoglobulin isoantigens (allotypes) in the mouse. I. Genetics and cross-reactions of the 7S γ_{2a} -isoantigens controlled by alleles at the Ig-1 locus. *J. Exp. Med.* **121**:415.
12. Porter, R. R. 1959. The hydrosis of rabbit globulin and antibodies with crystalline papain. *Biochem. J.* **73**:119.
13. Kawamura, A., Jr. 1969. *Fluorescent Antibody Techniques and Their Applications*. Univ. Tokyo Press and Univ. Park Press.
14. Nakane, P. K., and G. B. Pierce, Jr. 1967. Enzyme labeled antibodies for the light and electron microscopic localization of tissue antigens. *J. Cell Biol.* **133**:307.
15. Parrott, D. M. V., M. A. B. De Sousa, and J. East. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. Exp. Med.* **123**:191.