

## GROWTH OF B-LYMPHOCYTE COLONIES IN VITRO\*

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Semisolid culture systems have been developed which permit the proliferation of mouse hemopoietic progenitor cells to form colonies of neutrophils and macrophages (1, 2), erythropoietic cells (3), eosinophils (4), and megakaryocytes (5). These cloning systems have made it possible to analyse in detail many aspects of the nature and control of these hemopoietic populations.

With a cloning system for mouse plasmacytoma cells (6, 7) it was noted that the addition of erythrocytes or 2-mercaptoethanol usually increased the number and growth rate of plasmacytoma colonies. Because 2-mercaptoethanol increases the proliferation of antibody-forming cells and neoplastic lymphocytes in liquid cultures (8, 9), attempts were made to obtain colony formation in semisolid agar by mouse lymphoid cells using media containing 2-mercaptoethanol. This report describes a technique for growing B-lymphoid colonies in agar medium using cells from normal mouse lymphoid tissues and the nature and growth characteristics of these colonies. A preliminary report on this work was published elsewhere (10).

### Materials and Methods

*Mice.* Mice used were males and females aged 2-3 mo of the inbred strains C57BL/6J, NZB/B1 WEHI, CBA/CaH WEHI, BALB/c/An Bradley WEHI, and SJL/J and noninbred nu/nu (partially backcrossed to BALB/c mice).

*Culture Technique.* Lymph node, spleen, thymus, or marrow tissue was collected sterilely and dispersed cell suspensions prepared by teasing the tissue in Eisen's balanced salt solution using needles. Required numbers of cells were added to an equal volume mixture of double strength Dulbecco's modified Eagle's medium (at room temperature) and 0.6% agar (Difco Bacto-agar, boiled 2 min in double-distilled water and held at 37°C, Difco Laboratories, Detroit, Mich.). The composition of the double strength Dulbecco's modified Eagle's medium was: Dulbecco's modified Eagle's medium HG Instant Tissue Culture Powder H21 (13.47 g) (Grand Island Biological Co., Grand Island, N. Y.); double glass distilled water, 215 ml; 3 ml *l*-asparagine (20 µg/ml); 1.5 ml DEAE-dextran (75 µg/ml) (Pharmacia Fine Chemicals, Sweden, mol wt =  $2 \times 10^6$ /m = 0.70); 0.575 ml penicillin (200 U/ml); 0.35 ml streptomycin (200 U/ml); 175 ml NaHCO<sub>3</sub> (2.8% wt/vol); 250 ml unheated fetal calf serum (FCS).<sup>1</sup> Sufficient 2-mercaptoethanol was added to cell suspensions in

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<sup>1</sup> Abbreviations used in this paper: CSF, colony stimulating factor; DNP, 2,4-dinitrophenol; FCS, fetal calf serum; HEM, HEPES-buffered Eagle's medium; HGG, human gamma globulin; NIP, 4-hydroxy,3-iodo,5-nitrophenylacetic acid; POL, polymerized flagellin; RER, rough-surface endoplasmic reticulum; SDS, sodium dodecyl sulphate.

agar medium to give a final concentration of  $5 \times 10^{-5}$  M.

1-ml volumes of the cell suspension in agar medium were pipetted into 35-mm plastic petri dishes and allowed to gel. Materials for addition to the cultures were placed in the petri dish before the addition of the cell suspension in agar medium. Where erythrocytes were included in the cultures, these were added to the agar medium mixture before pipetting to the petri dishes. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO<sub>2</sub> in air. Cultures were scored routinely for clusters (discrete aggregates of 2–50 cells) after 4 days using an Olympus dissection microscope at  $\times 40$  magnifications (Olympus Corporation of America, New Hyde Park, N. Y.). For colony counts (aggregates of 50 or more cells), cultures were scored after 7 days.

*Harvesting of Colony Cells.* Colony cells were mass harvested by adding 0.5 ml of Eisen's balanced salt solution and rocking the dish until the agar detached from the dish walls, disrupted, and released some colony cells. A suspension of colony cells could then be harvested and washed. For micromanipulation of individual colonies, single discrete colonies were removed using a fine Pasteur pipette and placed in a droplet of HEPES-buffered Eagle's medium (HEM) containing 10% FCS (HEM-FCS) under paraffin oil. Colonies were trimmed under a compound microscope with sharp needles to ensure that no extraneous cells were present. Jets of HEM-FCS were injected into the center of the colony using a coarse micropipette (diam. ca. 25  $\mu$ m) controlled by a Leitz micromanipulator. This progressively released colony cells, which were then washed in microdrops of HEM-FCS.

*Detection of Fc Receptor-Bearing Cells.* Fc receptor-positive cells were identified by: (a) rosette formation with rabbit IgG antibody-sensitized sheep erythrocytes (11); (b) incubation of cells at room temperature for 30 min with 50  $\mu$ g/ml of aggregated human  $\gamma$ -globulin (HGG) (aggregates of size  $> 15 \times 10^6$  mol wt), followed by washing and incubation in ice with fluorescein-conjugated rabbit anti-HGG (Burroughs Wellcome & Co., Tuckahoe, N. Y.) (preabsorbed with mouse spleen and thymus cells); (c) binding of radiolabeled antigen-antibody complexes as described by Basten et al. (12).

*Detection of Membrane Bound Immunoglobulin.* Goat antimouse heavy chain specific sera conjugated with fluorescein ( $\mu$ ,  $\alpha$ ,  $\gamma_1$ , or  $\gamma_2$ ) (Meloy Laboratories, Springfield, Va.) were verified for specificity with immunofluorescence, using tumor cell lines expressing only one of these heavy chain types, and by radioimmunoassay analysis with <sup>125</sup>I-labeled purified mouse myeloma proteins. Sera were absorbed twice with an equal volume of packed mouse thymus cells. Before use, each sample was centrifuged at 3,000 g to remove any large aggregates. Mass harvested colony cells were washed twice with Eisen's balanced salt solution, cell pellets ( $10^3$ – $10^5$  cells) resuspended in 25  $\mu$ l of salt solution, and incubated for 45 min at 0°C with 25  $\mu$ l of a 1:5 dilution of each fluorescein-conjugated antiserum. The cells were washed twice in salt solution at room temperature. Cells with moderate to strong fluorescence were scored as positive.

