PROLIFERATION AND COLONY-FORMING ABILITY OF PERITONEAL EXUDATE CELLS IN LIQUID CULTURE*

By CARLETON C. STEWART, HSIU-SAN LIN,‡ AND CHERYL ADLES

(From the Section of Cancer Biology, Division of Radiation Oncology, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri 63110)

The multifunctional capabilities that have been attributed to cells of the mononuclear phagocyte system include interferon production (1, 2), the production of complement (C) intermediates (3), the phagocytosis of a variety of foreign substances (4–6), an accessory cell in antigen-trapping (7–9), antigen recognition (10, 11), antigen storage (12, 23), the production of immunologic RNA (14, 15), cytotoxic reactions with foreign cells (16–18), and the control of chronic infections in granulomas (19). "Activated" macrophages exhibit increased phagocytic activity, (5, 6, 20), increased bactericidal activity (6), and increased cytolytic activity against target cells (16–18). We recently showed that peritoneal exudate cells obtained after thioglycollate medium injection into the peritoneal cavity of mice formed colonies containing only "macrophages" when cultured in agar medium supplemented with L-cell-conditioned medium,[1] while cells from the unstimulated peritoneal cavity did not (21–23).

Since macrophages may be a heterogenous population of cells with regards to function, we have used the term to identify phagocytic mononuclear cells obtained from the peritoneal cavity of mice or which arise in culture. It may be possible by using colonies derived from a single precursor cell to determine if all progeny have similar function, or whether heterogeneity of function occurs within a colony. Since certain advantages, such as studying phagocytic activity, can be achieved if colonies form in the absence of agar, experiments were undertaken to determine if peritoneal exudate cells could form colonies in liquid medium. The kinetics of exudate cell proliferation and colony formation are described.

Materials and Methods

Mice. C3H/Anf mice, either male or female, 8–10 wk of age, obtained from Cumberland View Farms, Clinton, Tenn., were used throughout these experiments. Mice were injected intraperitoneally with 1.5 ml thioglycollate medium (Difco Laboratories, Detroit, Mich.), prepared according to the label directions.

Medium. α-Minimal essential medium (MEM) (Flow Laboratories, Inc., Rockville, Md.) (24) was the basic medium used for all these studies. Culture medium used for the preparation of exudate cells was α-MEM and 10% fetal calf serum. Growth medium used to demonstrate proliferation and

* This investigation was supported by U. S. Public Health Service grant CA13053.

‡ Recipient of a U. S. Public Health Service Research Career Development Award (CA-70730).

[1] *Abbreviations used in this paper:* L cell, L strain mouse fibroblasts; PBS, phosphate-buffered saline; PMN, polymorphonuclear; [^{3}H]TdR, tritiated thymidine.

colony formation was α-MEM, 10% fetal calf serum (Flow Laboratories, Inc.), 5% horse serum (Flow Laboratories, Inc.), and 10% L-cell-conditioned medium. All media were sodium bicarbonate buffered and supplemented with 100 U penicillin and 100 μg streptomycin/ml.

L-Cell-Conditioned Medium. Mouse L cells were seeded in 75 cm^2 Falcon culture bottles (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at 5×10^5 cells in 10 ml culture medium. 4 days later, the medium was removed and filtered through a 0.22 μm filter to insure a cell-free-conditioned medium. Conditioned medium was stored frozen until needed.

Harvesting the Exudate. Mice were killed by cervical dislocation, and injected intraperitoneally with 5 ml of culture medium containing 5 U heparin/ml. After 2–5 min, the medium was withdrawn from the flank opposite the injection site.

The medium containing exudate cells was collected and inspected with a phase-contrast microscope for contamination by erythrocytes or bacteria. Suspensions were then pooled and centrifuged at 200 *g* for 10 min, and recentrifuged with fresh culture medium. All procedures were carried out at 4°C to reduce the adherence of cells to the tubes. Even with this precaution, an adherence rate of 5–10% of the cells/h to "nonwettable" plastic tubes was found.

Culture Conditions. Unless otherwise noted, cells were cultured in 35-mm Falcon plastic tissue culture dishes (Falcon Plastics, Div. of BioQuest). Two culture dishes containing cells were then placed in a 100-mm Falcon Petri dish along with a third 35-mm culture dish containing distilled water to provide better humidity for the relatively long incubation periods required. These culture dishes were then placed in a humidified incubator having a 10% CO_2 in air atmosphere.

The number of peritoneal colony-forming cells in agar was determined by the method previously described (22). Cells were grown in 1 ml of agar medium which contained the same proportion of sera and L-cell-conditioned medium used for liquid culture in addition to Noble agar at a final concentration of 0.3%.

Cell Counting. All cell counts were performed using the pronase-cetrimide technique (25) and an electronic particle counter. With this technique, only viable cells which resist pronase digestion were counted. Cells to be counted, either adherent to culture dishes or in suspension, were first treated with 2.5 mg pronase/ml to digest dead cells. When cells were in suspension, 0.5 ml were treated for 10 min with 0.5 ml pronase (5 mg/ml) before addition of 10 ml of the detergent cetrimide. When cells were adherent to culture dishes, the medium from the dish was added to 10 ml cetrimide and the dish was then washed several times with the cetrimide.

Phagocytosis Assay for Adherent Cells. Phagocytosis of yeast and sheep erythrocytes was used to functionally identify adherent cells as macrophages. Heat-killed Baker's yeast was prepared at a concentration of 10^9 cells/ml in phosphate-buffered saline (PBS). To determine the percentage of cells that were phagocytic, 50 μl yeast suspension and 0.3 ml freshly prepared guinea pig C (1:2 saline), or 0.3 ml C3H/Anf mouse serum were added to each culture. These cultures were incubated for 30 min at 37°C, and then washed three times with PBS.

Antiserum to sheep erythrocytes was prepared by injecting C3H/Anf mice intraperitoneally with 10^8 washed (in PBS) sheep erythrocytes. 10 days later, mice were bled and serum was prepared. After heat inactivation at 56°C for 45 min, the serum was sterilized using a 0.22 μm filter and stored frozen. Just before determining the ability of adherent cells to phagocytize sheep erythrocytes, 10^9 sheep erythrocytes/ml were mixed with an equal volume of antiserum, and the cells were incubated 30 min at room temperature; 50 μl was then added to the 3-ml cultures. After 30 min incubation, the plates were washed three times with PBS.

Culture plates were either scored wet using phase-contrast optics or were stained with methylene blue. When colonies were not present, 200 cells were counted at random and the percent containing two or more particles of yeast or sheep erythrocytes was determined. When colonies of macrophages were present, all colonies were scored for phagocytic cells, counting a minimum of 20 cells in each. Cells found between colonies were also scored during the scan.

Culture of Cells for Autoradiography. Tritiated thymidine ([^{3}H]TdR) was used in some experiments to determine the fraction of cells in DNA synthesis. Exudate cells were grown on 22 × 22-mm sterile cover slips placed in 35-mm Falcon culture dishes. At appropriate times, 0.5 ml [^{3}H]TdR (7 μCi/ml in α-MEM, 3 Ci/mM) was added to each culture and incubated an additional 2 h. At 1.5 h, 50 μl yeast and 0.3 ml C solution were added to some cultures to determine the percentage of labeled adherent cells which were also phagocytic. After incubation, cover slips were removed from the dishes, washed three times in PBS, fixed in 10% formalin, and air dried. After mounting the cover

slips onto microscope slides, they were dipped into NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, N. Y.) and exposed for 2 wk. The slides were then developed in D-19 (Eastman Kodak Co.). and stained with May-Grünwald Giemsa.

