STUDIES ON ANTIBODY FORMATION BY PERITONEAL EXUDATE CELLS IN VITRO *

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Plates 48 to 52

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Previous studies of antibody formation *in vitro* carried out in this laboratory have utilized pieces of rabbit spleen as the synthesizing system (1, 2). Such a system had the disadvantages that antibody formation continued for only a few days and the cell type responsibile could not be determined. The system to be described utilized cells propagated in tissue culture and permitted more precise evaluation.

Materials and Methods

For the most part, the materials used were identical with those described earlier (2), and only variations will be recorded here.

Antigens.—(*a*) Bovine-γ-globulin (BGG), Armour's fraction II, lots P30008 and T30103. (*b*) Egg albumin (EA), twice recrystallized, Nutritional Biochemicals Co., Cleveland. (*c*) Casein, purified, Difco, Detroit. (*d*) Diphtheria toxoid, fluid and alum-precipitated, Merck Sharp & Dohme, Research Laboratories West Point, Pennsylvania, Lots 41901 and 51123, respectively.

Media.—TACPI is a complex amino acid–salt–vitamin mixture developed by Trowell. The material used here represents a modification made in this laboratory (1).

Mineral Oil.—Drakeol 350, Pennsylvania Refining Co., Butler, Pennsylvania.

Endotoxin.—Some of the purified lipopolysaccharide from *Salmonella typhosa*, prepared by the method of Webster *et al.* (3), was supplied by Dr. A. G. Johnson of the Department of Bacteriology, University of Michigan. The remainder was prepared in this laboratory from *S. typhosa* (strain 0-901) according to the method of Westphal *et al.* (4). Both preparations produced a 1.7°C. rise in body temperature of rabbits given 10 μg. intravenously and both produced similar enhancement of the antibody response *in vitro*.

Basic Procedure.—The procedure for obtaining the peritoneal exudate cells (PEC) was essentially that of Dixon *et al.* (5). Rabbits were injected intraperitoneally with 100 ml. of sterile mineral oil prewarmed to 37°C. Three days later the rabbits received an intraperitoneal

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injection of 300 ml. of sterile 0.15 M phosphate buffered saline at pH 7.3 (PBS). The animals were sacrificed by cardiac exsanguination or by a sharp blow to the base of the skull. The skin was removed from the torso, and the entire ventral body wall was swabbed with tincture of iodine followed by 70 per cent alcohol. A small midline incision was made just caudal to the xiphoid process and the peritoneal cavity opened aseptically. The milky fluid was withdrawn with a 50 ml. syringe fitted with a 15 gauge needle whose free end was fitted with a screen to prevent clogging. The aqueous and oily phases of the aspirated fluid were allowed to separate in a sterile separatory funnel at 1°C. for 45 minutes. Then the heavier aqueous phase was withdrawn and the cells deposited by centrifugation at 700 R.P.M. for 10 minutes at 4°C. The cells were resuspended in medium 199 and centrifuged at 700 R.P.M. for 3 minutes in a graduated tube. When the packed cell volume had been recorded, the cells were diluted appropriately in medium 199 and counted in a hemocytometer. There were approximately 5.3×10^6 packed cells/ml. Cells were diluted in the planting medium so as to number about

TABLE I

Differential Cell Counts of Peritoneal Exudate Cells Taken from Rabbits 3 Days after Heavy Mineral Oil Injection

Experiment No.	Monocytes*	Lymphocytes	Neutrophils
	per cent	*per cent*	*per cent*
175	71	27	2
180	68	31	1
215	78	19	3
220	75	20	5
225	71	21	8
230	75	19	6
241	80	20	0
245	73	20	7
253	73	23	4
254	76	19	5
Means	74	21.9	4.1

* Monocytes are defined as large mononuclear cells with horseshoe-shaped nuclei.

5×10^5/ml. Differential counts, made on 200 cells stained with Wright's stain, revealed approximately 74 per cent monocytes, 22 per cent lymphocytes, and 4 per cent neutrophils (Table I). These figures are in substantial agreement with Dixon *et al.* (5). Cells from animals which had various experimental treatments were counted but no differences in the differentials were noted. Since the lymphocytes and neutrophils did not attach to the glass in tissue culture, and were lost when the supernatant fluid was changed, the monocytes were considered as the cell under study and the terms peritoneal exudate cell and monocyte have been used interchangeably.