*Micromanipulation Method for Immunofluorescent Staining of Single Cells.* Single cells were manipulated into microdrops containing the relevant fluorescent reagent and left for 10 min at room temperature (ca. 25°C) or occasionally at 37°C. The reagents used were fluorescein-labeled goat antimouse globulin sera (Meloy Laboratories) at a final concentration of 1 in 10 in HEM-FCS or rhodamine-conjugated NIP (4-hydroxy,3-iodo,5-nitrophenylacetic acid) polymerized flagellin (NIP-POL-Ro) used at 100  $\mu$ g/ml. Cells were then washed three times in HEM-FCS by micromanipulation and examined at  $\times 1,000$  magnifications under both phase contrast and incident-light fluorescence using a Zeiss III RS fluorescence attachment (Carl Zeiss, Inc., New York).

*Preparation of Hapten-Specific Cells.* The method of Haas and Layton (13), as modified by Nossal and Pike (14), was used to prepare spleen cell suspensions enriched for B cells capable of binding the haptens NIP or DNP (2,4-dinitrophenol). The average yield was  $6 \times 10^4$  hapten-binding cells per  $10^6$  CBA spleen cells and enriched 500-fold for hapten-specific B cells.

*In Vitro Immunoglobulin Synthesis.* Cells were suspended at a viable cell concentration of  $1-10 \times 10^6$ /ml in Dulbecco's modified Eagle's Medium with  $10^{-5}$  M leucine and  $5 \times 10^{-5}$  M 2-mercaptoethanol. Tritiated leucine was added to 10–20  $\mu$ Ci/ml (sp act 55 Ci/mM), and the cultures were incubated 16–20 h in a CO<sub>2</sub> incubator at 37°C. Synthesized and secreted Ig was detected by binding the supernate to a specific anti-Ig immunoabsorbant which was an electrophoretic Ig fraction of a sheep antimouse IgG<sub>2b</sub> myeloma protein (with antigamma and antikappa activity), coupled to cyanogen-bromide-activated Sepharose 4B at a level of 2 mg/ml. Binding was carried out at room temperature with 100  $\mu$ l of supernate and 10  $\mu$ l of immunoabsorbant, mixed periodically over 1 h. The Sepharose was washed five times with 10 ml phosphate-buffered saline and the

eluate either counted directly or run in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (15) followed by slicing and counting. In both cases, samples were solubilized in 0.5 ml Soluene 350 (Packard Instrument Co., Downers Grove, Ill.) and counted, after addition of 8 ml toluene-based scintillation fluid, in a Packard liquid scintillation counter.

**Electronmicroscopy.** Mass-harvested colony cells were fixed in suspension in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.08 M sodium cacodylate buffer for 1 h at room temperature. The fixed cells were pelleted, postfixed, dehydrated, and embedded in epoxy resin and then uranium and lead stained. Ultrathin sections were examined using a Philips 300 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

### Results

In cultures of C57BL lymph node or spleen cells which lacked mercaptoethanol, extensive cell death occurred within hours, and from 24 h onwards such cultures exhibited no dividing cells. Only occasional cells survived and these were phagocytic macrophages.

In sharp contrast, cells cultured in agar medium containing  $5 \times 10^{-5}$  M 2-mercaptoethanol proliferated to form clusters and colonies. This concentration was optimal, and cultures containing  $1 \times 10^{-5}$  M or  $10^{-4}$  M mercaptoethanol exhibited little proliferation. As in cultures lacking mercaptoethanol, many of the original cultured cells died within the first 24 h. However, between 24 and 48 h, individual cells commenced proliferation to form clusters of daughter cells. As shown in Fig. 1, during the first 4–5 days of incubation there was a progressive increase in the number of cultured cells which commenced proliferation. On

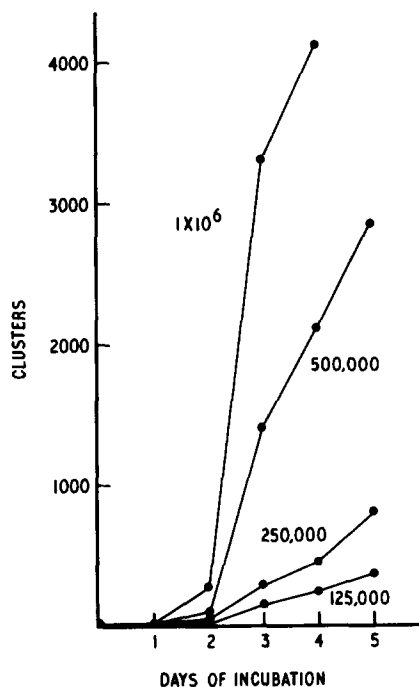


FIG. 1. Asynchronous onset of proliferation in cultures containing indicated numbers of C57BL mesenteric node cells. Shown are the total number of discrete aggregates of two or more cells at intervals after initiation.

continued incubation many of these clusters increased in size to form colonies of 50 or more cells. Colonies often contained a central region of tightly packed cells and a loose outer mantle of cells, but many were composed entirely of uniformly dispersed cells (Fig. 2).

In cultures of fewer than 100,000 cells per ml, colonies rarely exceeded 100 cells in size and exhibited extensive cell death before day 7 of incubation. Conversely, in cultures containing  $1 \times 10^6$  cells, colonies reached 200–800 cells by day 7, and cell death usually did not occur until after day 7. The maximum colony size observed in medium containing only mercaptoethanol was 2,000 cells at day 10 of incubation. Mitotic indices were highest early in the incubation period (3% at day 4), but fell to 1% or lower by day 7 of incubation. At all stages in the incubation period and size of colonies was markedly heterogeneous, and clusters of fewer than 50 cells outnumbered colonies by five to one.