Results

Definition of the Exudate. Fig. 1 shows the total number of cells obtained from the peritoneal cavity as a function of time after thioglycollate medium. These cells were first divided into the adherent and nonadherent cell populations. Adherent cells were defined as cells which would adhere to plastic tissue culture dishes after a 2 h incubation. The percentage of these adherent cells which would phagocytize yeast in the presence or absence of serum opsonin

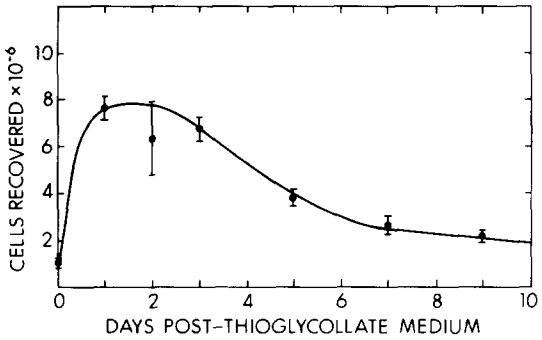

FIG. 1. Exudate cell yield as a function of time after thioglycollate medium. The cell yield per mouse is plotted as a function of time after intraperitoneal thioglycollate medium injection. The standard error from 30 mice in three experiments is shown.

and/or C, and the percent of the adherent cells which would phagocytize specific antibody-treated sheep erythrocytes were then determined. Adherent cells which exhibit these functions constitute our definition of macrophages. Table I shows the results of these studies. The cell population from the unstimulated peritoneal cavity contained 15% adherent cells of which 22% were phagocytic for yeast. The remaining cells which attached but were not phagocytic resembled those which were phagocytic, resembled fibroblasts, or they resembled lymphocytes, possibly B lymphocytes, which are known to be somewhat adherent (26). The nonadherent cells, comprising 84% of the population at this time, consisted mostly of small lymphocytes. By day 1, there was an extensive infiltration by polymorphonuclear (PMN) leukocytes into the exudate, representing 60–70% of the total cells. These cells almost completely disappeared by day 3. These results are consistent with those found by others (27).

The maximum number of adherent cells were found on days 2 and 3. Most of the nonadherent cells resemble lymphocytes at these times, as PMN leukocytes continue to decrease. Although the percentage of cells that were adherent decreased with time after day 3, the proportion of cells that were phagocytic remained higher than those found from the unstimulated peritoneal cavity. Most nonphagocytic adherent cells morphologically resembled macrophages. The percentage of adherent cells phagocytosing yeast was enhanced by adding serum opsonin and C, but the most striking effect observed was the greater than 10-fold

TABLE I
Functional Capacity of Adherent Peritoneal Cells

Day postinjection	Adherent	Adherent cells that were phagocytic					
		Yeast	Yeast with serum	Yeast with C	Yeast with serum and C	SRBC*	SRBC with* antiserum
	%	%	%	%	%	%	%
0	16 ± 2‡	5 ± 2	7 ± 1	18 ± 6	22 ± 3	0.8 ± 0.7	5.8 ± 1.5
1	41 ± 3	15 ± 2	18 ± 3	25 ± 2	37 ± 5	3.0 ± 2.6	11.6 ± 8.2
2	67 ± 3	58 ± 1	58 ± 6	75 ± 10	80 ± 10	8.5 ± 1.0	28.5 ± 9.7
3	64 ± 3	52 ± 10	65 ± 15	70 ± 14	72 ± 13	5.7 ± 1.3	61.7 ± 19.6
5	31 ± 3	65 ± 16	70 ± 8	76 ± 16	81 ± 5	3.8 ± 1.3	61.3 ± 7.1
7	23 ± 2	48 ± 12	55 ± 12	62 ± 12	71 ± 11	3.8 ± 0.1	77.5 ± 9.9
9	28 ± 1	20 ± 4	40 ± 12	42 ± 20	62 ± 12	1.3 ± .06	34.8 ± 9.1

* Includes rosettes.
‡ Standard deviation.

increase in the average number of yeast particles per cell in the presence of serum opsonin or C.

Another criterion used to identify macrophages was their facilitated ability to phagocytize sheep erythrocytes or form rosettes in the presence of specific antibody. In these experiments, sheep erythrocytes were pretreated with heat-inactivated syngeneic mouse antisheep erythrocyte serum. The results, using unstimulated and thioglycollate medium-stimulated adherent cells, are shown in Table I. Cells from the unstimulated peritoneal cavity were considerably less phagocytic than were those from peritoneal exudates. Antiserum enhanced the percentage of adherent cells which were phagocytic, or formed rosettes for sheep erythrocytes, and the number of erythrocytes phagocytized was greater than five times that found in phagocytic cells receiving untreated erythrocytes.

Kinetics of Cell Growth. The proliferative ability of exudate cells was measured at approximately 2×10^3, 2×10^4, and 2×10^5 cells/culture. Cells were cultured in 35-mm culture dishes containing 3 ml growth medium. Adherent cells were not separated from the nonadherent cells during culture. The kinetics of cell growth were then determined for 20 days by counting all the cells as a function of time after plating. Autoradiographs were also prepared of the adherent cells at various incubation times after a 2 h pulse with 2 μCi [^{3}H]TdR/ml.

The number of cells per culture as a function of time is shown in Fig. 2. Exudate cells at 2×10^3 cells/culture proliferated exponentially for 18 days with a mean doubling time of 68 h. After day 18, the number of cells per culture decreases. The percentage of cells having phagocytized yeast or antibody-treated sheep erythrocytes, the percent labeled cells, and the percent labeled cells with yeast are shown in Table II for this cell concentration. By day 2, the percentage of adherent cells which are phagocytic is maximal and remained high thereafter. This increase in phagocytic activity after culture, may be due to the recovery of cells from in vitro manipulations associated with the initial set up procedures.

In the initial suspension 4% of the cells were labeled after a 2 h pulse and added

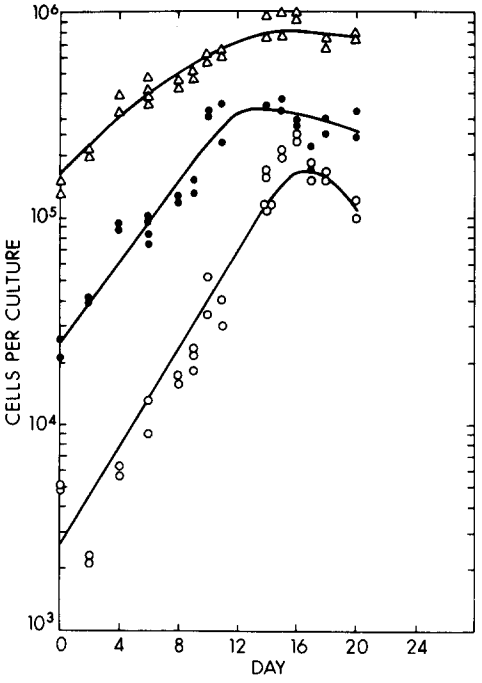

FIG. 2. Kinetics of exudate cell proliferation. 2.5 (open circles), 25 (closed circles), and 160 (triangles) $\times 10^3$ exudate cells were plated in 3 ml growth medium in 35-mm culture dishes. Note that the zenith in cellularity is reached earlier as the initial cell number is increased and that the highest concentration of cells grow slower.

