

MOUSE SPLEEN LYMPHOBLASTS GENERATED IN VITRO Their Replication and Differentiation in Vitro*

By RALPH M. STEINMAN,‡ STEVEN J. BLUMENCRANZ, BEVERLY G. MACHTINGER,
JERROLD FRIED, AND ZANVIL A. COHN

(From The Rockefeller University and the Memorial Sloan-Kettering Cancer Center, New York,
10021)

For many years it has been known that mouse spleen B cells can be stimulated to proliferate and secrete antibody when cultured in the presence of such substances as lipopolysaccharide (LPS)¹ and fetal calf serum (FCS; 1, 2). This stimulation is said to be "nonspecific" or "polyclonal" because large numbers of cells are activated, and individual antibody-forming cells (AFC) exhibit many different, probably noncross-reactive, specificities. The phenomenon of polyclonal B-cell activation or B-cell mitogenesis is thought to be a useful model for studying the normal immune response even though smaller proportions of specific lymphoid cells are activated.

Most studies of nonspecific B-lymphocyte activation have simply documented the behavior of whole cultures rather than individual cells. For example, we know little about the extent to which stimulated B cells divide in vitro; how many of the blasts mature into typical plasma cells as opposed to other cell types; or the life-span of the various cell types. We also have little information comparing the behavior of B cells in response to antigen-specific and nonspecific stimulation.

In this study, we take advantage of the availability of enriched populations of presumptive B lymphoblasts, obtained by floatation on dense bovine plasma albumin (BPA) columns as described in the accompanying paper (3). These populations consist almost entirely of blasts. They can be recovered in high yield in their first cycle of cell division in response to either LPS or FCS stimulation. By using these enriched populations, often in combination with a nontoxic [³H]thymidine radiolabeling technique, we show that the majority of mitogen-stimulated blasts mature into typical plasma cells. Maturation occurs after just two cell divisions, after which the plasma cell appears to withdraw from proliferative activity. The mature plasma cells are similar to those produced in response to antigen *in situ* in their cytologic features, content of abundant cytoplasmic immunoglobulin, diminished proliferative activity, and short life-span.

* Supported in part by grants AI 13013, AI 07012, and CA 16757 from the U. S. Public Health Service.

‡ Scholar of the Leukemia Society of America and an Irma T. Hirsch Fellow.

¹ Abbreviations used in this paper: AFC, antibody-forming cell; BPA, bovine plasma albumin; FCS, fetal calf serum; FMF, flow microfluorometry; HRP, horseradish peroxidase; Ig, immunoglobulin; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; RER, rough endoplasmic reticulum; TCA, trichloroacetic acid.

Materials and Methods

Enriched Populations of Mitogen-Stimulated Lymphoblasts. Lymphoblasts were obtained as described in the previous paper (3). Briefly, suspensions of 15×10^7 DBA 2/J mouse spleen cells were stimulated by LPS, 100 $\mu\text{g}/\text{ml}$ in the absence of serum, or by 5% vol/vol heat-inactivated FCS in RPMI-1640 medium supplemented with penicillin and 5×10^{-5} 2-mercaptoethanol (2-ME). After 24 h in vitro, the cells were harvested and cultured for an additional hour in fresh medium on a fresh Petri dish. During this hour, most of the typical macrophages in the suspension adhered to the fresh dish surface, and where necessary, the cells could be pulse radiolabeled with [^3H]thymidine (see below). The nonadherent cells were resuspended in dense BPA, final density of 1.080, and spun to equilibrium. More than 90-95% of cells proliferating in response to FCS and LPS in these cultures float under these conditions, and most (80% or more) of the low density cells are lymphoblasts by cytologic and other criteria.

Culturing Low Density Cells. We cultured $1-1.25 \times 10^6$ low density cells in 16-mm diameter tissue culture wells (Linbro Chemical Co., New Haven, Conn.) in 1 ml of RPMI-1640 supplemented with penicillin, 2-ME, 30-100 $\mu\text{g}/\text{ml}$ LPS (for LPS-induced blasts), and 5% FCS. We have examined a number of variables in this culture procedure, e.g., kinds of serum, cell density, presence or absence of LPS and/or 2-ME. These variables had relatively little effect on the major findings reported here. Other supplements have been used, such as 1-2.5% heat-inactivated isologous mouse serum, 5% heat-inactivated horse serum (Grand Island Biological Co., Grand Island, N. Y.), and 0.05% lactalbumin hydrolysate (Grand Island Biological).

Cell Viability. Generally, cells were stained in suspension with a final concentration of 0.08% trypan blue and counted in a hemocytometer. We also looked for profiles of dead cells in Giemsa-stained smears and in electron micrographs, and we followed the disappearance of [^3H]thymidine incorporated into lymphoblast DNA at the start of the cultures (see below).

Cell Smears, Autoradiography, and Electron Microscopy. See accompanying paper (3).

Radiolabeling with [^3H]Thymidine. Radiolabeling was used to follow the level of proliferative activity in lymphoblast cultures. Cells were harvested with a Pasteur pipette, washed, and exposed to [^3H]thymidine (spec act 6.0 Ci/mM; Schwartz Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.) for 1-2 h at a final concentration of 1-2 $\mu\text{Ci}/\text{ml}$. Cell smears were prepared for autoradiography (see below), and/or [^3H]thymidine uptake was quantitated by liquid scintillation counting. For the latter, the labeled cells were harvested directly onto glass fiber filters (no. 5601, Whatman, Inc., Clifton, N. J.) using a multisample harvester, or the preparations were precipitated in ice-cold 5% trichloroacetic acid (TCA) for several hours and the washed precipitates counted. Liquid scintillation counting was done in Aquasol-2 (New England Nuclear, Boston, Mass.) using a Mark II counter (Nuclear-Chicago, Des Plaines, Ill.) operating at 40% efficiency.

[^3H]Thymidine radiolabeling was also used to follow the fate of the cultured lymphoblasts that were in S phase at the time of the pulse. Cells were pulsed with 0.3 $\mu\text{Ci}/\text{ml}$ of label for 1 h, washed, and returned to culture for up to 3 days. [^3H]Thymidine incorporation in the cell nucleus can be accompanied by significant radiotoxicity (reviewed in 4). However, we found that cultures labeled by this protocol behaved similarly to nonradiolabeled ones with respect to viable cell recoveries; proliferative activity assessed by the uptake of [^3H]thymidine at varying times in culture; and number of plasma cells detected immunocytochemically as cells showing dark staining for cytoplasmic Ig (see below). Decreasing the labeling regimen to 0.1 $\mu\text{Ci}/\text{ml}$ did not alter the observations obtained with 0.3 $\mu\text{Ci}/\text{ml}$, but increasing the dose to 1.0 $\mu\text{Ci}/\text{ml}$ produced detectable decreases in [^3H]thymidine uptake and plasma cell numbers. Reutilization of radiolabel (5), e.g. from dying cells, was prevented by maintaining the cells in 20 μM "cold" or nonradioactive thymidine (Sigma Chemical Co., St. Louis, Mo.). This dose did not alter cell behavior but could completely block the uptake of that amount of free [^3H]thymidine initially incorporated into labeled blasts. (10^6 Low density cells incorporate 0.01 μCi of label during a 1-h exposure to 0.3 $\mu\text{Ci}/\text{ml}$ of [^3H]thymidine.)

Flow Microfluorometry. At various times in culture, samples were obtained for determination of relative DNA content by flow microfluorometry as described in the accompanying paper (3). The DNA histograms were analyzed mathematically by the method of Fried (6-8) and graphically by G6hde's procedure (9). In the latter method, the percentage of cells in S phase is estimated first. A rectangle is constructed within the DNA histogram in which the height is equal to the minimum ordinate value between the left- and right-hand peaks of the histogram; the width is

equal to the distance between the channels corresponding to the maxima of these peaks. The sizes of the G_1 and $G_2 + M$ components, respectively, are proportional to the relative areas of the remaining portions of the histogram.