In Giemsa-stained preparations day 4 colonies were composed of a uniform population of very large mononuclear cells with bulky, intensely basophilic, cytoplasm (Fig. 3). However, colonies from cultures incubated for 5–9 days contained cells of variable size. Some were as small as 7–10  $\mu\text{m}$  in diameter and had the appearance of immature plasma cells with an excentric nucleus, basophilic cytoplasm, and prominent Golgi region (Fig. 3). Less commonly, cells had the morphology of small or medium lymphocytes. Classical mature plasma cells or small lymphocytes with eosinophilic cytoplasm were not observed. In some cells from colonies in which cell death was commencing, the nucleus was divided into lobes by deep clefts, giving the nucleus a clover leaf appearance (Fig. 3 c).

In the electronmicroscope, the majority of 5D colony cells had the general morphology of lymphoid cells. These included a small proportion of typical small

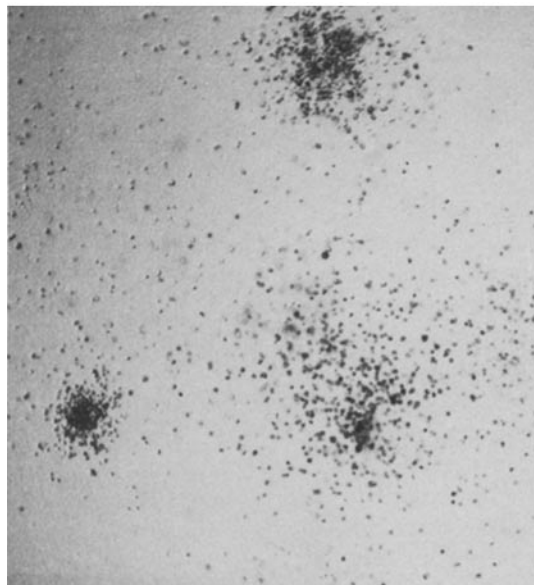


FIG. 2. A 7-day culture of C57BL lymph node cells. Three colonies are visible, one with loosely dispersed cells.

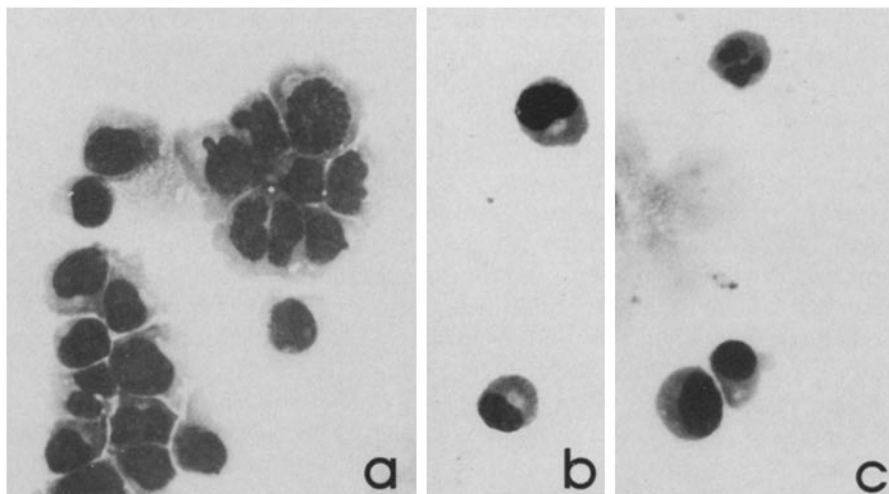


FIG. 3. Colony cells grown from C57BL lymph node cells: (a) large undifferentiated mononuclear blast cells in 4-day colonies, (b, c) more differentiated cells resembling immature plasma cells in 7-day colonies, (c) a degenerating cell with a clover-leaf nucleus. May-Grunwald-Giemsa.

lymphocytes having a round nucleus and sparse cytoplasm containing few organelles. Large blast-like cells with voluminous cytoplasm, many polyribosomes, a few cisternae of rough-surfaced endoplasmic reticulum (RER), and a nucleus with a prominent nucleolus were common (Fig. 4 a). Also seen were cells with the morphology of early plasma cells. These ranged from cells that resembled the blasts in overall structure, except for their greater content of RER (Fig. 4 b), to cells with the structure of typical immature plasma cells. These latter cells (Fig. 4 c) had extensive cytoplasm containing numerous flattened cisternae of RER, a few polyribosomes (Fig. 4 d), and a large Golgi zone (Fig. 4 c). The nucleus of these latter cells was large, often excentric, and contained a prominent nucleolus. C-type virus particles were present in colony cells but were rare.

*Effect of Red Cells and other Additives on Colony Formation.* In cultures containing progressively fewer lymph node or spleen cells it was observed that the incidence of clusters or colonies was not linear with respect to the number of cells cultured (Fig. 5). Some improvement in linearity of colony and cluster formation was observed after the addition of  $1 \times 10^6$  thymus cells or  $1 \times 10^6$  preirradiated spleen cells (2,000 rad) to cultures containing from 20,000 to 200,000 lymph node cells.