TABLE II

Phagocytosis and Thymidine Incorporation by Adherent Peritoneal Cells

Day	Phagocytic		Labeled cells	Fraction of the labeled cells with yeast
	Yeast	SRBC		
	%	%	%	
0	74 ± 13*	45 ± 29	4	1.00
2	95 ± 5	81 ± 15	11.5	0.87
4	99 ± 1	95 ± 2	18	0.94
6	90 ± 7	64 ± 28	20	1.00
8	99 ± 2	—	34.5	0.98
9	99 ± 2	—	28.5	1.00
10	100	—	22.5	1.00
11	100	—	30	1.00
14	93 ± 1	84 ± 12	30	1.00
16	99 ± 1	—	15.5	1.00
17	96.5 ± 3	—	5	1.00
18	95	—	7	1.00
21	95 ± 8	79 ± 18	2	1.00

* Standard deviation.

2 h after plating. The fraction of labeled cells increases to 34% by day 8, and fluctuated between 22 and 34% until day 14. Thereafter, labeled cells continued to decrease. The labeled cells were also phagocytic, indicating this function occurs during cell cycle.

When 2×10^4 cells are initially seeded (Fig. 2), exponential proliferation occurs for only 12 days. The growth rate is almost identical to that for 2×10^3 cells. These cultures appeared somewhat confluent in the sense that accumulations of cells were found on most areas of the culture dish even though some areas were devoid of cells. In one experiment using 2×10^4 cells/culture, nonadherent cells were separated from the adherent cells and the two were counted separately. In this experiment, it was found that the increase in cellularity observed was due solely to the adherent cell fraction.

At 10^5 cells/culture, exponential proliferation occurred for about 10 days, and the doubling time was 81.6 h. These cultures were confluent by day 10.

The most extensive proliferative response was seen at a cell density of 2×10^3 cells/dish. The overall cell increase was 64-fold when the initial number cultured was 2×10^3; 17.5-fold for 2×10^4, and 5-fold for 2×10^5 cells.

Studies were also undertaken to determine under what conditions exudate cells would proliferate in liquid culture. Cells were seeded at 10^4 cells/culture in various media and the extent of proliferation was determined at 2 wk. Table III shows the results of this study. It can be seen that there was a significant increase in cellularity only when conditioned medium was added. L-cell-conditioned medium had a greater stimulating effect than medium obtained from 4-day old nearly confluent macrophage cultures. Conditioned medium used in the absence of fetal calf serum and horse serum was also ineffective in inducing proliferation.

Colony Formation by Peritoneal Exudate Cells

COLONY FORMATION IN LIQUID MEDIUM. Mouse peritoneal exudate cells were obtained 2 or 3 days after thioglycollate stimulation, washed once in medium, and 10^3 cells in 3 ml growth medium were plated into 35-mm culture dishes. Each week, for 3 wk, colony counts were obtained on replicate plates. A few cell clusters containing 4–8 cells could be seen in plates examined after 2 h incubation. The frequency of clusters seemed to increase by 48 h but by 96 h both

TABLE III
Effect of Different Medium on Exudate Cell Proliferation

Medium	Fractional increase in cells at 2 wk
α-MEM	1.0
α-MEM + 10% LCM	0.7
α-MEM + 10% MCM*	1.4
α-MEM + 10% FCS*	1.7
α-MEM + 10% FCS + 5% HS*	1.9
α-MEM + 10% FCS + 5% HS + 10% LCM	26
α-MEM + 10% FCS + 5% HS + 10% MCM	9

* MCM, macrophage-conditioned medium; FCS, fetal calf serum; and HS, horse serum.

an increased frequency of cell clusters, and an increase in the number of cells per clusters (4–12) could easily be found. Between the 1st and 2nd wk a number of cell clusters, ranging in size from 4–40 cells, were observed. During this period a number of multinucleated cells appeared. Colonies (defined as cell clusters which have more than 50 cells) were distinct by the 2nd wk in culture. Although plating efficiencies as high as 20% and as low as 1% have been occasionally observed, the usual range is between 4–12%. After 20 days in culture, colonies were seen to degenerate, resulting in a loss of cells from the culture dish during the washing procedure which preceded staining. Colonies formed only in the presence of conditioned medium.

A typical colony at 14 days is shown in Fig. 3 *a* and a small macrophage colony growing beside a large fibroblast colony is shown in Fig. 3 *b*. Generally one fibroblast colony was observed per 50 macrophage colonies. Fig. 3 *c* shows a higher power phase-contrast photomicrograph of cells within a macrophage colony.

COLONY FORMATION AS A FUNCTION OF CELLS PLATED. In order to determine if the colonies arose from a single cell, mouse peritoneal exudate cells obtained 3 days after injection of thioglycollate medium were prepared at different cell concentrations. 3 ml of each cell concentration (ranging between 10^2 to 3×10^3 cells) were plated into 35-mm culture dishes. After 14 days incubation colonies were counted. The results, shown in Fig. 4, indicate a linear relationship between the number of cells plated and the number of colonies formed. We interpreted this to mean that each colony results from the proliferation of a single colony-forming cell. The average plating efficiency for 1,000 cells cultured in 3 ml medium in these experiments was found to be 6.6%.

COLONY FORMATION IN LIQUID AND SOFT AGAR MEDIUM. In order to compare the cells capable of forming colonies in liquid culture medium with those that form colonies in soft agar, exudate cells were plated under both culture conditions and the number of colonies were counted on day 14 for liquid culture and day 28 for soft agar cultures. The data from 26 different experiments are shown in Fig. 5. Although a significant discrepancy in terms of the plating efficiency was occasionally noted between the two conditions, colonies formed were, in general, parallel to each other. The mean value for the number of colonies per 1,000 exudate cells was 48 ± 28 (1 SD) in liquid culture and 54 ± 32 in soft agar culture.

Colony Formation by Adherent and Nonadherent Peritoneal Exudate Cells. To determine whether the colony-forming cells are adherent cells, peritoneal exudate cells were obtained 3 days after thioglycollate medium and 1,000, 3,000, or 10,000 cells in 3 ml of growth medium were plated onto 35-mm dishes. After allowing the adherent cells to attach for 2 h, the culture dishes were vigorously washed and the nonadherent cells collected and replated onto new culture dishes. After 14 days, all plates were observed for colonies.

Only the adherent cell population formed colonies (Fig. 6). Colonies were rarely found in cultures of the nonadherent cell fraction (0 to $1/10^4$ cells). These colonies which looked identical to those formed by the adherent cell fraction were probably due to contamination by adherent cells. Furthermore, the number of colonies formed by the adherent cells (20 colonies/1,000 cells with a range of 14–30) was not significantly different from those formed by the unseparated population (18 colonies/1,000 cells with a range of 9–40).