Tissue Culture Techniques.—Cells from the various sources to be described later were seeded and grown on glass at 37°C. in, (*a*) Smith bottles with a surface area of 72 $cm.^2$ in a fluid volume of 20 ml.; (*b*) 1 liter Blake bottles with a surface area of 180 $cm.^2$ in a fluid volume of 100 ml.; (*c*) 1 x 12 cm. roller tubes; (*d*) on 11 x 22 mm. coverslips in 1 x 7 cm. Leighton tubes. When roller tubes were used, the cells were planted in 1 ml. of medium and the tubes were incubated in Drummond racks for 48 to 72 hours. After this initial period of incubation, the medium was replaced with 2 ml. of fresh medium, and the tubes were rotated at 15 R.P.H. in a roller drum.

Antibody assays were carried out on freshly harvested fluids with tanned formalinized sheep

red cells as previously described (2). The cells were coated with BGG unless otherwise noted. The erythrocytes used during these experiments gave the sharpest end points after 2 hours at room temperature and were read at that time instead of following overnight incubation at 1°C. The standard diluent (SD) was 1 per cent normal rabbit serum (inactivated) in 0.9 per cent saline. Cell controls consisted of cells in SD as the negative control and cells in antisera to BGG or EA as the positive controls. These antisera each had a mean titer of 12,800 during the course of the experiments. All hemagglutination titers (HA) are reported as the reciprocal of the highest tissue culture fluid dilution which showed complete agglutination. Hemaglutination inhibition tests were carried out by dissolving 0.025 mg./ml. of BGG or casein or 0.1 mg./ml. of EA in SD and using these as the diluents. The sensitized cells were added immediately after serial dilutions were made; prior incubation for 1 hour as previously recommended (2) was not required.

EXPERIMENTAL

Preliminary Experiments.—The early experiments were designed to determine the most suitable cell type for culture, the best medium, and the best route for administration of the antigens.

Rabbits 2-77 and 2-78 each were given 100 ml. of sterile mineral oil intraperitoneally. Two days later each rabbit received 10 μg. of endotoxin intravenously. Rabbit 2-77 received 40 mg. of alum-precipitated bovine-γ-globulin (AP-BGG) intravenously, while rabbit 2-78 received the same antigenic dose intraperitoneally, 6½ hours after the endotoxin. Each animal received an injection of 300 ml. phosphate buffered saline (PBS) intraperitoneally 18 hours after the antigen. The peritoneal exudates and the spleens were removed under sterile conditions. The spleens were diced in Hanks' solution, mildly agitated in 0.25 per cent trypsin in Hanks' solution for 3 hours at room temperature, and washed. Splenic cells and PEC were planted at 5×10^5/ml. in stationary roller tubes in various media supplemented with 30 per cent normal rabbit serum (NRS). The serum used in culture media was not inactivated. Media were changed every 3 days and the pooled culture fluids assayed for antibody.

The results, given in Table II, show that antibody synthesis occurred in both splenic cells and monocytes through the 7th day in all media. After this time, Hanks' solution was inadequate to support any cellular proliferation despite the presence of 30 per cent NRS. In addition, splenic cells, although replicating, seemed no longer capable of antibody formation after the 9th day. However, TACPI and medium 199 permitted longer cell survival and prolonged synthesis of antibody. Both monocytes and splenic cells from the animal that received the antigen intravenously produced higher titered antibody than did cells from the intraperitoneally injected animal. Normal monocytes did not form antibody to BGG.

The ability of peritoneal exudate cells, trypsinized splenic cells, and bone marrow cells to form antibodies after secondary antigenic stimulation[1] was tested in the following experiment.

[1] In this paper the following definitions are used: A *primary* antibody response was that obtained after a single injection of the specific antigen, usually in a 40 mg. dose. A *secondary* antibody response was that obtained after the second injection of specific antigen 1 to 2 months after the first. The first dose was usually 40 mg. the second 4 mg. A *hyperimmune* antibody response was that obtained after multiple doses over a 3 week period.

Rabbit 2-85 was injected with 30 mg. of AP-BGG and 6 weeks later was given a secondary injection of 10 mg. intravenously. Three days later the animal was given 100 ml. of mineral oil intraperitoneally. The animal was sacrificed 3 days after the injection of oil, and the cells from the three sources were maintained in medium 199 70 per cent:NRS 30 per cent.

It may be seen from the curves in Text-fig. 1 that cells from all three sources produced antibody for at least 6 days. Splenic cells were passed successfully 4 times before dying. Monocytes in the 3rd pass were lost to contamination, while bone marrow cells did not pass. The splenic cells and PEC produced antibody over a period of 24 days.

TABLE II

Hemagglutination (HA) Titers in Culture Fluids of Cells Grown in Various Media, Supplemented with 30 Per Cent Normal Rabbit Serum.