As shown in Fig. 6, addition of 0.1 ml of 5% washed sheep red cells markedly enhanced colony and cluster formation when low cell numbers were cultured. Colony and cluster formation were relatively linear with cell doses from  $1 \times 10^3$  to  $1 \times 10^6$  cultured cells. Colony growth rates were also enhanced by the addition of red cells, and colonies containing up to 3,000 cells were observed. This growth enhancement was more obvious when small numbers of cells were cultured, and colony size in such cultures was essentially similar to that in cultures containing  $1 \times 10^6$  cells. Potentiation of colony formation was stronger with 10% sheep red cells, although 0.5 ml of 3% acetic acid had to be added to the cultures to lyse the

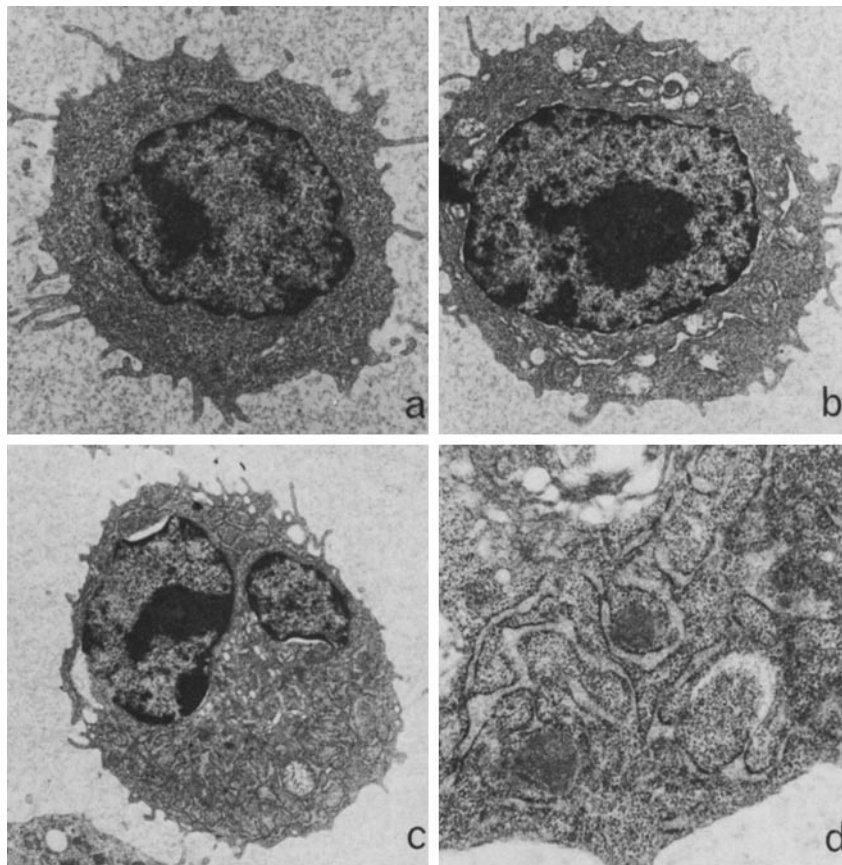


FIG. 4. Electronmicroscopic morphology of 5-day colony cells grown from C57BL spleen cells. (a) A typical blast-like lymphoid cell ( $\times 7,500$ ). (b) Early plasmablast ( $\times 6,000$ ), (c) A relatively well-differentiated plasma cell ( $\times 7,500$ ). (d) A detail of the cytoplasm of a plasma cell similar in its differentiation to that illustrated in (c) ( $\times 19,000$ ).

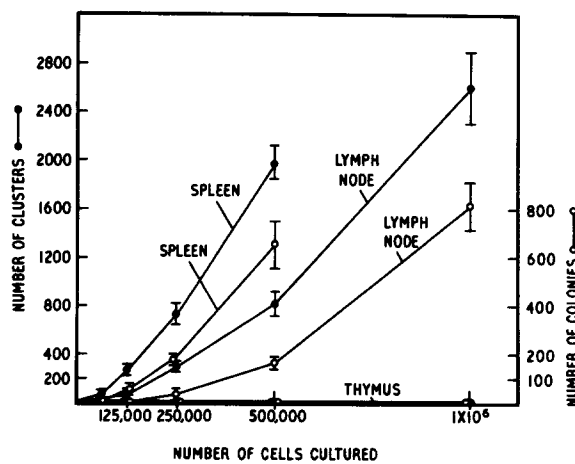


FIG. 5. Relationship between the number of C57BL spleen, mesenteric lymph node, or thymus cells cultured and the number of 7-day colonies  $\circ$ — $\circ$  and 4-day clusters  $\bullet$ — $\bullet$  developing. Note the nonlinearity of the culture system. Vertical bars are standard deviations of values from four replicate cultures.

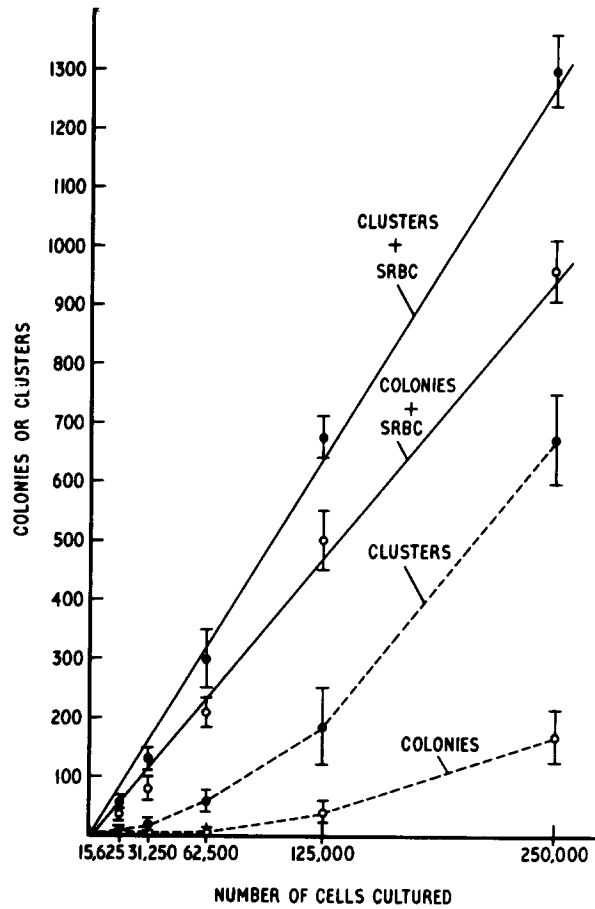


FIG. 6. Improvement in linearity in cultures of C57BL mesenteric lymph node cells containing 0.1 ml 5% sheep red cells (SRBC) and increased frequency of colonies and clusters. Vertical bars are standard deviations of values from four replicate cultures.

red cells before colony counting was possible. In the absence of mercaptoethanol, sheep red cells were unable to stimulate cell proliferation.