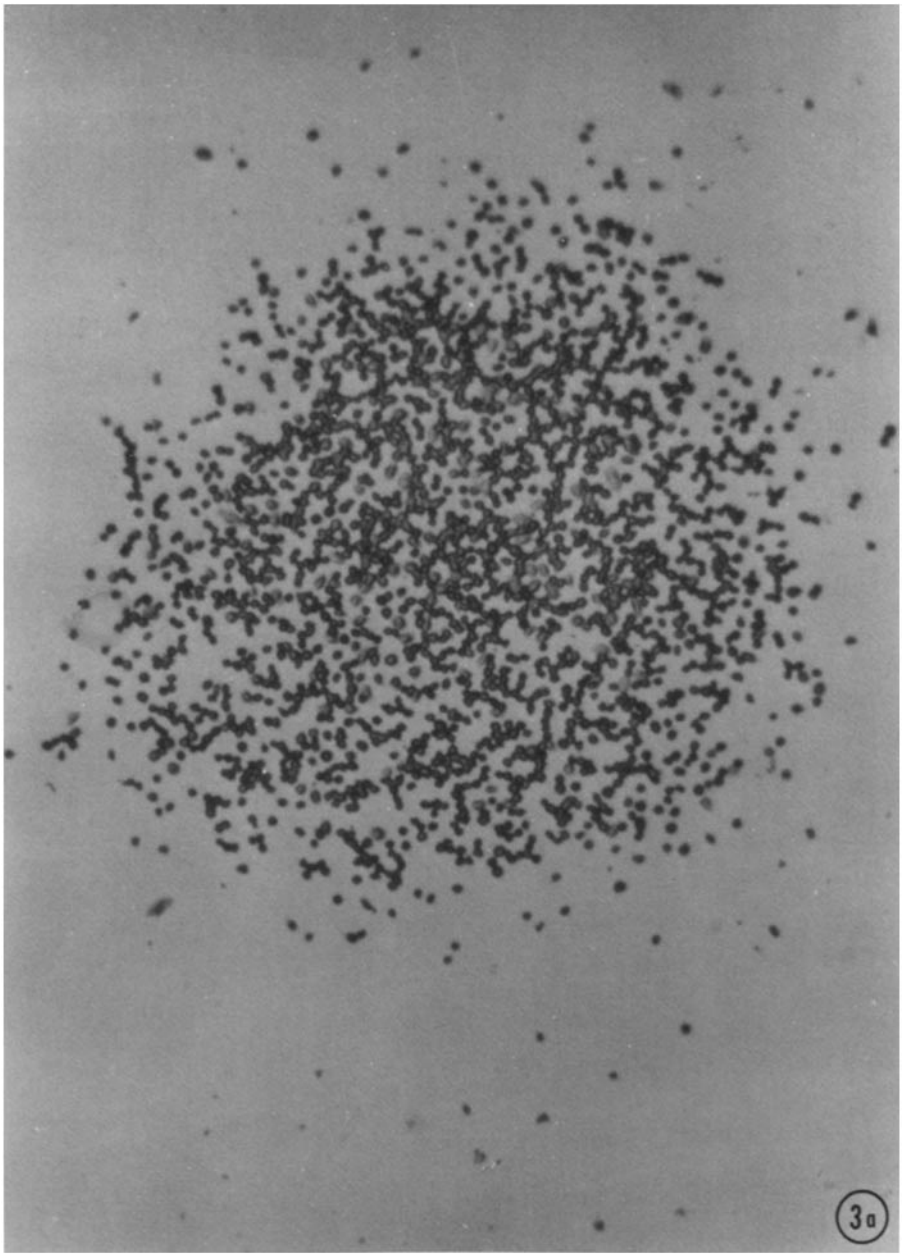

FIG. 3. Photomicrographs of colonies after 14 days culture. Cells were obtained 3 days after intraperitoneal thioglycollate medium injection and plated at 10^3 cells/culture. (*a*) Colony stained with methylene blue. (× 40).

Identification of Cells in Colonies. Cells in colonies were identified as macrophages by their ability to phagocytize yeast and sheep erythrocytes. Each day an assay was performed, cultures were incubated for 30 min with either yeast and C, or sheep erythrocytes pretreated with antiserum.

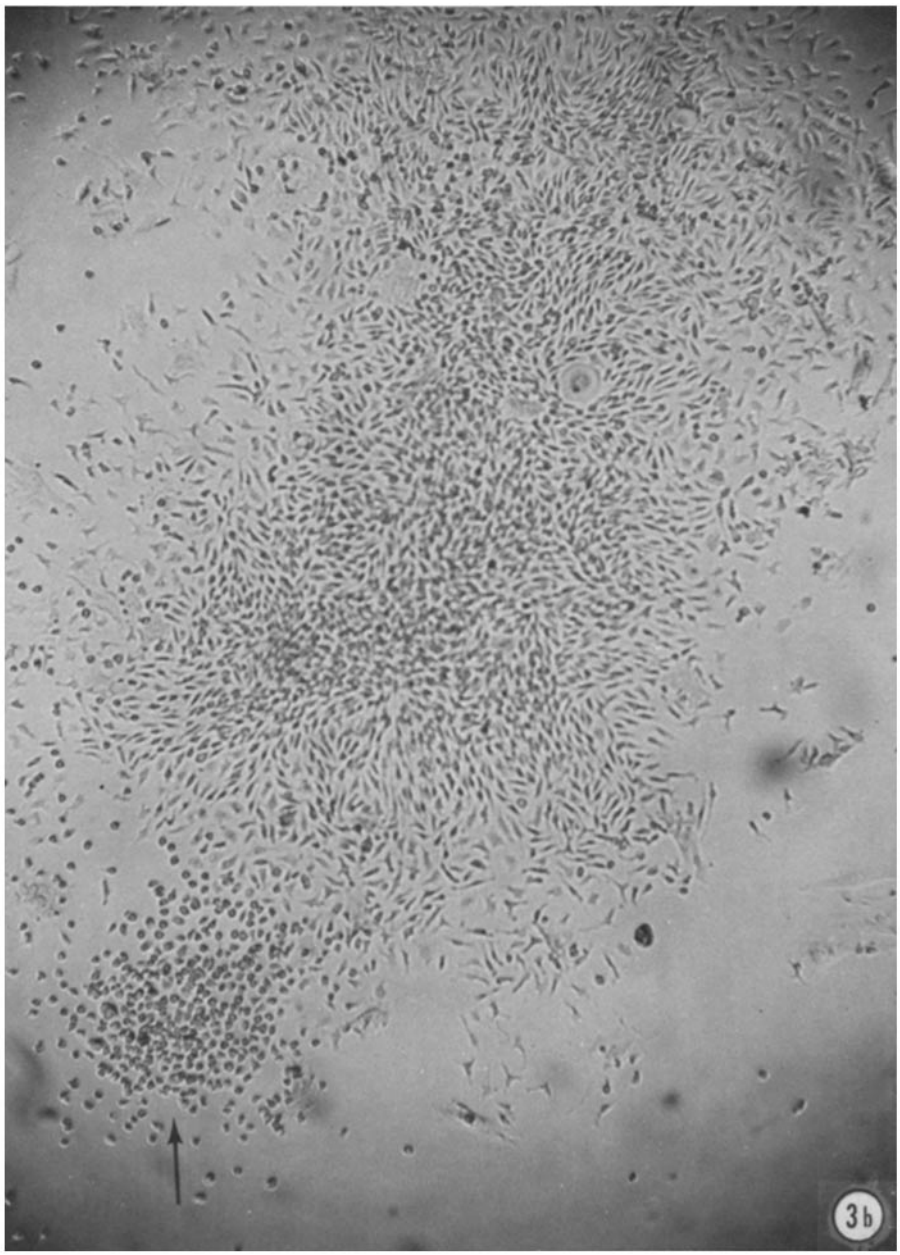

FIG. 3 *b*. See legend under Fig. 3 *a*. (*b*) Appearance of methylene blue stained fibroblast colony and macrophage colony (arrow) growing next to it. Notice the two different morphologies. (× 40).