Immunocytochemical Visualization of Cytoplasmic Immunoglobulin (Ig). Cell smears were prepared by centrifuging cells onto coverslips that were previously coated with 25 $\mu\text{g}/\text{ml}$ polylysine (type VII B, Sigma Chemical) in phosphate-buffered saline. The coverslips with cells were drained and immersed directly into 5 ml of cold 1% glacial acetic acid-96% ethanol fixative, for 30 min, followed by several rinses in phosphate-buffered saline. 80% of the added cells were recovered on the coverslips. Other fixation regimens (60% alcohol-40% ether; 100% methanol; 100% ethanol; or 3% formaldehyde followed by acetone or methanol extraction), with or without air drying, gave poorer results in that the staining was absent, variable, or required larger quantities of reagents. To stain, we used a sandwich technique in which the primary reagent was a rabbit antimouse- κ , and the secondary reagent a sheep antirabbit Ig coupled to horseradish peroxidase (HRP) by either the Avrameas-Ternynck (10) or Nakane-Kawasi (11) procedures. The rabbit anti- κ was kindly supplied to us by Dr. U. Hammerling (Sloan Kettering Institute, N. Y.) and subsequently prepared independently by his protocol which involves biweekly injections of $F(ab)'_2$ fragments of an IgG myeloma ($\kappa \gamma_1$) in complete Freund's adjuvant followed by incomplete Freund's adjuvant. This very potent antiserum could be used directly or after immunoselection of κ -chains insolubilized with cyanogen bromide on Sepharose-4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). The sheep antirabbit Ig was generously supplied by Dr. S. Silverstein, The Rockefeller University, N. Y., and was immunoselected on a rabbit Ig Sepharose column before peroxidase coupling. The staining procedure involved sequential 30-min incubations of the fixed smears in a 1:1,000 dilution of the primary reagent followed by a 1:80 dilution of the peroxidase conjugate. The bound HRP was then visualized by the Graham and Karnovsky procedure (12) using a 5-min incubation at room temperature in 50 mg/100 ml diaminobenzidine tetrahydrochloride (Sigma Chemical) and 0.01% hydrogen peroxide in 0.1 M tris buffer pH 7.6. Increasing or decreasing the concentration of the anti- κ reagent threefold did not alter the result, and substitution of the anti- κ with anti-HRP or anti-BPA (generated in rabbits in Freund's adjuvant) abolished staining for cytoplasmic Ig.

For combined immunocytochemistry and autoradiography, we substituted a rhodamine-coupled, goat antirabbit Ig reagent (kindly provided by Dr. J. Unkeless, The Rockefeller University) for the HRP-coupled sheep antirabbit Ig. Diaminobenzidine staining for visualizing the latter reagent produces nonspecific autoradiographic grains, so that the fluorescent secondary reagent was required. The smears were stained before autoradiography and visualized in a Zeiss Photomicroscope II equipped with a Zeiss Precision Line Interference Green 546 \pm 2 nm excitation filter (no. 46-78-08), a 580-nm barrier filter (no. 46-78-69), and a 580-nm dichroic reflector for epifluorescence (no. 46-63-05; Morrell Instruments, Syosset, N. Y.).

Results

The aim of these experiments was to follow the fate of a homogeneous population of presumptive B blasts, obtained in their first proliferative cell cycle by floatation in dense BPA columns. This method, and the characterization of the blast population, were outlined in the accompanying paper (3). Most of our detailed work, and much of the data in this paper, relates to the behavior of LPS-stimulated blasts floated from 1-day cultures of mouse spleen. In all respects, FCS-induced blasts behaved similarly. We presume that the starting blasts are primarily B cells, because LPS and FCS are predominantly B-cell mitogens (1, 2), and because most of them develop into plasma cells in vitro (see below).

Three aspects of the behavior of B blasts in vitro will be described: proliferative activity, differentiation, and viability.

Proliferative Activity of Cultured Lymphoblasts

[^3H]THYMIDINE PULSE RADIOLABELING AND FLOW MICROFLUOROMETRY. Cultures were pulse radiolabeled with [^3H]thymidine for 1 h on successive days. At

TABLE I
Proliferative Activity in LPS-Induced Low Density Cultures
[³H]Thymidine Pulse Radiolabeling Studies

Time in culture	Viable cells ($\times 10^{-6}$)	Thymidine up- take (cpm/cul- ture)	Labeling index
<i>h</i>			%
0.5	1.0	19,173	24
17	1.2	40,376	36
41	1.1	19,012	19
65	0.8	7,420	10

Enriched lymphoblast preparations were floated from mouse spleen cells stimulated 26 h with 100 μ g/ml LPS (no serum). The low density cells were maintained 3 days *in vitro* with LPS and 5% FCS. On each day, the cells were pulse labeled for 1 h with [³H]thymidine, 1 μ Ci/ml. Proliferative activity was assessed by liquid scintillation counts (cells were harvested onto fiber filters) or by autoradiography (percent of viable cells with nuclear labeling in Giemsa-stained smears).

the start of the culture, some 20–25% of the cells were in S phase (Table I). As was shown previously (3), the other blasts were in G₁, and were many hours from entering their first S phase. After a day of culture, total cell numbers increased 20–50%, thymidine uptake increased two- to threefold, and the percentage of labeled cells (labeling index) increased to 30–40%. On days 2 and 3, thymidine uptake and the autoradiographic labeling index fell, reaching a level of 10–15% by day 3. We conclude that many cells proliferating at day 1 must have withdrawn from active proliferative activity upon further culture.

Flow microfluorometry (FMF) provided information on the number of cells in each of the main phases of the cell cycle: G₁, S, and G₂+ M. At the start of culture, most of the cells were in G₁ with only 2% having reached their first G₂+ M (Fig. 1; Table II). After a day in culture, the microfluorograms looked like typical cultures in logarithmic growth (Fig. 1 B) with some 40–50% of the cells showing more than the 2n level of fluorescence, corresponding to S and G₂+ M cells. By day 3, (Fig. 1 C), the number of cells in S and G₂+ M fell considerably, although we had difficulty performing a mathematical analysis on the day 3 data (Fig. 1 C legend).

We conclude that B blasts proliferate actively during the first day of culture, but then most stop dividing. If S phase represents a constant proportion of the proliferative cell cycle, then the 50–70% fall in S phase cells (Tables I and II) means that at least 50–70% of the cells proliferating at day 1 have withdrawn from cell cycle by day 3. This figure is probably an underestimate because the viability of proliferating cells seems to be greater than the nonlabeling ones (see below).

FATE OF LYMPHOBLASTS TAGGED WITH [³H]THYMIDINE AT THE START OF CULTURE. To obtain more detailed and direct information on the proliferative potential and fate of cultured lymphoblasts, we tagged those blasts that were in S phase at the start of the culture with [³H]thymidine. As outlined in Materials and Methods, radiolabeling could be accomplished without detectable toxicity using an exposure of 0.3 μ Ci/ml for 1 h, spec act 6.0 Ci/mM. In