*Frequency of Colony-Forming Cells.* A survey of the incidence of colony-forming cells in C57BL organs was carried out using cultures supplemented by mercaptoethanol and 0.1 ml of 10% sheep red cells. All cultures contained only 25,000 nucleated cells to permit more accurate colony counts. Use of this low number of cultured cells also prevented the formation of granulocytic and macrophage cluster and colony formation in cultures of bone marrow cells due to mercaptoethanol-stimulated production of colony-stimulating factor (CSF) by marrow lymphocytes (5). The frequency of colony-forming cells varied between 1 in 50 and 1 in 200 cells in both spleen and lymph nodes (Table I), but the frequency of colony-forming cells in the thymus was extremely low. The incidence of colony-forming cells in bone marrow populations was consistently lower than in lymphoid organs. High levels of colony-forming cells were observed in both the blood and thoracic duct lymph. Significant numbers of colony-forming cells were present in both peritoneal and pleural cavity cells.

*Membrane Characteristics of Colony Cells.* Analysis of mass-harvested colony cells from cultures of spleen or lymph node cells (Table II) indicated that 23–39% exhibited Fc receptors. Positive control cells were pooled 7-day granulocytic and macrophage colony cells grown from C57BL bone marrow cells (1), 175/304 (58%) positive; C57BL peritoneal macrophages, 75/99 (76%) positive; and S49 lymphoma cells, 114/116 (98%) positive. Negative controls were C57BL thymus cells, 0/100 (0%) positive and W232 (T cell) lymphoma cells, 2/300 (1%) positive. Using the more sensitive radioiodinated immune complex method (fowl  $\gamma$ -globulin-rabbit, antifowl  $\gamma$ -globulin), up to 94% of colony cells were labeled. Control C57BL thymus cells reharvested 1 h after culture in agar medium showed 0/57 (0%) labeling, and fresh C57BL marrow cells showed 38/200 (19%) labeled cells.

The presence of membrane immunoglobulin was examined using anti- $\mu$ , anti- $\alpha$ , anti- $\gamma_1$  and anti- $\gamma_2$  sera on colony cells mass harvested from 7-day cultures of C57BL mesenteric node or Peyer's patch cells. The results (Table III) indicated that 61–69% of cells reacted with anti- $\mu$ -sera and 4–11% with anti- $\gamma_2$ -sera. Although a few reactive cells were noted with anti- $\alpha$  and anti- $\gamma_1$ -sera, the numbers were too low to assess the significance of this observation. In this context it was notable that Peyer's patch-derived cells did not exhibit an unusual incidence of positive cells with anti- $\alpha$ -serum. The pattern of reactivity of colony cells was very similar to that of adult spleen cells, with the exception of a relative deficiency in  $\alpha$ -bearing cells. Negative control cells in these experiments were adult thymus cells and pooled 7-day bone marrow-derived neutrophil and macrophage colony cells (2/443 positive). As a positive control, cells from the IgG<sub>2</sub>-bearing 2PK3 lymphoma were used, and 194/220 (88%) of these cells were found to be reactive using the anti- $\gamma_2$ -serum.

The median intensity of staining of colony cells with anti- $\mu$ -sera was less than that for spleen B lymphocytes. Capping was observed with 70% of colony cells when incubated in the presence of these antisera at 37°C, and pinocytosis of

TABLE I  
*Frequency of Colony-Forming Cells in C57BL Lymphoid Organs as Assessed in Cultures Supplemented with Sheep Red Cells\**

Tissue	Number of pools cultured	Colony-forming cells per 10 <sup>6</sup> cells
Spleen	15	8,290 ± 4,530
Subcutaneous lymph nodes	6	5,500 ± 2,800
Mesenteric lymph nodes	6	5,400 ± 2,640
Peyer's patches	3	11,560 ± 3,740
Peripheral blood	3	3,760 ± 3,090
Thoracic duct	8	4,700 ± 3,800
Thymus	5	9 ± 9
Bone marrow	5	1,110 ± 420
Peritoneal cells	3	2,670 ± 1,360
Pleural cells	2	5,540 ± 3,370

\* Pooled cells from 2 to 3 C57BL mice. Cultures contained 25,000 cells per dish or 1 × 10<sup>6</sup> cells in case of thymus. All cultures were supplemented by 0.1 ml of 10% sheep red cells. Mean colony counts from four replicate cultures on day 7 ± SD.



TABLE II  
Assays for Fc Receptors on Colony Cells

Type of colony cells	Technique	Number of positive cells/number tested
4-day C57BL mesenteric node colonies	EA rosettes	57/243 (23%)
4-day C57BL spleen colonies	Aggregated HGG	53/192 (28%)
6-day NZB mesenteric node colonies	Aggregated HGG	12/31 (39%)
6-day C57BL mesenteric lymph node colonies	<sup>125</sup> I FGG-anti-FGG	632/700 (91%)
7-day C57BL spleen colonies	<sup>125</sup> I FGG-anti-FGG	90/96 (94%)

FGG, fowl  $\gamma$ -globulin; EA, sheep erythrocyte-antisheep erythrocyte serum rosettes.

TABLE III  
Heavy Chain Specificity of Membrane Immunoglobulin on C57BL Lymph Node-Derived Colony Cells

Cells	Number of cells positive/number tested			
	anti- $\mu$	anti- $\alpha$	anti- $\gamma_1$	anti- $\gamma_2$
7D C57BL mesenteric node colony cells	233/336 (69%)	11/454 (2%)	8/336 (2%)	48/446 (11%)
7D C57BL Peyer's patch colony cells	78/128 (61%)	6/196 (3%)	1/136 (<1%)	6/146 (4%)
Normal C57BL spleen	68/206 (34%)	12/202 (6%)	7/227 (3%)	25/274 (10%)
Normal C57BL thymus	0/200 (<1%)	0/240 (<1%)	0/200 (<1%)	1/201 (<1%)
2PK3 lymphoma	2/242 (1%)	0/200 (<1%)	0/284 (<1%)	194/220 (88%)

fluorescein-conjugated anti- $\mu$  appeared to be particularly rapid. Although the staining with the anti- $\gamma_2$ -sera was unequivocal, the intensity of this staining was consistently less than with the anti- $\mu$ -sera.