Cells in colonies phagocytized yeast as shown in Fig. 3 *d*, and sheep erythrocytes as shown in Fig. 3 *e*. Multinucleated cells were also phagocytic. Accumulations of fibroblasts were rarely observed in culture (less than 1 fibroblast cluster/50 macrophage colonies). The fibroblasts were never found to

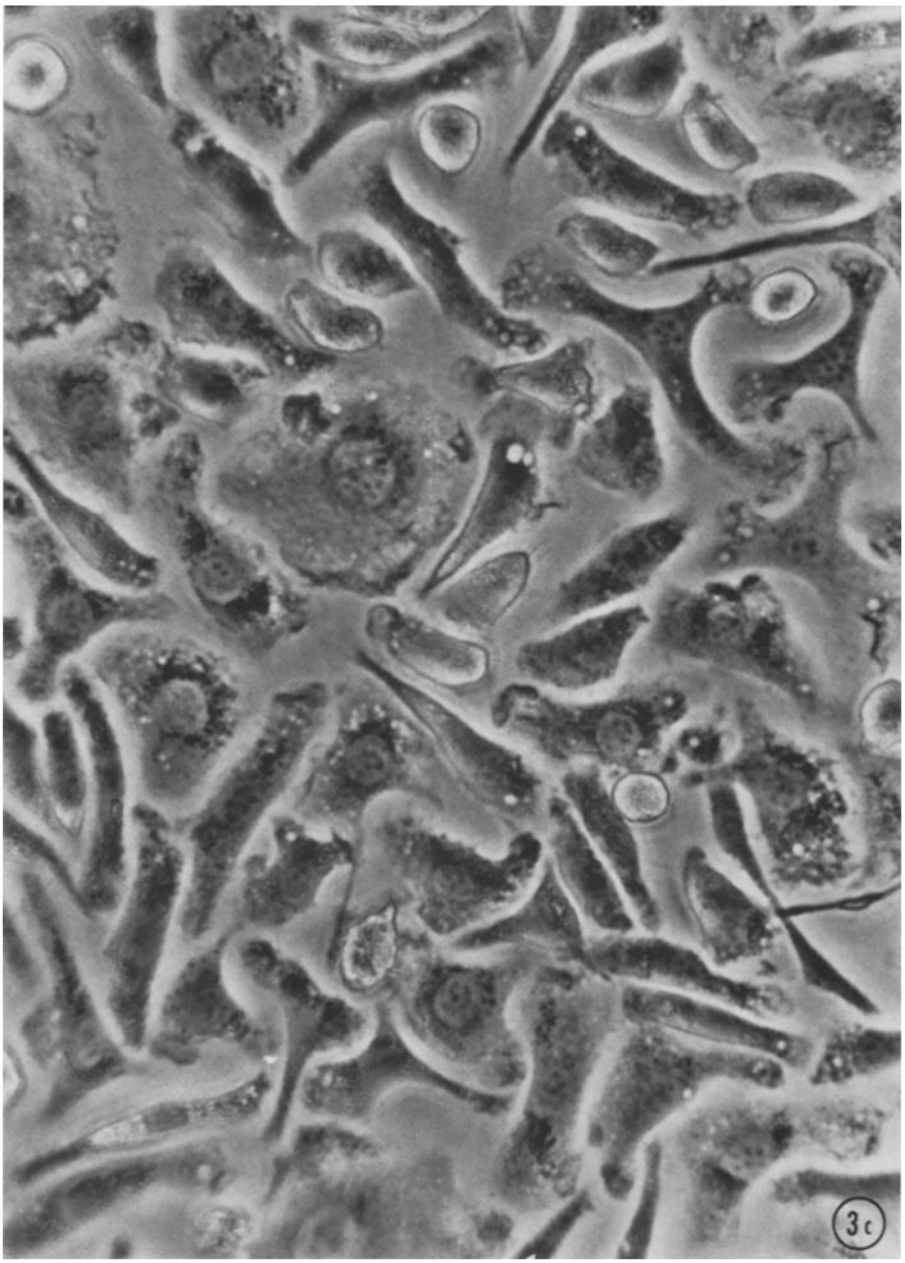

FIG. 3 *c*. See legend under Fig. 3 *a*. (*c*) Phase-contrast photomicrograph of cells within a colony. (× 200).

contain phagocytized particles, and they were morphologically different from the macrophages. It was not uncommon, however, to find macrophages mixed in with the fibroblasts. Mixed fibroblasts and macrophages were not scored as colonies. Colonies containing granulocytes have never been observed in these cultures.

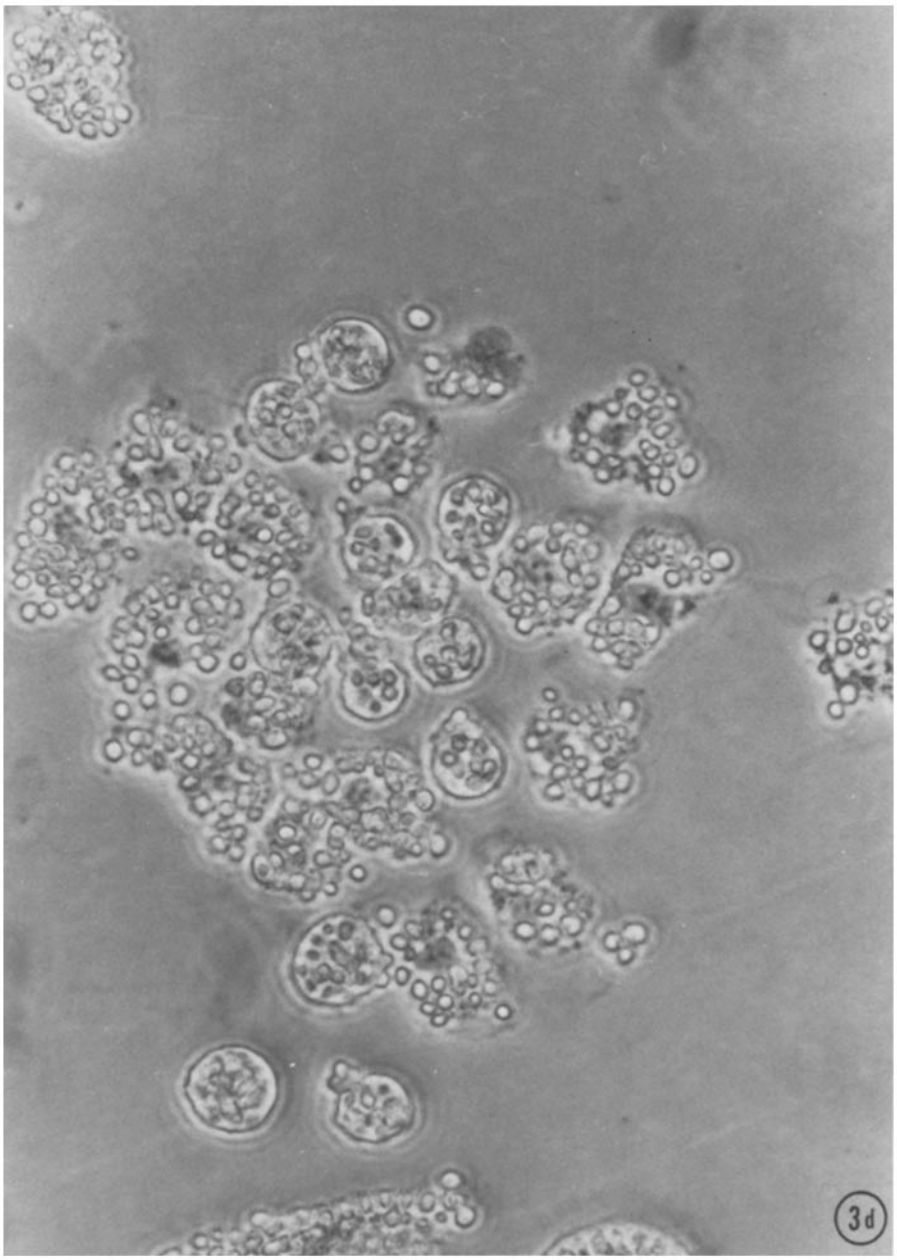

FIG. 3 *d*. See legend under Fig. 3 *a*. (*d*) Phase-contrast photomicrograph of a cell cluster after 1 wk of culture and treated 30 min with yeast and C. (× 200).