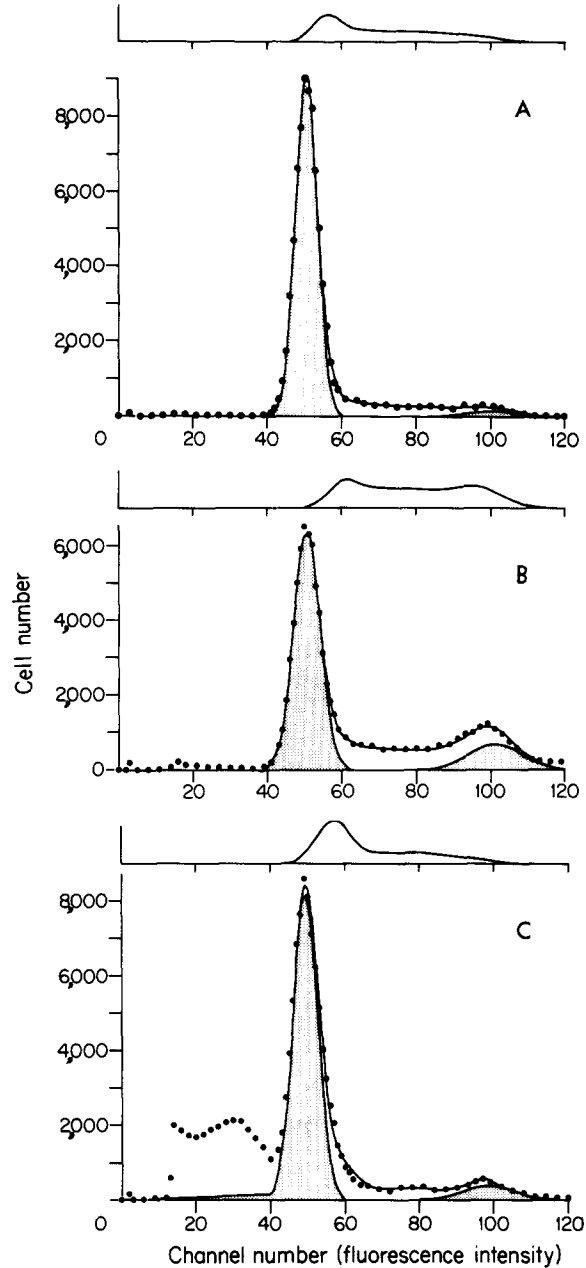


FIG. 1. Laser FMF distributions of LPS-induced propidium iodide-stained lymphoblast cultures at times 0 (A), 20 (B), and 60 (C) h. The points are the actual data, and the continuous lines represent the least-squares fit to the data. The shaded portions of the histograms represent the mathematically derived distributions of G_1 and $G_1 + M$ cells, and the unshaded regions between them correspond to S phase cells. The latter are more easily visualized if redrawn with a horizontal base line, as shown in the upper portions of each figure. At 0 h, most cells were in G_1 , whereas at 20 h, substantial S and $G_2 + M$ components were evident. By the time of the 60-h sample, the percentages of cells in S and $G_2 + M$ decreased, and a large low-fluorescent component appeared. This probably represented dead cells or cell fragments. Because the fluorescence of this component overlaps with that of intact cells to an unknown extent, the mathematical analysis of this histogram may be disturbed. This may be responsible for the lack of agreement between the mathematically derived estimate of S phase fraction and the $[^3H]$ thymidine labeling index for this sample (Table II).

TABLE II
FMF Analysis of Lymphoblast Cultures

Time in cul- ture	Percent cells in different phases of the cell cycle				
	G ₁	S			G ₂ + M
		FMF Math	FMF Graph	[³ H]Thymidine	
<i>h</i>					
0	81	18	17	19	2
20	59	29	29	32	12
60	79	24	14	12	7

LPS-induced lymphoblasts were cultured for 60 h. At 0, 20, and 60 h, the cells were harvested and stained with propidium iodide, a DNA-specific fluorescent reagent appropriate for FMF (Fig. 1). Aliquots also were exposed for 1 h to [³H]thymidine for autoradiographic analysis. The percentage of cells in S phase was obtained by three techniques: mathematical and graphical analysis of the FMF data (see Materials and Methods) and autoradiographic labeling index. Reasons for the discrepancy between the [³H]thymidine labeling index and the mathematically derived estimate of S phase percentage of the 60-h sample were given in the legend to Fig. 1. The portion of the histogram interpreted as early S phase cells in the mathematical method would be included within the G₁ compartment in the graphical procedure.

employing this tag, we assumed that the bulk of the label incorporated initially into DNA was metabolically stable. A major loss of label per cell (indicated by the grain count in autoradiographs) could only occur by cell division, whereas a major loss in the number of labeled cells could only occur by cell death.

After a day in culture (Table III), the number of labeled cells doubled, and the grain count per cell halved (Table III, Fig. 2). The labeling index increased, probably because cells labeled initially divided sooner than those not so labeled (i.e. G₁ cells). On day 2, the number of labeled progeny changed little, but the grain count fell considerably, almost 50%. At day 3, the number of labeled progeny fell, whereas the grain counts showed only a slight decrease—even if the autoradiographs were exposed for prolonged periods to try to pick up lightly labeled cells. Visual examination of the grain count histograms showed that the entire distribution had shifted from day 0 to day 1 to day 2. Also, the ratio of the standard deviation of the distribution to the mean grain count was constant, indicating that all cells participated in the grain count dilution process. Taken together, these data indicate that labeled lymphoblasts divide only twice in vitro, and that many of the progeny must die because the number of labeled cells never expands to the extent predicted by the grain count dilution data (fourfold).

Colchicine blockade was also used to follow mitotic activity in cultured lymphoblasts. After 10⁻⁷ M drug at 0–10 h of culture, many colchicine metaphases were evident, and 80% were radiolabeled (Table IV). The high percentage meant that cells not tagged with [³H]thymidine initially (G₁ cells) have not yet reached mitosis. When colchicine was given at 20–30 h, more metaphases were noted, but now only 25% were labeled. This indicated that cells at all

TABLE III
Fate of Lymphoblasts Radiolabeled with [³H]Thymidine at the Start of 3-Day Cultures

Time in culture	Viable cells ($\times 10^6$)	[³ H]Thymidine-labeled cells		Mean grain count/cell/day autoradiographic exposure
		Labeling index	Total ($\times 10^{-6}$)	
<i>h</i>		%		
0	1.25	24.2	0.30	15.0
20	1.67	35.1	0.59	7.4
44	1.30	35.4	0.46	4.3
69	1.00	24.2	0.24	3.4

LPS-induced low density cells were radiolabeled for 1 h with [³H]thymidine at 0.3 μ Ci/ml, spec act 6.0 Ci/mM. The cells were washed and recultured in medium supplemented with LPS, FCS, 2-ME, and 20 μ M cold thymidine. At each time-point, the cultures were harvested and the following measurements made: (a) viable cells (trypan blue negative); (b) [³H]thymidine-labeled cells (total cells \times labeling index); and (c) mean grain count of the labeled cells in autoradiographs exposed for sufficiently long periods to detect all labeled progeny (grain count development was linear with time; and the background after 4 days of development was 0-2 grains/nucleus).

phases of the cell cycle initially (of which 20% were in S phase) were equally likely to be in mitosis. The mean grain count of the metaphases fell from 28.8 (0-10 h colchicine treatment) to 17.1 (20-30 h), so that many of the cells that were in their first mitosis at 0-10 h had entered a second mitotic cycle. Few cells were arrested in metaphase in the 40- to 50-h pulse, confirming that most cells were no longer mitotically active.

The use of [³H]thymidine as a tag for following the fate of cultured lymphoblasts does not require that proliferating cells be enriched, i.e., blasts labeled at day 1 in unfractionated spleen cultures behaved identically to floated blasts (data not presented).

Differentiation of the Cultured Lymphoblast

GIEMSA-STAINED CELL SMEARS. At the start of the culture, the cells were predominantly blasts of varying size (see Fig. 1 A, accompanying paper). After a day in culture, most of the cells were large lymphocytes with large nuclei and abundant basophilic cytoplasm (Fig. 3 A). By day 2 and 3, most of the surviving cells were smaller in size, especially nuclear size (Fig. 3 B-D). In preparations that were tagged with [³H]thymidine at the start of culture, it was evident that the small cells arose from the labeled blasts (Fig. 3 B, C). However, if the cells were pulse labeled with [³H]thymidine at day 2 or 3, most of the small progeny did not label (Fig. 3 D). These cells with smaller nuclei could be distinguished from small lymphocytes by their more abundant and strongly basophilic cytoplasm. In some instances (Fig. 3 D), the preparations were adequate to demonstrate clear cut "perinuclear halos" corresponding to Golgi zones. Plasma cells are the only lymphoid cells known with all of these cytologic features, so that most of the progeny of the starting blasts must be plasma cells. By day 3, a small percentage of typical Mott cells were seen.