Spleen cells from various strains of mice were cultured in the presence of mercaptoethanol and added sheep red cells. At 5 days mass-harvested colony cells showed the following reactivity with anti- $\gamma_2$ -serum: C57BL, 10%; BALB/c, 6%; SJL, 14%; nu/nu, 6%; NZB, 3%; CBA, 5%. Thus spleen cells from a number of strains generated colonies with a generally similar percentage of  $\gamma_2$ -positive cells. It was noteworthy that nu/nu cells (from congenitally athymic mice) were able to generate  $\gamma_2$ -positive cells in the probable absence of significant numbers of T lymphocytes.

**Antigen-Binding by Colony Cells.** Cultures were prepared of 30–50,000 CBA spleen cells per dish in agar medium containing  $5 \times 10^{-5}$  M mercaptoethanol and 0.1 ml of 10% sheep red cells. Three types of spleen cells from unprimed CBA mice were used: (a) unfractionated cells, (b) adherent cells bound to NIP-gelatin

dishes and, (c) adherent cells bound to DNP-gelatin dishes. NIP-adherent cell suspensions contained 20–30% NIP-binding cells. Each cell suspension was cultured in three types of dishes: (a) containing 0.1 ml saline, (b) containing 300 ng of NIP-POL, or (c) containing 300 ng of DNP-POL. Few NIP-binding cells were present in colonies grown from normal spleen cells or cells bound to DNP-gelatin plates (Table IV). However, 19–26% of colony cells grown from cells adherent to NIP-gelatin plates bound rhodamine-conjugated NIP. The addition of NIP-POL to the cultures did not significantly affect the percentage of NIP-binding cells nor did it significantly increase the number or average size of the colonies developing.

The distribution of NIP-binding cells within colonies was investigated by micromanipulation of individual colonies and tests on colony cells for NIP-rhodamine fluorescence. Cultures were prepared of 10–30,000 CBA spleen cells which were adherent to NIP-gelatin plates. Cultures contained  $5 \times 10^{-5}$  M mercaptoethanol, 0.1 ml 10% sheep red cells, and 300 ng NIP-POL. Examination of mass-harvested colony cells from replicate culture dishes indicated that a mean of 23% of colony cells were NIP binding (84% moderately fluorescent and 16% strongly fluorescent).

A total of 157 cells was examined from 16 colonies, and the results indicated that 107 (68%) were negative and 50 (32%) were NIP binding. Of the NIP-binding cells, 26% were strongly fluorescent. Analysis of individual colonies (Table V) indicated that there was a distinctly greater homogeneity within individual colonies than for the whole population. This homogeneity was also evident from the strength of fluorescence of individual cells within a single colony, e.g., colonies 10, 12, and 15 vs. colonies 8 and 13. 103 of the 107 negative cells came from 11 colonies that in total had only one fluorescent cell. In contrast, 45 of the

TABLE IV  
*Binding of Rhodamine-Conjugated NIP by Mass-Harvested Colony Cells Grown from Various Types of CBA Spleen Cells\**

Type of spleen cells cultured	Material added to culture dish	Immunofluorescent cells
		%
Unfractionated	Saline	0
	NIP-POL	0
	DNP-POL	0
NIP-binding	Saline	19.2
	NIP-POL	24.1
	DNP-POL	26.5
DNP-binding	Saline	4.4
	NIP-POL	0
	DNP-POL	1.4

\* 10,000–30,000 spleen cells per culture dish. All cultures contained  $5 \times 10^{-5}$  M mercaptoethanol and 0.1 ml 10% sheep red cells. Various groups contained in addition 0.1 ml saline, 300 ng NIP-POL, or 300 ng DNP-POL. Colony cells mass-harvested at 6 days of incubation.

50 fluorescent cells came from four colonies that in total had only a single negative cell. Only one colony (No. 8) appeared to show a mixed population of reactive and unreactive cells. However, the reactivity of positive cells in this colony was weak (original scoring; 4+; 2±; 1-), and all cells may well have been weakly reactive. The proportion of DNP-binding cells was highest in colonies grown from the DNP-gelatin adherent cells and again was not influenced significantly by the addition of DNP to the culture dish.

*Analysis of Membrane Immunoglobulin on Cells from Individual Colonies.* 26 colonies from NIP-binding or unfractionated CBA spleen cells were sampled from 4 to 7 days of incubation. In each case, some cells were removed from the colony by micromanipulation and tested for reactivity to fluorescein-conjugated anti- $\mu$ -serum. A further group of cells was freed from the colony and reacted with fluorescein-conjugated anti- $\gamma_2$ -serum.

The data are shown in Table VI. The clonal nature of the colonies is well shown by colonies 1-4 which were examined in some detail. Colonies 1-3 appeared to be composed of  $\mu$ -bearing cells, whereas in colony 4 the cells reacted both with anti- $\mu$  and anti- $\gamma_2$ . For colonies 5-26, fewer cells were examined, but a clonal pattern of reactivity was again evident. 19 of the 26 colonies contained cells bearing  $\mu$ -chains but not  $\gamma_2$ -chains, and only one colony had cells which bore neither  $\mu$ - or  $\gamma_2$ -chains. Note that in no case was a colony observed in which  $\gamma_2$ -bearing cells were present but  $\mu$ -bearing cells absent ("pure"  $\gamma_2$  colony), and that  $\gamma_2$ -positive cells were observed at the earliest culture time sampled (4 days) and were not confined to older cultures. This agrees with data from the mass-harvested cultures which did not indicate a progressive rise in  $\gamma_2$ -positive cells on continued incubation.