Survival of the Colony-Forming Precursor. Since colonies do not form unless conditioned medium is present, the survival of the colony-forming precursor in the absence of conditioned medium was estimated by adding the conditioned medium after various incubation times. 3-day old peritoneal exudate cells were plated at 1,000 cells/culture in medium containing 10% fetal calf serum and 5%

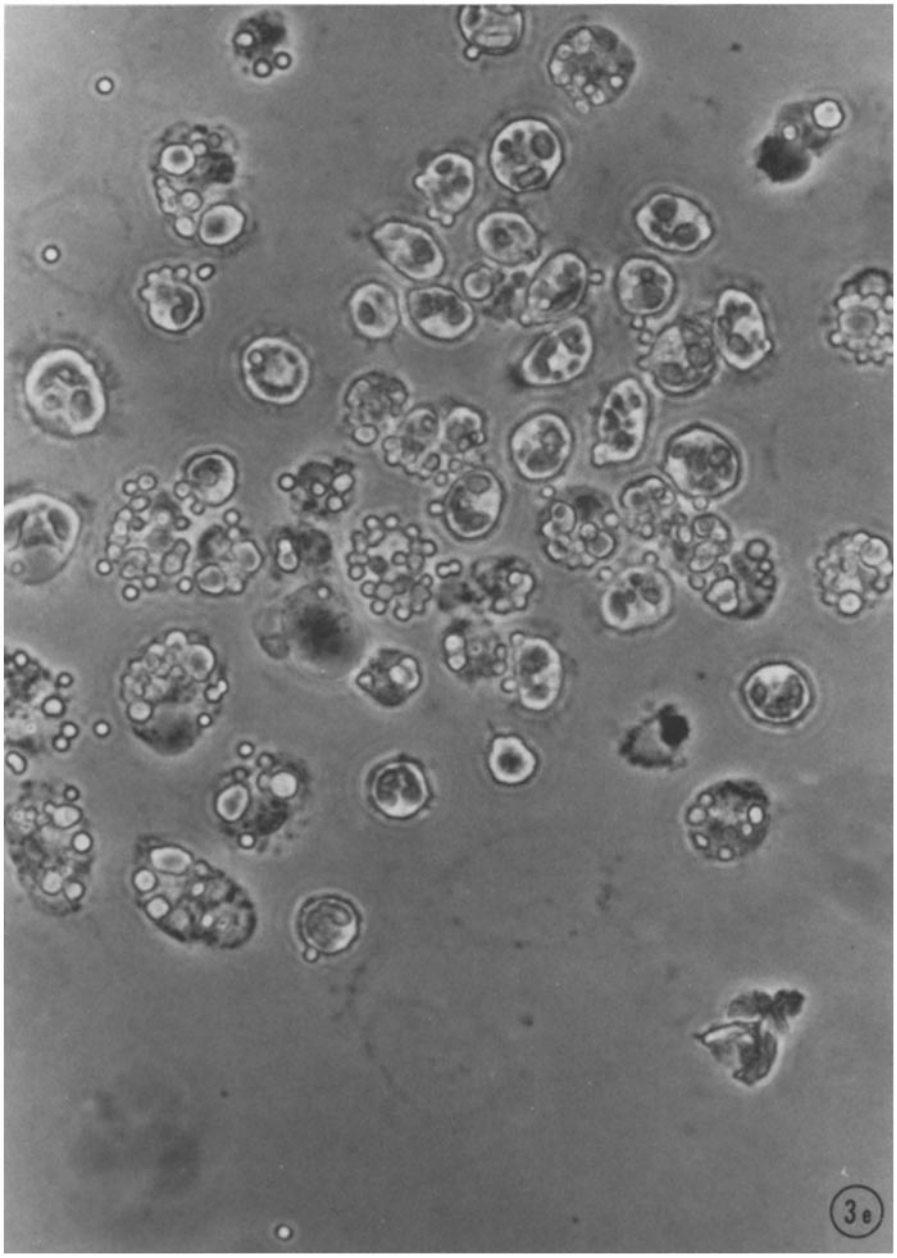

FIG. 3 *e*. See legend under Fig. 3 *a*. (*e*) Small colony at 2 wk incubated 30 min with sheep erythrocytes treated with specific antiserum. Note phagocytosis as well as rosetting. (× 200).

horse serum. At various times thereafter, L-cell-conditioned medium was added to appropriate cultures to make a final concentration of 10%. Colonies were then scored 2 wk after adding the conditioned medium. Control cultures contained no conditioned medium.

The results are shown in Table IV. It can be seen that the colony-forming

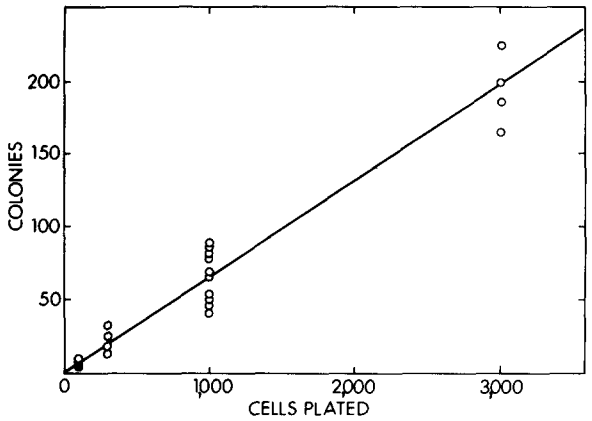

FIG. 4. Colonies formed as a function of cells plated. Each point represents a separate culture. Average plating efficiency for this study was 6.6%.

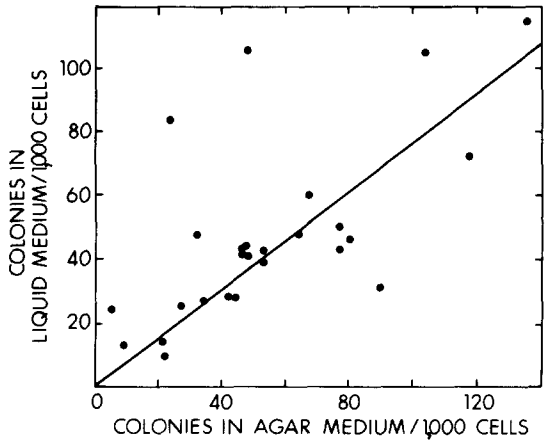

FIG. 5. Colony formation in agar and liquid medium. Exudate cells were cultured at 1,000 cells/culture in growth medium with or without agar.

precursors exhibit a good survival for at least 14 days in the absence of conditioned medium. There was a slight increase in colonies formed in cultures without conditioned medium at 28 days. This might be due to an ability of the exudate cells to produce a limited amount of colony-stimulating factor as suggested by the results of Table III.

Appearance of Colony-Forming Cells in the Peritoneal Cavity After Stimulation by Thioglycollate Medium. Groups of mice were injected with thioglycollate medium, and various times thereafter, exudate cells were prepared at 1,000 cells/culture. After 14 days, cultures were washed and stained, and the number of colonies counted. These data are shown in Table V. It can be seen that colony-forming cells increased slightly over the sample from unstimulated mice after 4 h. By 8 h, they had significantly increased in number and by day 3 the maximum number of colony-forming cells were found. Although intervals between 10 and 32 days were not tested, by 32 days prestimulation values had

again been achieved. During the initial phases of the inflammatory response, the colony-forming cells might be diluted by the infiltrating PMN leukocytes. Thus, the number of colony-forming cells per peritoneal washing was calculated and the data are also shown in Table V. The absolute number of colony-forming cells in