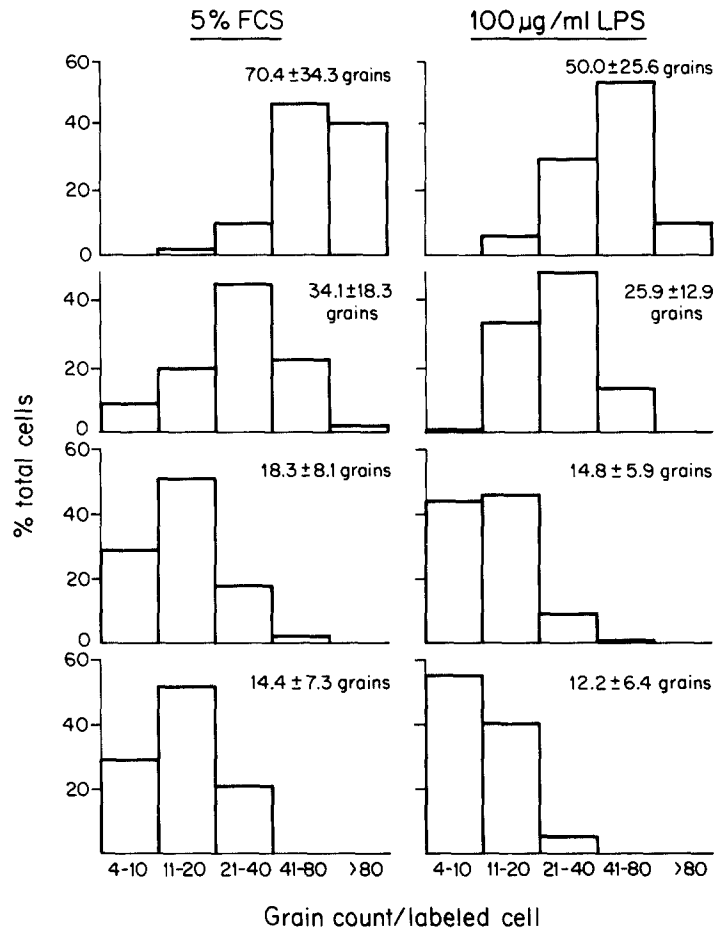


FIG. 2. Histograms of the grain count distributions in cultures that were pulse radiolabeled at the start of the experiment and then followed in vitro for 3 days. In descending order, the distributions were obtained at 0, 20, 44, and 66 h of culture. Autoradiographs were exposed for 4 (data shown) and 11 (data not shown) days. Background grains ranged from 0-2/nucleus, so that a threshold of four grains was used. For both FCS- and LPS-induced blasts, the grain count fell markedly over the first 2 days, but little on the third. The entire grain count distribution appears to shift at the different time-points, and the ratio of the standard deviation to the mean grain count remains constant.

These are a variant of the plasma cell lineage in which the cytoplasm contains droplets of varying size lined by a basophilic rim. In addition to intact cells, many anucleate dead cells were evident in the smears, and these contained silver grains in preparations tagged with [^3H]thymidine at the start of the culture (Fig. 3C). Macrophages, granulocytes, and other hematopoietic cell types were rarely observed at any time among the progeny of the starting lymphoblasts.

ELECTRON MICROSCOPY. At the start of culture, and at day 1, most of the cells were typical blasts, i.e. the nuclei and nucleoli were large, there was relatively little condensed chromatin or heterochromatin, and the abundant

TABLE IV
*Colchicine Metaphases in [³H]Thymidine-Labeled, LPS-Induced,
 Low Density Cultures*

Time of colchicine pulse	Number of colchicine metaphases/culture	Metaphases radiolabeled	Mean grain count of labeled metaphases
<i>h</i>		%	
0-10	156,000	82	28.8
20-30	228,000	26	17.1
40-50	36,000	6	ND*

LPS-induced, low density cells were [³H]thymidine pulse radiolabeled initially and then exposed to 10⁻⁷ colchicine for 10-h intervals at varying times in culture. All measurements were made from autoradiographs of cell smears.

* Not determined, too few labeled metaphases to record.

cytoplasm contained many polyribosomes, most nonmembrane-bound (see Fig. 2 in the accompanying paper and Fig. 4A in this paper). At days 2 and 3, the majority of the cells took on features of the plasma cell lineage, particularly well-developed rough endoplasmic reticulum (RER; Fig. 4). The RER cisternae contained an electron-dense content and were dilated to a variable extent. Some cells (Fig. 4D) exhibited large dilated cisternae corresponding to Mott cell droplets (13, 14). A sample differential of a typical 3-day culture of LPS blasts is presented in Table V. Only profiles with adequate amounts of nucleus and cytoplasm were analyzed, using cytologic criteria outlined in Fig. 4.

Other cell types were present at days 2 and 3 in addition to plasma cells. Some typical lymphoblasts persisted, as did cells that we classified as medium lymphocytes (Fig. 4E). The latter had larger nuclei and more cytoplasm than small lymphocytes; similar profiles have been detected as AFC in hemolytic plaques (15). Small lymphocytes, i.e., cells with abundant nuclear heterochromatin and single cytoplasmic particles, comprised at most 5% of the viable profiles at all days of culture. As predicted from the cell smears, myeloid and erythroid progeny were rarely noted.

In addition to viable cells, profiles of dead cells were found, i.e., cells with pyknotic nuclei and lacking in cytoplasmic organelles or dense cytoplasmic matrix (Fig. 4F). Dead cell profiles, even when detected at day 1 of culture, frequently contained ribosome-lined vesicles, so we suspect that they had been plasma cells.

IMMUNOCYTOCHEMISTRY. The maturation of lymphoblasts into plasma cells was assessed immunocytochemically using a sandwich technique. Application of a rabbit, antimouse, κ -reagent was followed by an HRP-conjugated, sheep, antirabbit reagent and diaminobenzidine staining. This technique reveals four categories or cells in a preparation of freshly isolated, mouse spleen cells: small, diffusely stained B lymphocytes; nonstaining small cells; plasma cells; and granulocytes.

In days 0 and 1 cultures, 3-6 and 8-12% of the cells, respectively, were typical mature plasma cells with strong cytoplasmic staining (Fig. 5A). Most of the other low density cells appeared to have weak cytoplasmic staining (Fig.