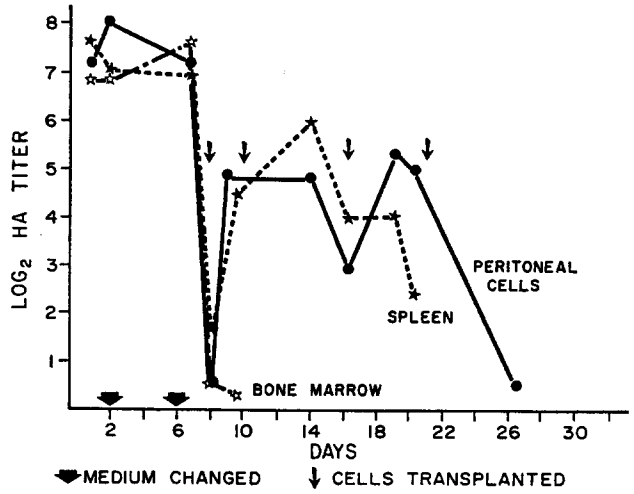
Time in culture	No antigen	Antigen intravenously						Antigen intraperitoneally					
	Monocytes	Spleen			Monocytes			Spleen			Monocytes		
Medium	199	TACPI	199	Hanks'	TACPI	199	Hanks'	TACPI	199	Hanks'	TACPI	199	Hanks'
days													
2	2	32	32	32	64	32	32	16	16	8	16	8	8
3	<2	32	16	16	32	32	16	16	8	8	16	16	16
7	2	64	32	16	32	32	32	8	16	8	8	16	8
9	2	32	16	2*	64	64	4*	8	16	2*	16	8	4*
11	<2	<2	<2	—	64	64	—	<2	<2	—	16	2	—
14	4	2	<2	—	64	32	—	<2	2	—	<2	<2	—
21	<2	<2	<2	—	16	32	—	<2	<2	—	<2	<2	—

* Cells dead.

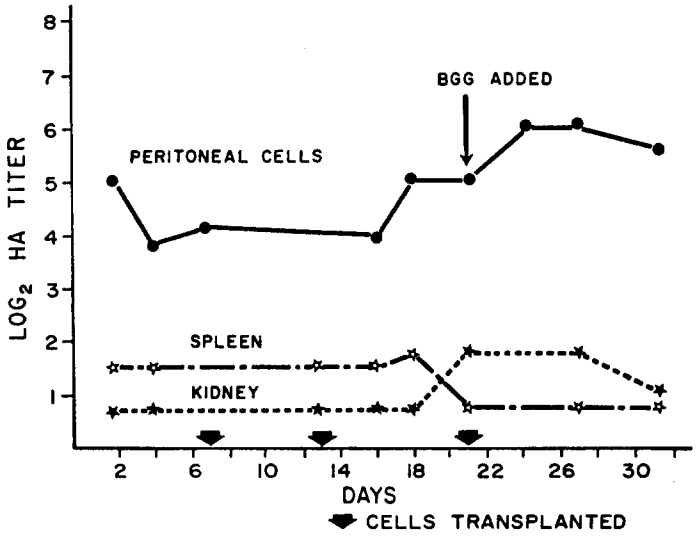
In another experiment, rabbit 3-22 was sacrificed 4 days after a single intravenous injection of 40 mg. of AP-BGG and 3 days after the injection of oil. The spleen and right kidney were diced and mildly agitated in 0.25 per cent trypsin in Hanks' solution at 1°C. overnight. The entire kidney was used to obtain fibroblastic outgrowth since cortical tissue alone gives rise primarily to epithelial cells in tissue culture. The splenic cells, kidney cells; and PEC were seeded at 5×10^5/ml. in medium 199 70 per cent:NRS 30 per cent.

The antibody-producing capacities of these cells may be seen on inspection of Text-fig. 2. Once again the PEC produced antibody through 3 passages over 30 days. However, the addition of BGG to the cultures on the 21st day did not result in an increase in antibody. It is felt that the failure of the splenic cells was due to prolonged contact with trypsin, even though prolonged trypsinization gave a higher cell yield.

The cells in the early experiments were grown in various sized containers,—from roller tubes with 0.5 ml. of medium through 1 liter Blake bottles with 100 ml. of medium. Results indicated that the most consistent growth with far less bacterial contamination was obtained in roller tubes.



TEXT-FIG. 1. Formation of antibody *in vitro* by three tissues from a secondary rabbit.



TEXT-FIG. 2. Formation of antibody *in vitro* by three tissues from a primary rabbit.

It became evident that Hanks' solution was not adequate for cellular growth, but that either TACPI or medium 199 was suitable. The more ready availability of medium 199 indicated its use in the system.

It was also necessary to establish the role of serum in cellular growth and in antibody synthesis. Two rabbits were used to study the effects of serum and tissue extracts on the secondary response of the PEC, and two were used to study the primary response.