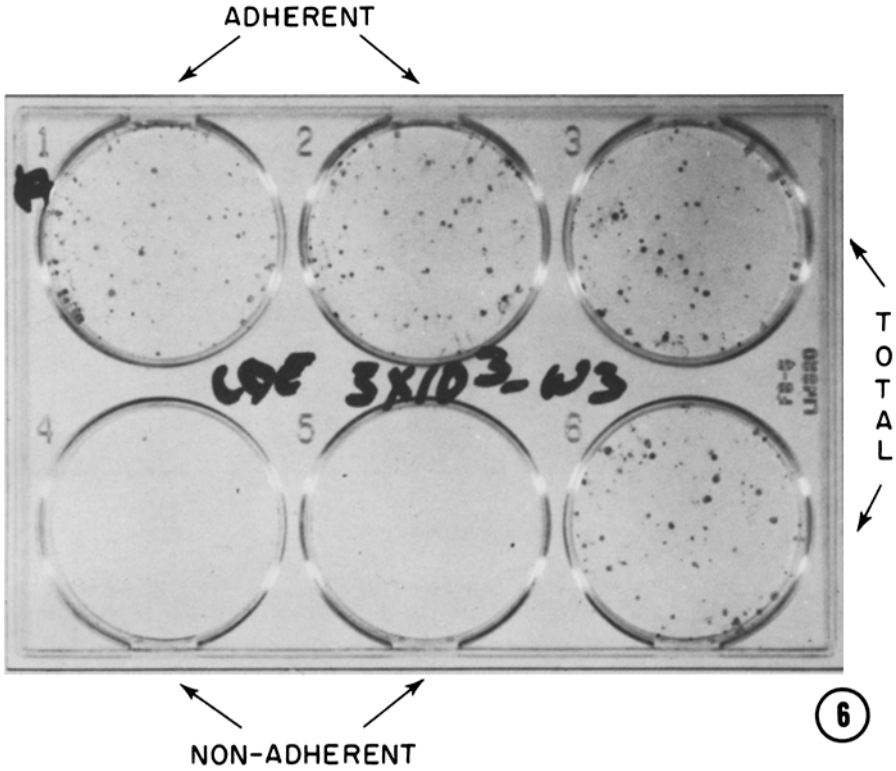

FIG. 6. Colony formation by adherent cells. Exudate cells were cultured at 3,000 cells/plate. After 2 h, the dishes labeled "adherent" were vigorously washed with medium and the cells were transferred to the plates labeled "non-adherent". After the cultures were incubated for 14 days, the growth medium was removed and the plates were stained with methylene blue.

TABLE IV
Survival of Colony-Forming Cells In Vitro in the Absence of L-Cell-Conditioned Medium

Day LCM added	Day assay	Colonies/1,000 exudate cells	Controls‡
0	14	68 ± 5*	0
1	15	41 ± 8	0
4	18	38 ± 7	0.5
7	21	47 ± 7	1
14	28	54 ± 8	6

* Standard error.
‡ Cells were cultured in α-MEM containing 10% FCS and 5% HS only.

TABLE V
Appearance of Colony-Forming Cells (CFC) after Stimulation by Thioglycollate Medium

Time post-thioglycollate medium	Cells per peritoneal washing	Colonies per 1,000 cells*		Total CFC‡ per peritoneal washing
		Mean	Range	
Hours				
0	1×10^6	0.5	0–1	500
2	1×10^6	0.5	0–1	500
4	1.2×10^6	1	0–2	1,200
8	1.3×10^6	14	10–18	18,200
16	1.7×10^6	40	36–44	68,000
24	7.5×10^6	17	25–27	127,500
Days				
2	7.5×10^6	30.7	11–84	230,250
3	6.8×10^6	82.4	42–127	557,600
4	5.3×10^6	40.1	27–55	212,530
6	3.1×10^6	45	42–44	138,500
8	2.4×10^6	38	37–40	91,200
10	2.2×10^6	36	36–37	79,200
32	1×10^6	1	0–1	1,000
44	1×10^6	3	2–4	3,000

* Determined after 14 days of culture.
‡ Total CFC = (no. colonies × no. cells/exudate)/1,000.

the exudate continuously increased after a latent period of 4 h after injection of thioglycollate medium.

Discussion

The results of these experiments show that peritoneal exudate cells obtained after thioglycollate medium injection will proliferate in the presence of L-cell-conditioned medium. The increase in cellularity was found to be due to the proliferation of the adherent cell fraction. This is in good agreement with the finding by Virolainen and Defendi who demonstrated that "macrophages" (adherent cells) proliferate in vitro in the presence of L-cell-conditioned medium (28). They also report the appearance of a relatively high percentage (20%) of multinucleated cells which is consistent with our findings.

The extent of the increase in cellularity was also found to be dependent upon the initial cell density and concentration. Since essential nutrients are depleted very quickly by exudate cells (24) it seems reasonable to believe that a sustained proliferative response would be increasingly compromised as the cell density and cell concentration are increased. This may account for the decrease in the time an exponential increase in cellularity was noted as the cell concentration was increased. At 2×10^3 cells/culture, nutrient depletion would not express itself in the early days of culture, but by day 18 the increased cellularity noted could also have resulted in sufficient nutrient depletion and cells enter a plateau phase even though they may not be confluent. Alternatively, cell proliferation may stop either because they became contact inhibited or the precursors are capable of

only a limited number of divisions. Further work on these possibilities are in progress.

The results also show that medium conditioned by L cells is necessary for exudate cell proliferation. Medium obtained from cultures of peritoneal exudates also showed some stimulating activity suggesting that some cells in the exudate might be capable of producing the same factor. The addition of conditioned medium alone without additional serum supplements was insufficient to produce an increase in cellularity even though its addition to α-MEM would have produced a final concentration of 1% serum. Thus, the serum supplements were also important for exudate cell proliferation. As shown in Table III, however, the number of exudate cells can be maintained for at least 2 wk in the absence of serum.

The results of this study indicate that peritoneal exudate cells obtained after thioglycollate medium injection are capable of forming colonies in liquid medium while cells from peritoneal lavages of normal mice (unstimulated) only rarely form colonies. In addition to morphology, three functional criteria identified the cells in colonies as macrophages: glass adherence (6, 29), phagocytosis promoted by C and opsonin (30–32), and the presence of surface gamma globulin receptors (33–35).

The results for the colony-forming cells reported here show some similarities and some differences with those previously reported for the colony-forming cell in agar (22). To demonstrate colonies using either liquid medium or medium containing agar, the addition of L-cell-conditioned medium is necessary and cells from the stimulated peritoneum are required. Both show a linear increase in colonies formed as a function of cells plated and the number of colonies formed per 1,000 exudate cells seeded are also nearly identical. The time for colonies to reach visible size, however, are different. Whereas colonies are clearly visible after 10 days when cultured in liquid medium, 15–20 days are required when agar medium is used. The longer time required to see colonies in agar may be due to a longer initial lag period before proliferation begins. The survival of the precursor cell in the absence of L-cell-conditioned medium also appears to be considerably better in liquid medium than in agar medium (22). Whether these differences are due merely to the growth conditions or to the proliferation of two separate precursor cells is not yet known.

The data suggest that both stimulation of the peritoneal cavity and L-cell-conditioned medium are essential to demonstrate the presence of colony-forming precursors. The inflammatory-inducing agent may act to trigger resident cells or to attract the precursors to the peritoneum. Once being activated or migrating to the peritoneal cavity, they can be stimulated to divide by a factor present in the L-cell-conditioned medium. It is not possible from this study to determine if colony-forming cells are resident in the peritoneum or migrate to it after stimulation.

We conclude that the precursor cell for macrophages can be activated or attracted to the peritoneal cavity by an inflammatory stimulus to produce individual colonies when grown at low cell density in conditioned medium. These colonies may provide cells which can be used to study macrophage function. Since it would be expected that progeny within the clone would be devoid of all

exogenously bound substances such as cytophilic antibody, they may also provide a convenient source of macrophages to study other macrophage functions such as gamma globulin receptors, antigen-binding capabilities, and other defense functions. Furthermore, the system provides a model for studying the proliferation and differentiation kinetics of non-neoplastic cells which exhibit an easily quantitatable function.