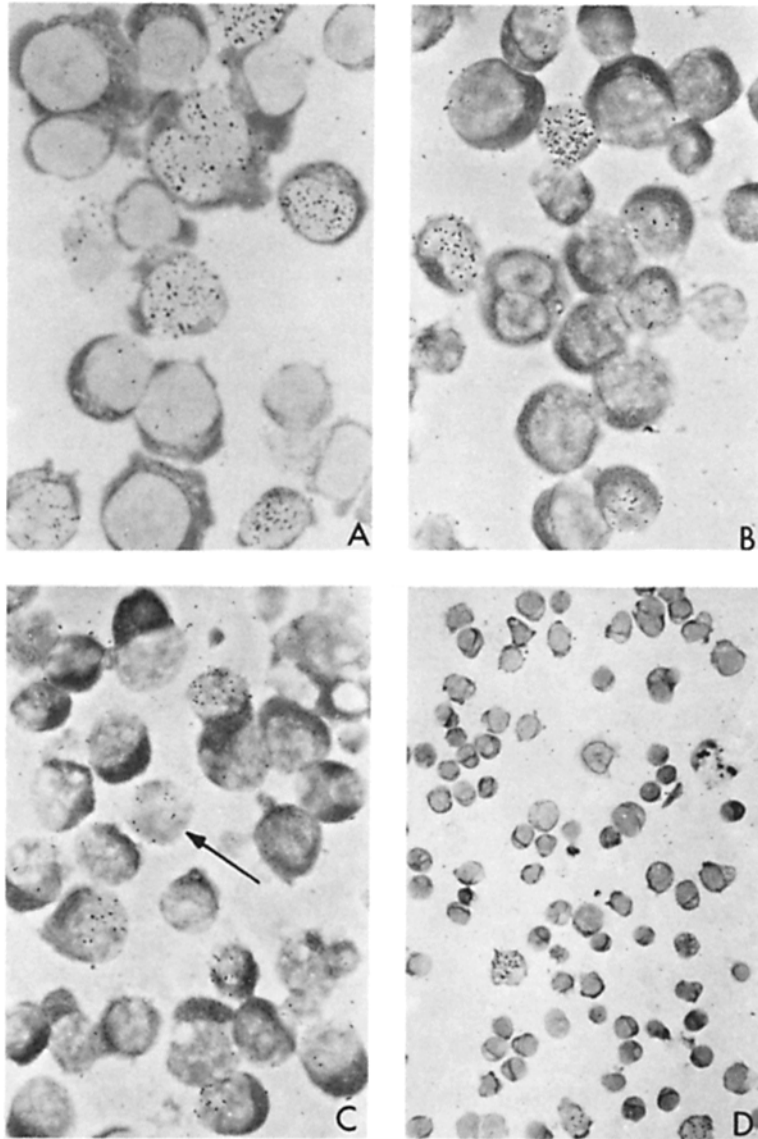


FIG. 3. Giemsa-stained autoradiographs of LPS-induced low density cells maintained for varying times *in vitro* in medium supplemented with LPS, 2-ME, and FCS. Fig. 2 A-C ($\times 1,600$) are of 1-, 2-, and 3-day cultures that were tagged with [^3H]thymidine at the start of the culture. Small but discrete silver grains (Ilford L4 emulsion; Ilford Ltd., Ilford, Essex, Eng.) are evident over a subpopulation of the progeny. It is evident that the number of grains per cell decreases considerably between day 1 (A) and day 2 (B), but less so at day 3 (C). The progeny of the blasts are primarily large lymphocytes at day 1 and plasma cells at day 2 and 3, i.e., cells with small eccentric nuclei, and prominent, basophilic cytoplasm. D ($\times 365$) is a 3-day culture radiolabeled at day 3. Most of the smaller plasma cells, here with striking perinuclear halos, do not radiolabel. The few cells that do label are large lymphocytes.

5A), but this staining was largely diminished by trypsinization of the cells before fixation. Presumably, we were visualizing surface rather than cytoplasmic Ig. At days 2 and 3 (Fig. 5B), larger numbers of darkly staining cells were found (15–30% of the total). The number of cells exhibiting trypsin-resistant, cytoplasmic Ig immunocytochemically was always two- to threefold less than the number of cells that appeared to be plasma cells in smears and in electron micrographs. We conclude that most blast progeny are B cells because they stain for surface and intracellular Ig. However, plasma cells must vary considerably in their content or concentration of cytoplasmic Ig.

Combined autoradiography and immunocytochemistry on cells labeled at time 0 showed that 50% of the [³H]thymidine-labeled progeny at day 2 appeared to be typical plasma cells, and at least 50% of the plasma cells were radiolabeled. The latter value fell to 20–30% at day 3, which we think means that cells in S phase at the start of the culture mature more rapidly into plasma cells than cells initially in G₁. If cultures were pulse radiolabeled at days 2 or 3, only 10% of the immunocytochemically reactive cells incorporated [³H]thymidine, indicating that most plasma cells had withdrawn from active proliferative activity.

We conclude from all these morphologic approaches that mitogen-induced lymphoblasts differentiated into typical mature plasma cells. Some blasts persisted, but small lymphocytes or other cells were not detected among the progeny.

Viability of Cultured Lymphoblasts. Trypan blue staining (Tables I, II, and VI) is a measure of cell recovery, instead of viability. Initial labeling of the culture with [³H]thymidine provides a more direct and quantitative approach to the question of viability, or longevity, of the cultured lymphoblast. The assumption is that cell death is the only means whereby a major loss in cell-associated label could occur.

During the first day of culture some 10–15% of the radiolabel was lost from the cells into the culture medium (Table VI). Using the fiber filter assay, cell-associated label then fell another 40–50% on each of the second and third days of culture. At days 2 and 3, the amount of TCA-precipitable, cell-bound label was larger than obtained by the filter technique (Table VI). Counts in the culture medium were largely (85%) TCA soluble.

By autoradiography (Table VI), the percentage of cell-associated silver grains remaining with time paralleled the liquid scintillation data of cells harvested onto fiber filters. We presume that TCA precipitation recovers many dead cells that carry [³H]thymidine (or silver grains—Fig. 3C), and thus falsely elevates the value for viable cell-associated counts.

The viable cell count (trypan blue negatives) could be corrected by using the quantitative data on [³H]thymidine losses as an estimate of the actual extent of cell death in the entire culture. We then found (Table VI) that viable cell numbers would have expanded some fourfold over a 3-day culture period. This is what one would expect from the grain count dilution data which indicated that the blasts had divided twice, as long as labeled and unlabeled blasts behaved similarly.

We tested the culture medium at varying times to see whether it contained some toxic factor responsible for the fall in cell viability. Cultures harvested at