*Immunoglobulin Synthesis by Colony Cells.* The ability of colony cells to

TABLE V  
*Analysis of Cells from Individual Colonies for Capacity to Bind Rhodamine-Conjugated NIP\**

Colony number	Number of cells		
	Negative	Moderate immuno-fluorescence	Strong immuno-fluorescence
1, 2, 3, 5, 6, 7, 9, 11, 14, 16	96	0	0
4	7	1	0
8	3	4	0
13	0	12	0
10	1	8	4
15	0	4	3
12	0	8	6
Total	107	37	13

\* All cultures contained 30,000 NIP-gelatin adherent spleen cells from unprimed CBA mice in agar medium containing  $5 \times 10^{-5}$  M mercaptoethanol, 0.1 ml of 10% sheep red cells, and 300 ng NIP-POL. Individual colonies sampled on days 6 or 7 of incubation. Colony size 50-200 cells.

TABLE VI  
*Analysis of Antiglobulin Staining of Cells from Individual Colonies*

Colony number	Age and origin of Colonies*	Added to culture	Number of cells						Result
			Anti- $\mu$			Anti- $\gamma_2$			
			Negative	Moderate	Strong	Negative	Moderate	Strong	
1	7D NIP enriched	NIP-POL	1	3	6	14	0	0	$\mu^+\gamma^-$
2	"	"	1	2	8	37	0	0	$\mu^+\gamma^-$
3	"	"	0	3	5	17	0	0	$\mu^+\gamma^-$
4	"	"	1	2	8	1	14	2	$\mu^+\gamma^+$
5	4D NIP enriched	NIP-POL	1	2	6	9	0	0	$\mu^+\gamma^-$
6	"	"	0	5	0	7	2	0	$\mu^+\gamma^\pm$
7	"	"	0	6	0	0	3	0	$\mu^+\gamma^+$
8	5D NIP enriched	NIP-POL	0	2	4	5	0	0	$\mu^+\gamma^-$
9	"	"	0	1	4	9	0	0	$\mu^+\gamma^-$
10	"	"	0	2	2	6	0	0	$\mu^+\gamma^-$
11	"	"	0	2	1	3	0	0	$\mu^+\gamma^-$
12	"	"	0	0	3	6	0	0	$\mu^+\gamma^-$
13	6D NIP enriched	NIP-POL	0	0	3	2	2	0	$\mu^+\gamma^\pm$
14†	5D NIP enriched	NIP-POL	8	0	0	12	0	0	$\mu^-\gamma^-$
15	"	"	1	3	0	3	0	0	$\mu^+\gamma^-$
16	7D CBA spleen	Saline	0	0	8	10	0	0	$\mu^+\gamma^-$
17	"	"	0	0	6	6	0	0	$\mu^+\gamma^-$
18	6D NIP enriched	NIP-POL	0	2	9	8	0	0	$\mu^+\gamma^-$
19	7D CBA spleen	Saline	0	0	3	4	0	0	$\mu^+\gamma^-$
20§	"	NIP-POL	0	3	2	1	6	0	$\mu^+\gamma^+$
21	7D NIP enriched	Saline	0	9	0	7	0	0	$\mu^+\gamma^-$
22	"	"	0	0	6	7	0	0	$\mu^+\gamma^-$
23	"	"	0	6	0	6	0	0	$\mu^+\gamma^-$
24	"	"	0	4	3	5	0	0	$\mu^+\gamma^-$
25	"	"	0	5	1	3	0	0	$\mu^+\gamma^-$
26	"	NIP-POL	0	0	5	0	6	1	$\mu^+\gamma^+$
Total			13	62	93	188	43	3	19 $\mu^+\gamma^-$ ; 1 $\mu^-\gamma^-$ 4 $\mu^+\gamma^+$ ; 2 $\mu^+\gamma^\pm$

\* NIP enriched, virgin CBA spleen cells adherent to NIP-gelatin and containing 20-30% NIP-binding cells; CBA spleen, unfractionated virgin CBA spleen cells.

† This colony was also tested with anti- $\alpha$  and four cells were negative. It was definitely of lymphoid morphology.

§ This colony was further tested. Against  $\gamma_1$  it gave one negative and four moderate; against  $\alpha$  it gave one neg. five mod, and one strong; against a sheep antirabbit globulin, nonabsorbed, it gave 4 moderate. Against a nonabsorbed rabbit antigoat globulin it gave four strong. The questions of cross-reactivity and Fc receptors have not yet been resolved.

|| This colony gave four negative cells with anti- $\alpha$  but one faint positive. With anti- $\gamma_1$  it gave four pos. (moderate) and three neg. In view of the very definite anti- $\gamma_1$  activity (the most definite we have had) and the exceptionally strong anti- $\mu$ , cross-reactions cannot be excluded.

synthesize and secrete immunoglobulin was demonstrated in short-term studies on the incorporation of [<sup>3</sup>H]leucine. The amounts of [<sup>3</sup>H]leucine-labeled secreted material bound to the anti-immunoglobulin immunoabsorbant are shown in Table VII. Cells mass harvested from agar colonies, as well as established plasmacytomas, regularly secreted measurable amounts of immunoglobulin.

The nonspecific binding under these conditions approached machine background, as shown in the case of P-815, a mastocytoma, which does not secrete detectable amounts of immunoglobulin. The values in this table do not necessarily represent the relative amounts of material secreted, as the cell inputs varied considerably, and the relationship between cell concentration and secretion has not yet been established. Fig. 7 shows two representative polyacrylamide gel electrophoresis patterns, the results of which are summarized in the right hand column of Table VII. All the cultures of colony cells (both from lymph nodes and

TABLE VII  
Synthesis of [ $^3\text{H}$ ]Leucine Labeled Ig by Cultured Cells

Cells*	Bound‡	Chains detected§
	<i>cpm</i>	
Mesenteric node colony cells	3,400	$\mu$ , L
Mesenteric node colony cells	1,248	$\mu$ , L
Peyer's patch colony cells	1,521	$\mu$ , L
HPC-158	9,900	$\gamma$ , L
HPC-6	1,715	L
P-815	13	—

\* HPC-158, IgG<sub>2</sub> myeloma; HPC-6, L-chain producing myeloma; P815, mastocytoma.