Summary

Peritoneal exudate cells, obtained from mice injected with thioglycollate medium and cultured in medium containing L-cell-conditioned medium will proliferate in an exponential fashion for 18 days with a doubling time of 68 h. After a 2 h pulse of tritiated thymidine, labeled adherent cells increased to a maximum of 22–34% during the 1st and 2nd wk of culture. Increasing the cell concentration from 2×10^3 to 2×10^5 cells/culture reduced exponential growth to 10 days and the doubling time was increased to 81.6 h.

Under these culture conditions, peritoneal exudate cells were shown to form colonies on the surface of culture dishes when plated at low density. The cells within the colony were shown to be macrophages using yeast and antibody-coated sheep erythrocytes as a test for phagocytic function. The plating efficiency ranged between 4 and 12% of the total viable cells plated and it appears that the colonies arose from a single precursor cell. The adherent cell population contains the colony-forming precursors. These precursors can be stimulated to form colonies for at least 2 wk by the addition of conditioned medium to cultures at various times after plating. While very few colony-forming cells could be demonstrated in the unstimulated peritoneal lavage, their numbers begin to increase in the exudate 4 h after injection of thioglycollate medium and reach a maximum by day 3 and then decrease. Isolated colonies may be useful in studying the function of macrophages.

Received for publication 18 November 1974.

References

1. Smith, T. J., and R. R. Wagner. 1967. Rabbit macrophage interferon. I. Conditions for biosynthesis by virus-infected and uninfected cells. *J. Exp. Med.* **125**:559.
2. Kono, Y. 1967. Rapid production of interferon in bovine leukocyte culture. *Proc. Soc. Exp. Biol. Med.* **124**:155.
3. Stecher, V. J. 1970. Synthesis of proteins by mononuclear phagocytes. *In* Mononuclear Phagocytes. Ralph van Furth, editor, F. A. Davis Co., Philadelphia, Pa. 133.
4. Rabinovitch, M. 1970. Phagocytic recognition. *In* Mononuclear Phagocytes. Ralph van Furth, editor, F. A. Davis Co. Philadelphia, Pa. 299.
5. Whaley, K., and H. Singh. 1973. *In vitro* studies on the phagocytosis of *Staphylococcus aureus* by peritoneal macrophages of New Zealand mice. *Immunology.* **24**:25.
6. Stubles, M., A. V. Kuhner, E. A. Glass, J. R. David, and M. L. Karnovsky. 1973. Metabolic and functional studies on activated mouse macrophages. *J. Exp. Med.* **137**:537.
7. Ada, G. L., C. R. Parish, G. J. V. Nossal, and A. Abbot. 1967. The tissue localization, immunogenic, and tolerance inducing properties of antigens and antigenic fragments. *Cold Spring Harbor Symp. Quant. Biol.* **32**:381.

8. Uhr, J. W., and G. Weissmann. 1968. The sequestration of antigens in lysosomes. *J. Reticuloendothel. Soc.* **5**:243.
9. North, R. J. 1968. The uptake of particulate antigens. *J. Reticuloendothel. Soc.* **5**:243.
10. Argyris, B. F. 1968. Role of macrophages in immunological maturation. *J. Exp. Med.* **128**:459.
11. Mitchison, N. A. 1969. The immunogenic capacity of antigen taken up by peritoneal exudate cells. *Immunology.* **16**:1.
12. McDevitt, H. O. 1968. The cellular localization of antigen. *J. Reticuloendothel. Soc.* **5**:256.
13. Campbell, D. H., and J. S. Garvey. 1968. Nature of retained antigen and its role in immune mechanisms. *Adv. Immunol.* **3**:261.
14. Gottlieb, A. A. 1968. Antigens, RNA's and macrophages. *J. Reticuloendothel. Soc.* **5**:270.
15. Friedman, H., editor. 1973. RNA in the immune response. *Ann. N. Y. Acad. Sci.* **207**:1.
16. Evans, R., and P. Alexander. 1972. Role of macrophages in tumour immunity. I. Co-operation between macrophages and lymphoid cells in syngeneic tumour immunity. *Immunology.* **23**:615.
17. Evans, R., and P. Alexander. 1972. Role of macrophages in tumour immunity. II. Involvement of a macrophage cytophilic factor during syngeneic tumour growth inhibition. *Immunology.* **23**:627.
18. Kramer, J. J., and G. A. Granger. 1972. The *in vitro* induction and release of a cell toxin by immune C57Bl/6 mouse peritoneal macrophages. *Cell. Immunol.* **3**:88.
19. Spector, W. G. 1969. The granulomatous inflammatory exudate. *Int. Rev. Exp. Pathol.* **8**:1.
20. Donald, K. J. 1972. The mechanism of enhanced clearance of colloidal carbon from the blood of rabbits stimulated with a tubercule bacillary lipid. *J. Pathol.* **198**:97.
21. Lin, H., and C. C. Stewart. 1973. Colony formation by mouse peritoneal exudate cells *in vitro*. *Nat. New Biol.* **243**:176.
22. Lin, H., and C. C. Stewart. 1974. Peritoneal exudate cells. I. Growth requirements of cells capable of forming colonies in soft agar. *J. Cell. Physiol.* **83**:369.
23. Lin, H. 1974. Peritoneal exudate cells. II. Kinetics of appearance of colony-forming cells. *J. Cell. Physiol.* **84**:159.
24. Stewart, C. C. 1973. Nutrient utilization by peritoneal exudate cells. *J. Reticuloendothel. Soc.* **14**:322.
25. Stewart, C. C., S. F. Cramer, and P. G. Steward. 1975. The response of human blood lymphocytes to phytohemagglutinin: determination of cell numbers. *Cell. Immunol.* **15**:1.
26. Rosenthal, A. S., J. M. Davie, D. L. Rosenstreich, and J. T. Balke. 1971. Depletion of antibody-forming cells and their precursors from complex lymphoid cell populations. *J. Immunol.* **108**:269.
27. Slonecker, C. E. 1971. The cellular composition of an acute inflammatory exudate in rats. *J. Reticuloendothel. Soc.* **10**:269.
28. Virolainen, M., and V. Defendi. 1967. Dependence of macrophage growth *in vitro* upon interaction with other cell types. Growth regulating substances for animal cells in culture. *Wistar Inst. Symp. Monogr.* **7**:67.
29. Mackaness, G. B. 1964. The immunological basis of acquired cellular resistance. *J. Exp. Med.* **120**:105.
30. Filkins, J. P., and N. R. DiLuzio. 1969. Influence of opsonins and heparin on intravascular carbon clearance after endotoxin. *J. Reticuloendothel. Soc.* **6**:287.

31. Vaughn, R. B. 1965. The discriminative behavior of rabbit phagocytes. *Br. J. Exp. Pathol.* **46**:71.
32. Carpenter, R. R., and P. B. Barsales. 1967. Uptake of mononuclear phagocytes of protein coated bentonite particles stabilized with a carbonimide. *J. Immunol.* **98**:844.
33. Berben, H., and B. Benacerraf. 1968. Sedimentation properties of antibody cytophilic for macrophages. *J. Immunol.* **100**:1219.
34. Rabinovitch, M. 1968. Effect of antiserum on the attachment of modified erythrocytes to normal or trypsinized macrophages. *Proc. Soc. Exp. Biol. Med.* **127**:351.
35. Huber, H., and H. H. Fudenberg. 1968. Receptor sites of human monocytes for IgG. *Int. Arch. Allergy. Appl. Immunol.* **34**:18.