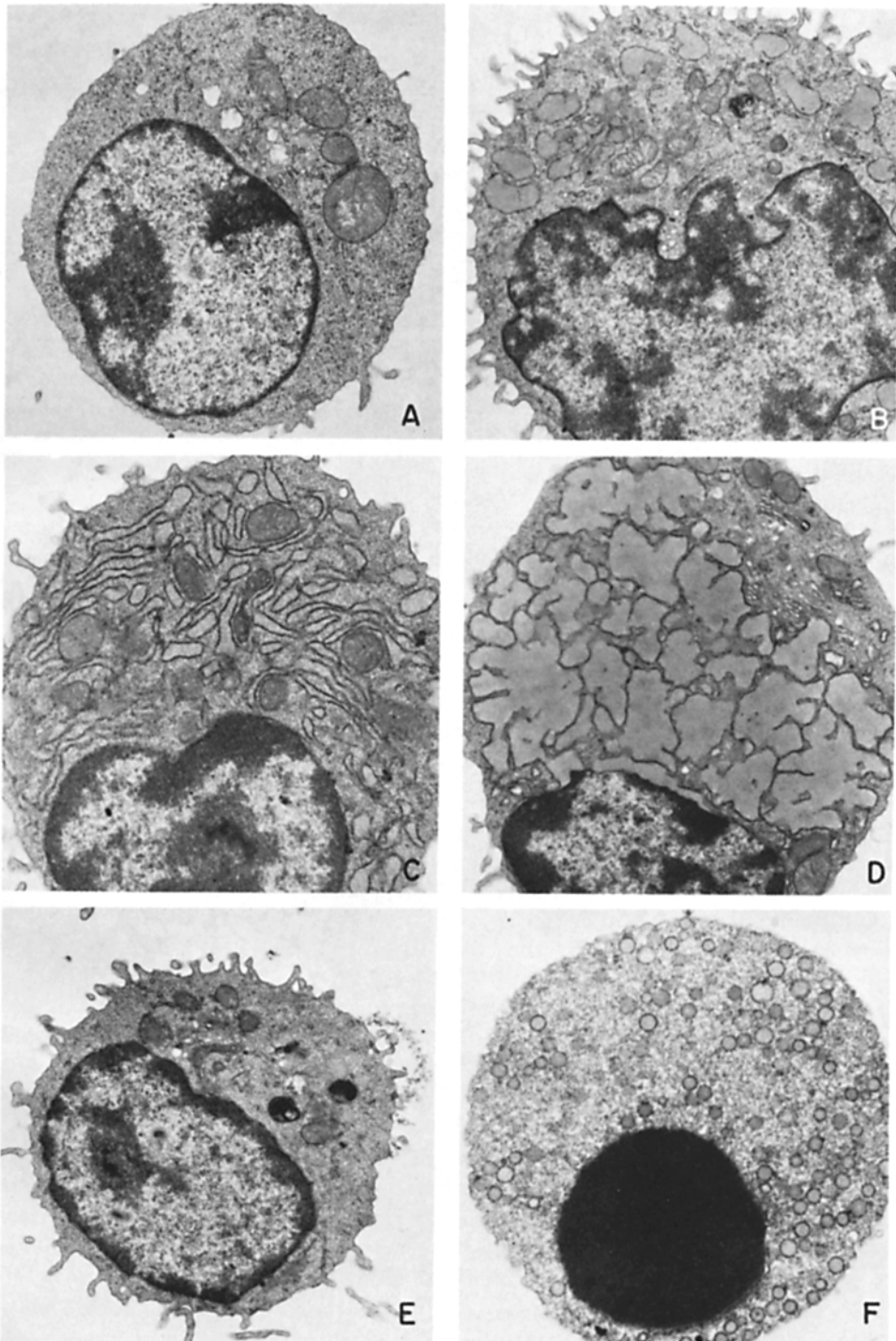


TABLE V
*Classification of Cells by Electron Microscopy—3-Day
 Lymphoblast Cultures*

Cell type	
Lymphoblasts	20.0
Immature plasma cells	28.4
Mature plasma cells*	34.2
Medium lymphocytes	9.8
Other‡	7.6

LPS-induced blasts were cultured for 3 days in medium supplemented with both LPS and FCS. 247 viable cell profiles were analyzed in a random thin section and classified by criteria exemplified in Fig. 5.

* Includes Mott cells.

‡ Primarily small lymphocytes and dendritic cells. Less than 1% of the cultures were macrophages.

day 2 showed similar losses in trypan blue-negative cells and cell-associated label when cultured for an additional day in fresh or conditioned medium. Freshly isolated lymphoblasts exhibited excellent viability in fresh medium and medium harvested from day 3 cultures. We conclude that the marked fall in viability at days 2 and 3 is primarily a property of the cells.

Discussion

We have made several changes in emphasis in the study of LPS- and FCS-induced AFC development. The first is that we begin our studies with homogeneous populations of mitogen-induced lymphoblasts (3) rather than with whole spleen cells. Because they are proliferating cells, the blasts that are in the S phase of the cell cycle can be tagged with [³H]thymidine, and can then be followed continuously in vitro. Secondly, we are trying to follow events in vitro more directly than previously. We reason that if we want to identify the cellular changes and mechanisms involved in the development of AFC from B lymphocytes, we must be able to do more than measure the magnitude of an immune response at any one time-point. According to current thinking, the size of a response would include four main components with respect to B-cell behavior: the number of B lymphocytes that are blast transformed; the extent to which individual cells expand their numbers by cell proliferation; the time

FIG. 4. Examples of the various cell types present in day 3 lymphoblast cultures are: (A) A lymphoblast (? plasmablast). The cytoplasm contains free rather than membrane-bound polyribosomes. The nucleus and nucleolus are large, and there is relatively little heterochromatin. ($\times 8,100$). (B) An immature plasma cell. The term "immature" is used to describe either the nucleus (as in this case because it shows evidence of mitotic activity) or the cytoplasm (i.e., many nonmembrane-bound polyribosomes are present—though not in this example). ($\times 8,100$). (C) A mature plasma cell. The cell's nucleus (small with abundant heterochromatin) and cytoplasm (membrane-bound polyribosomes) are both mature. ($\times 13,000$). (D) Mott cell with extremely dilated RER. It would appear that these large cisterns may be formed both by dilatation and fusion. ($\times 9,400$). (E) Presumptive medium lymphocyte. These cells contain more cytoplasm than small lymphocytes, but have relatively little RER or free polyribosomes. ($\times 8,300$). (F) Dead cells with a pyknotic nucleus and residual vesicles of RER. ($\times 12,000$).

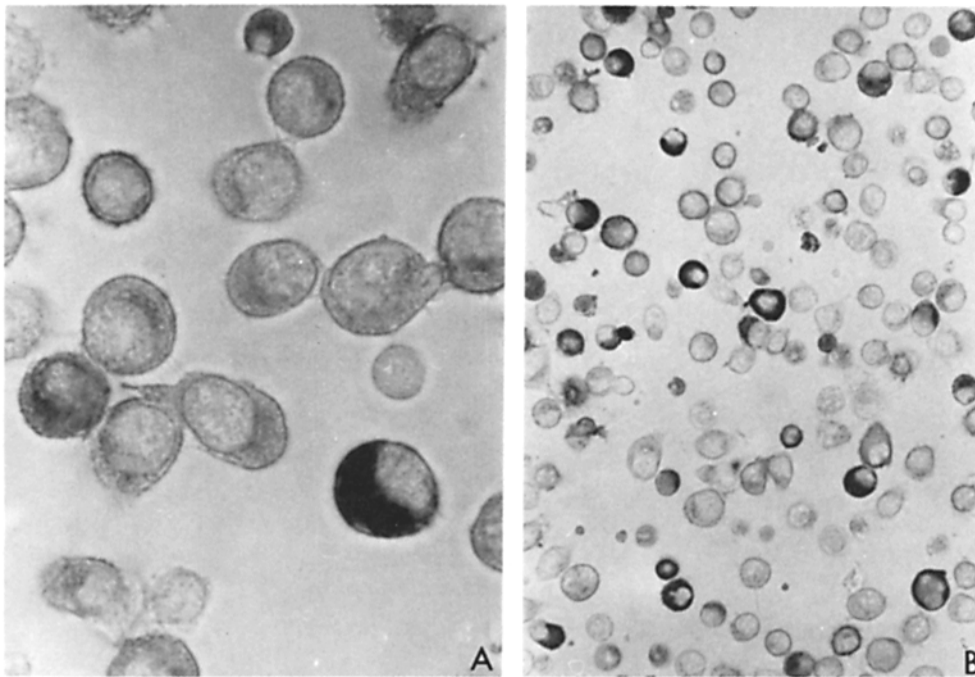


FIG. 5. Immunocytochemical visualization of cellular Ig. Alcohol-acetic acid-fixed smears were exposed successively to rabbit antimouse- κ ; HRP-conjugated, sheep antirabbit Ig; and diaminobenzidine staining. (A) LPS-induced blasts cultured for 1 day in vitro. About 10% of the cells are dark-staining plasma cells. The others have weak, probably surface, reactivity. ($\times 860$). (B) LPS blasts after 2 days in vitro. 20/92 cells have strong reactivity and would be classified as mature plasma cells, whereas most others have weak staining that is sensitive to trypsinization before fixation (250 $\mu\text{g}/\text{ml}$, 30 min). Ig-negative cells are readily detected in the presence of plasma cells by this immunocytochemical procedure, e.g., 3-day progeny of Con A blasts (3) are at least 80% Ig-negative cells and 10% typical plasma cells. ($\times 345$).