‡ Total counts per minute bound to immune-adsorbent, these figures do not necessarily reflect relative secretion (see text).

§ Based on apparent molecular weights in SDS-polyacrylamide gel electrophoresis.  $\mu$ , 76,000; L, 23,000;  $\gamma$ , 53,000.

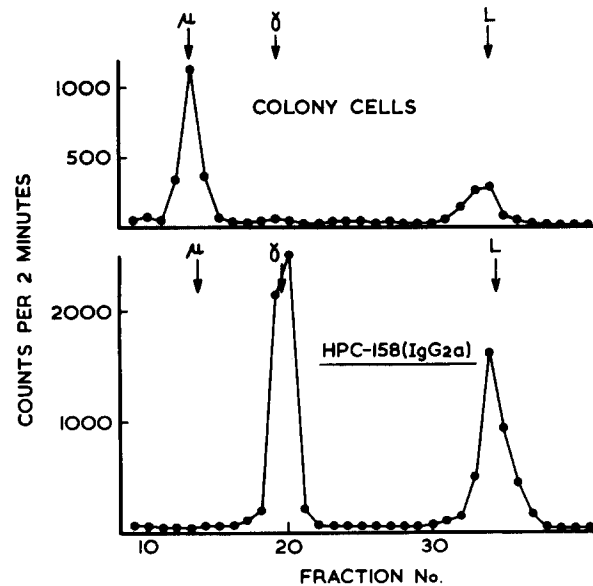


FIG. 7. SDS polyacrylamide electrophoresis of labeled material secreted by C57BL mesenteric node colony cells. Note radiolabeled peaks corresponding to  $\mu$ - and L-chains. Control material secreted by HPC-158 cells (an IgG<sub>2</sub>-producing myeloma).

Peyer's patches) produced a  $\mu$ -like heavy chain and light chain, the two myelomas producing IgG and free light chain, respectively. The secreted  $\mu$ -like heavy chain migrated in SDS gels exactly as did heavy chains from an IgM myeloma (MOPC-104), although its serological properties have not yet been determined.

### Discussion

The present experiments have shown that by adding mercaptoethanol to otherwise conventional agar cultures, it is possible to obtain survival and proliferation in vitro of cells from all major mouse lymphoid organs with the notable exception of the thymus. Colonies grown from these cells were composed of mononuclear cells which on maturation showed the light and electronmicroscopic morphology of immature lymphoid and plasma cells. Colony cells exhibited membrane Fc receptors, but lacked demonstrable C3 receptors (10). Colony cells exhibited membrane immunoglobulin in most cases of Ig-M type, but 5-10% of colony cells showed membrane IgG<sub>2</sub>. Mass-harvested colony cells were shown to be capable of synthesizing IgM-like heavy and light chains.

It is concluded that these colonies are composed of B-lymphoid cells, the majority of which appear to differentiate, if incompletely, in the plasma cell pathway rather than towards small lymphocytes. As is true of other hemopoietic colonies grown in vitro, differentiation was incomplete compared with that occurring in vivo (1, 4, 5).

The evidence, so far, strongly indicates that colonies are clones derived from single-forming cells. Colonies grown from spleen cell suspensions enriched for NIP-binding cells contained a corresponding proportion of NIP-binding cells, and individual colonies were composed of either NIP-binding or nonbinding cells. There was also a notable homogeneity between cells from individual colonies in the avidity of such binding. Further evidence for the clonality of colonies was obtained from an analysis of membrane immunoglobulin. Where colony cells exhibited reactivity with anti- $\gamma_2$ -serum, all cells from that colony appeared to exhibit similar reactivity. Analysis of cells from early versus late colonies failed to document a switch in membrane immunoglobulin from IgM to IgG, since  $\gamma_2$ -positive cells could be found in early colonies and such cells at all stages were also  $\mu$ -positive.

The present lymphocyte colony system appears to differ from all other hemopoietic colony-forming systems in vitro in not requiring a specific macromolecule for cellular proliferation. It may be that mercaptoethanol substitutes for such a specific macromolecule or alternatively mercaptoethanol may actually induce the production of a specific macromolecule by other cells in the culture dish. In this context it should be noted that mercaptoethanol has been shown to induce the production by mouse lymphoid cells of large amounts of CSF and the specific factors required for growth in vitro of eosinophilic and megakaryocytic cells (5).

While there is need to determine the capacity of B-lymphoid colony cells to secrete antibody and to characterize their properties on reinjection in vivo, the present cloning system offers a powerful new tool for analysing B-lymphoid populations which should have many uses in investigating regulatory factors controlling the response of this population to antigenic stimulation.

### Summary

In semisolid agar cultures containing mercaptoethanol, cells from the spleen, lymph nodes, marrow, peritoneal cavity, thoracic duct, and blood of normal mice generated clusters and colonies of up to 3,000 cells. Colony numbers and growth were markedly enhanced by the addition of sheep red cells. The frequency of colony-forming cells in the spleen or lymph nodes was 0.5–2.0%, and cluster-forming cells were approximately five times more numerous. The mononuclear cells comprising these colonies had the electronmicroscopic morphology of immature lymphoid and plasma cells. The majority of the cells possessed Fc receptors, 61–69% reacted with anti- $\mu$ -serum and 4–11% with anti- $\gamma_2$ -serum. Analysis of single cells from individual colonies indicated a higher frequency of the cells with membrane immunoglobulin and a clonal pattern of anti- $\mu$  or anti- $\gamma$  reactivity. The clonal nature of colonies was supported by an analysis of NIP-binding cells in colonies grown from CBA spleen cells enriched for NIP-binding cells. Mass-harvested colony cells synthesized immunoglobulin in short-term liquid cultures. It is concluded that the colonies are clones of functionally active B-lymphoid cells.

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