TABLE VI
Viability of [^3H]Thymidine-Tagged LPS Blasts

Time in culture	^3H Thymidine (cpm/culture)			^3H Thymidine (autoradiography) (% initial value)	Viable cell recovery		"Corrected" viable/culture
	Cell associated (% initial value)		Culture medium		$(\times 10^{-6}/\text{culture})$		
	Fiber filter	TCA insoluble			-	+	
<i>h</i>							
1	16,970 (100)	16,780 (100)	0	100	1.05	0.02	1.05
20	14,180 (84)	14,990 (89)	1,640	95	1.50	0.25	1.80
44	8,620 (51)	11,830 (71)	4,210	43	1.45	0.45	2.86
67	4,890 (29)	9,790 (58)	6,720	19	1.10	0.95	3.80

Low density, LPS-induced blasts were radiolabeled with a 1-h pulse of 0.3 $\mu\text{Ci}/\text{ml}$ [^3H]thymidine (6.0 Ci/mM). At varying times, the cultures were harvested with Pasteur pipettes, and the cells spun at 150 g for 10 min. Counts were measured in the culture medium (85% of which were TCA soluble), and in the cell pellet. Cell-associated cpm were measured by harvesting aliquots directly on fiber filters, or after TCA precipitation (less than 10% of the cell-associated cpm were TCA soluble). The percentage of initially incorporated [^3H]thymidine remaining with time was also assessed by autoradiography, using the mean grain count/viable cell times the number of labeled cells/culture. Viable cell recoveries were determined by trypan blue staining and then corrected for losses due to cell death by using the percentage of cell-associated [^3H]thymidine remaining per culture (fiber filter assay).

at which maturation into an AFC occurs (which in turn may depend on the particular assay for AFC that is used); and the life-span of the AFC. All the accessory cells and/or factors currently thought to be important in AFC development could act at any of these points. We think that by concentrating on the lymphoblast, we can gain direct access to some of these components in a B-cell response. Finally, we have concentrated on the so-called maturation of AFC, rather than on antibody secretion. We looked for the development of mature plasma cells *in vitro* using as criteria those properties that have been established *in situ*. These include distinctive cytologic features such as eccentric small nuclei and basophilic cytoplasm by light microscopy (16) and abundant RER by electron microscopy (15, 17, 18); strong immunocytochemical reactivity for cytoplasmic Ig (16, 19); withdrawal from proliferative activity (16, 20, 21) and short life-span (22).

We feel the main finding in this paper concerns the proliferative activity of mitogen-stimulated B blasts. Most develop into typical mature plasma cells after just two cycles of cell division, within 2-3 days of culture, and even in the continued presence of LPS and FCS. Tagging the blasts at the start of the culture and then following the number and grain counts of labeled progeny was the most useful approach for following proliferative activity. Comparable information would be difficult to obtain with current techniques in which proliferative activity and AFC numbers are measured at any one time-point. These methods are insufficient for answering questions on the extent of cell proliferation in systems where cells may mature rapidly into end stage cells, cell death is frequent, the cell cycle of the proliferating cells is unknown, and new cells can be recruited continually into the mitogen response.

Clonal selection theory implies that individual B cells divide many times during an AFC response, producing large clones of genetically identical progeny (23). Actually we know little about the proliferative potential of B cells responding to antigens *in situ*. A large number of experimental systems have been devised, however, to get B cells (24-29) and T cells (30-33) to proliferate much more extensively than documented in this paper, both to antigens and mitogens. Most involve the maintenance of responding cells at very low densities usually in the presence of large numbers of appropriate nonresponding "feeder" and/or "filler" cells *in vitro* or in lethally irradiated spleen *in situ*. It is not clear how these systems relate to the physiologic behavior of B lymphoblasts or whether the proliferating cells represent a special subclass of the starting population. We simply wish to emphasize that the opposite extreme to large clones does exist.

Also, it has often been assumed that the failure of lymphocytes to proliferate continuously *in vitro* reflects poor tissue culture conditions. However, it may be that rapid maturation of the blast to an end stage cell limits proliferation. For example, Schooley's data (22) indicate that at least 80% of mature plasma cells *in situ* may live less than 12 h. What we need to know is whether some physiologic counterpart to "clones" of four mature plasma cells exists. That is, in a normal immune response there may exist a pathway or mechanism whereby typical plasma cells develop after just two rounds of cell division, and there may be other mechanisms whereby proliferative activity is accelerated

and/or extended. In current terminology, the former might represent a "suppressor" effect because the number of AFC would be relatively reduced.

A second surprising finding was that we did not detect small lymphocytes arising in our cultures of B blasts. There is evidence that T blasts arising in transplantation and mitogen responses can revert back to small lymphocytes, with memory function (34), but we were unable to accomplish this with mitogen-induced B blasts. We did observe some lymphoblasts persisting after 3 days of culture, however. The numbers of these cells could be increased by using FCS rather than other sera to supplement the cultures, by frequent changes in the culture medium, by maintaining the cultures at lower cell densities, and by keeping the number of macrophages low – less than 5% of the total cells (data not presented). We do not know whether these persistent blasts represent a variant in an otherwise homogeneous population of mitogen-induced cells, or whether they are a true subpopulation with different capacities. One possibility, assuming that they are B blasts, is that they are cells stimulated by antigens in the animal before sacrifice, rather than by mitogen *in vitro*. Another possibility relates to the work of Zauderer and Askonas (35) who have shown that IgG- and IgA-secreting AFC arise in mitogen-stimulated cultures maintained for 1-2 wk *in vitro*.

Cultures of mitogen-induced B blasts may be useful for characterizing the function of the various cell types in the plasma cell lineage. It is usually assumed that the mature plasma cell is the main secretory element in the lineage. This is based on cytologic criteria, namely, abundant RER and increased immunocytochemical reactivity for cytoplasmic Ig. In fact, all presumptive stages in the development of plasma cells have been detected as antibody-secreting cells in plaque assays (e.g. reference 15). Active Ig secretion is found very early in LPS-stimulated whole spleen cultures at a time when mature plasma cells have not evolved (36), and even Fagraeus pointed out that her splenic fragments secreted antibody more actively when they contained immature rather than mature plasma cells (37). It is also unclear why many AFC exhibit such dilated RER in our cultures. It has been suggested that extensive RER dilatation may be associated with decreased antibody secretion (14), and we wonder whether dilated RER results in the strongest staining for cytoplasmic Ig in immunocytochemical assays. It is possible that the maturation into typical plasma cells functions to limit the amount of Ig secreted in an immune response. If proliferating plasma cell precursors actively secrete antibody, then maturation to an end stage cell might limit the number and life-span of antibody-secreting units.

We are currently applying the concepts and techniques used in this study to the cellular events involved in the development of antigen-specific AFC. It is quite possible that antigen-induced blasts behave very differently from mitogen-induced ones. In fact, *in vitro* conditions can be devised whereby AFC (or their precursors) divide five to six times during the development of an antisheep red blood cell response. As in the case of mitogen-induced cells, the response may be terminated by the production of typical, short-lived, mature plasma cells.

Summary

Mouse spleen lymphoblasts induced with lipopolysaccharide and fetal calf

serum were obtained in high yield and purity in their first proliferative cell cycle by floatation in dense bovine plasma albumin columns (3). The blasts were maintained *in vitro* for 3 more days. The cultures were examined in bulk on each day, and in addition, those cells in S phase initially were tagged with [³H]thymidine and followed continuously *in vitro*.

Grain count dilution data indicated that most blasts divided but twice over a 2- to 3-day interval *in vitro*. [³H]Thymidine pulse radiolabeling and flow microfluorometry suggested that at least 50–70% of the proliferating blasts withdrew from proliferative activity after 2–3 days of culture.

Morphologic studies demonstrated that lymphoblasts persisted as such for 1–2 days *in vitro* and then matured into typical plasma cells. Many of the blast progeny had small nuclei and considerable basophilic cytoplasm on Giemsa-stained cell smears; abundant rough endoplasmic reticulum by electron microscopy; and readily detectable cytoplasmic Ig by immunocytochemistry. Reversion of blasts to small lymphocytes could not be detected; however, some blasts persisted even after 3 days of culture.

The viability of the cultured lymphoblast was followed by initially tagging the cells with [³H]thymidine as well as several other techniques. Little cell death was documented during the first day of culture. The number of labeled progeny increased twofold whereas the grain count halved. But 40–50% of the cell-associated label was lost during each of the second and third days, and fewer labeled progeny than predicted by grain count dilution were identified. The culture medium could not be implicated in this loss of lymphoblast progeny, and we suggest that the maturation of the lymphoblast to a short-lived plasma cell was responsible.

Therefore mitogen-stimulated B blasts seem to mature into typical plasma cells after just two cycles of cell division. The plasma cells resemble those produced *in situ* during an immune response in their cytologic features, withdrawal from active proliferative activity, and short life-span.

The authors are indebted to Doctors S. Silverstein and J. Unkeless for providing the labeled antirabbit Ig reagents used in the plasma cell stains; to J. Adams for valuable help with the electron microscopy; to Dr. B. Clarkson for stimulating discussion; and to L. Weller for preparing the several drafts of this manuscript.

Received for publication 28 July 1977.

References

1. Andersson, J., G. Möller, and O. Sjöberg. 1972. Selective induction of DNA synthesis in T and B lymphocytes. *Cell. Immunol.* 4:381.
2. Coutinho, A., E. Gronowicz, G. Möller, and H. Lemke. 1976. Polyclonal B cell activators (PBA). *In Mitogens in Immunobiology.* J. J. Oppenheim and D. L. Rosenstreich, editors. Academic Press, Inc., N. Y. 173.
3. Steinman, R. M., B. G. Machtinger, J. Fried, and Z. A. Cohn. 1977. Mouse spleen lymphoblasts generated *in vitro*. Recovery in high yield and purity after floatation in dense bovine plasma albumin solutions. *J. Exp. Med.* 147:279.
4. Wimber, D. E. 1964. Effects of intracellular irradiation with tritium. *Adv. Radiat. Biol.* 1:85.
5. Rieke, W. O. 1962. The *in vivo* reutilization of lymphocytic and sarcoma DNA by cells growing in the peritoneal cavity. *J. Cell Biol.* 13:205.

6. Fried, J. 1976. Method for the quantitative evaluation of data from flow microfluorometry. *Comput. Biomed. Res.* 9:263.
7. Fried, J., X. Yataganas, T. Kitahara, A. G. Perez, R. Ferguson, S. Sullivan, and B. Clarkson. 1976. Quantitative analysis of flow microfluorometric data from asynchronous and drug treated cell populations. *Comput. Biomed. Res.* 9:277.
8. Fried, J. 1977. Analysis of DNA histograms from flow cytofluorometry: estimation of the distribution of cells within S phase. *J. Histochem. Cytochem.* 25:942.
9. Göhde, W. 1973. Zellzyklusanalysen mit dem impulszytrophotometer. Thesis. Münster University. W. Germany.
10. Avrameas, S., and T. Ternynck. 1971. Peroxidase labeled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochemistry.* 8:1175.
11. Nakane, P. K., and A. Kawasi. 1974. Peroxidase-labeled antibody: a new method of conjugation. *J. Histochem. Cytochem.* 22:1084.
12. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291.
13. Bessis, M. C. 1961. Ultrastructure of lymphoid and plasma cells in relation to globulin and antibody formation. *Lab. Invest.* 10:1040.
14. Zagury, D., J. Bernard, S. Lemieux, J. C. Mazie, S. Avrameas, and A. E. Bussard. 1976. The relationship between storage and secretion of specific antibody by immune lymphoid cells: ultrastructural localization of anti-peroxidase antibodies in plaque-forming cells of the rabbit popliteal lymph node. *Eur. J. Immunol.* 6:194.
15. Harris, T. N., K. Hummeler, and S. Harris. 1966. Electron microscopic observations on antibody producing lymph node cells. *J. Exp. Med.* 123:161.
16. Balfour, B. M., E. H. Cooper, and E. L. Alpen. 1965. Morphological and kinetic studies on antibody-producing cells in rat lymph nodes. *Immunology.* 8:230.
17. Avrameas, S., and E. H. Leduc. 1970. Detection of simultaneous antibody synthesis in plasma cells and specialized lymphocytes in rabbit lymph node cells. *J. Exp. Med.* 131:1137.
18. Movat, H. Z., and N. V. P. Fernando. 1965. The fine structure of the lymphoid tissue during antibody formation. *Exp. Mol. Pathol.* 4:155.
19. Leduc, E. H., A. H. Coons, and J. M. Connolly. 1955. Studies on antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit. *J. Exp. Med.* 102:61.
20. Cooper, E. H. 1961. Production of lymphocytes and plasma cells in the rat following immunization with human serum albumin. *Immunology.* 4:219.
21. Nossal, G. J. V., and O. Mäkelä. 1962. Autoradiographic studies on the immune response. I. The kinetics of plasma cell proliferation. *J. Exp. Med.* 115:209.
22. Schooley, J. C. 1961. Autoradiographic observations of plasma cell formation. *J. Immunol.* 86:331.
23. Burnet, F. M. 1959. *The Clonal Selection Theory of Acquired Immunity.* Cambridge University Press, Cambridge, Eng.
24. Andersson, J., A. Coutinho, W. Lernhardt, and F. Melchers. 1977. Clonal growth and maturation to immunoglobulin secretion *in vitro* of every growth-inducible B lymphocyte. *Cell.* 10:27.
25. Askonas, B. A., and A. R. Williamson. 1972. Factors affecting the propagation of a B cell clone forming antibody to the 2,4-dinitrophenol group. *Eur. J. Immunol.* 2:487.
26. Bosma, M., and E. Weiler. 1970. The clonal nature of antibody formation. I. Clones of antibody forming cells of poly-D-alanine specificity. *J. Immunol.* 104:203.
27. Kennedy, J. C., J. E. Till, L. Siminovitch, and E. A. McCulloch. 1966. The

- proliferative capacity of antigen sensitive precursors of hemolytic plaque-forming cells. *J. Immunol.* 96:973.
28. Marbrook, J., and J. S. Haskill. 1974. The *in vitro* response to sheep erythrocytes by mouse spleen cells: segregation of distinct events leading to antibody formation. *Cell. Immunol.* 13:12.
 29. Metcalf, D., G. J. V. Nossal, N. L. Warner, J. F. A. P. Miller, T. E. Mandel, J. E. Layton, and G. A. Gutman. 1975. Growth of B-lymphocyte colonies *in vitro*. *J. Exp. Med.* 142:1534.
 30. Bernheim, J. L., and J. Mendelsohn. 1975. The kinetics of DNA synthesis in human lymphocytes stimulated by phytohemagglutinin (PHA). *Clin. Res.* 23:401A. (Abstr.)
 31. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J. Exp. Med.* 145:508.
 32. Stewart, C. C., S. F. Cramer, and P. G. Steward. 1975. The response of human peripheral blood lymphocytes to phytohemagglutinin: determination of cell numbers. *Cell. Immunol.* 16:237.
 33. Teh, H.-S., E. Harley, R. A. Phillips, and R. G. Miller. 1977. Quantitative studies on the precursors of cytotoxic lymphocytes. I. Characterization of a clonal assay and determination of the size of clones derived from single precursors. *J. Immunol.* 118:1049.
 34. Andersson, L. C., and P. Häyry. 1974. Generation of T memory cells in one-way mixed lymphocyte culture. I. Selective recovery of activated cells and their reversion to "secondary" lymphocytes. *Scand. J. Immunol.* 3:461.
 35. Zauderer, M., and B. A. Askonas. 1976. Several proliferative phases precede maturation of IgG-secreting cells in mitogen stimulated cultures. *Nature (Lond.)*. 260:611.
 36. Andersson, J., and F. Melchers. 1974. Maturation of mitogen-activated bone marrow derived lymphocytes in the absence of proliferation. *Eur. J. Immunol.* 4:533.
 37. Fagraeus, A. 1948. Antibody production in relation to the development of plasma cells: *in vivo* and *in vitro* experiments. *Acta Med. Scand. Suppl.* 204.