Preparation of Tissue Extracts.—16 normal rabbits were exsanguinated and the spleens and livers were removed. The organs were homogenized as a 50% W/V suspension in Hanks' solution in a high speed Waring blendor for 15 minutes at 4°C. The tissue homogenates were clarified at 5000 R.P.M. for ½ hour and finally at 15,000 R.P.M. for 1 hour at 1°C. Each supernate was filtered through a sintered glass UF filter and stored at −40°C.

For studies of the secondary response, rabbits 2-85 and 2-86 each were injected intravenously with 10 mg. of AP-BGG. The rabbits were sacrificed 5 days after the antigen injection and 3 days after the oil injection.

TABLE III

Geometric Mean HA Titers (GMT) in Culture Fluids of Peritoneal Exudate Cells From Secondary Rabbits Grown in Various Supplements

Medium	HA titer on day				
	1	2	7	8	14
199 and 30 per cent NRS	32	24	32	32	64
199 and 10 per cent NRS	8	2	<2	<2	<2
199 and 5 per cent NRS	4	<2	<2	<2	<2
199 and 5 per cent NRS and 5 per cent liver extract	32	Dead			
199 and 5 per cent NRS and 5 per cent spleen extract	64	32	23	16	32

TABLE IV

GMT in Culture Fluids of Peritoneal Exudate Cells from Primary Rabbits Grown in Various Supplements

Medium	HA titer on day						
	1	3	5	7	9	14	17
199 and 25 per cent NRS	64	48	8	32	24	32	16
199 and 5 per cent NRS	8	4	2	<2	2	<2	<2
199 and 5 per cent NRS and 5 per cent spleen extract	32	32	8	6	2	<2	<2

Geometric mean titers (GMT) of antibody produced are given in Table III.

Rabbits 5 and 2-0, used for the primary response studies, were sacrificed 1 week after a single intravenous injection of 40 mg. of AP-BGG and 4 days after the injection of the mineral oil. Geometric mean titers for this experiment are given in Table IV.

On examination of Table III, it may be see that medium 199 supplemented with 5 per cent NRS and 5 per cent spleen extract proved as good a medium as medium 199 supplemented with 30 per cent NRS for antibody synthesis. In contrast, 5 per cent liver extract was toxic to the cells. Serum concentrations of 10 per cent and 5 per cent did not admit of antibody formation, although the cells multiplied, albeit at a slower rate.

It may be seen from the data in Table IV that medium 199 supplemented with 25 per cent NRS proved to be the most suitable medium for antibody

synthesis by the PEC, in a primary response to antigenic stimulation. Again 5 per cent NRS in medium 199 was not an adequate medium for antibody formation.

The medium in both these sets of experiments were changed every 2nd day, at which time assays for antibody were carried out on the pooled supernates. The data show a net synthesis of antibody in the high serum media, and approximately the same synthesis in media containing spleen extract.

As a result of these preliminary experiments, it was decided: (*a*) to use peritoneal exudate cells exclusively for subsequent experiments, (*b*) to use medium 199 supplemented with 25 per cent fresh frozen NRS as the medium of choice both for cellular replication and antibody synthesis, (*c*) to use roller tube cultures for ease of handling, since no differences were found among various sized vessels, and (*d*) to use the intravenous route for the administration of antigens, since somewhat higher titers were obtained by the use of this route (Table II).

Antibody Responses of Monocytes from Primary, Secondary and Hyperimmune Rabbits and from Normal Rabbits Exposed to BGG in Vitro.—The next three series of experiments to be described compare antibody synthesis in tissue culture by PEC taken from primary, secondary, and normal animals.

For the primary response studies, rabbits 2-77, 5, 3-22, 3-23, and 3-61, each were given 40 mg. of AP-BGG intravenously and sacrificed 4 days after the antigen injection. In the secondary response studies, rabbits 2-85, 2-86, 3-31, and 2-49, each were given 4 mg. of AP-BGG intravenously 6 weeks after a 40 mg. dose of the same antigen injected by the same route. These animals were sacrificed 4 days after the secondary antigenic injection. Rabbits 2-57, 3-60, 3-74, 3-75, 14, and 3-77, which had had no previous experience with BGG, were used for the completely *in vitro* response studies. All animals received 100 ml. of sterile mineral oil intraperitoneally 3 days prior to sacrifice.

As an antigen control for the experiments initiated *in vivo*, 4 rabbits received 4 mg. of alum-precipitated casein intravenously 6 weeks after a primary intravenous dose of 40 mg. of the same antigen.

For the antibody responses initiated *in vitro*, the experiments were controlled as follows. When the cells had been harvested from the peritoneal cavities, ½ was incubated for 1 hour at 37°C. in medium 199 in which was dissolved 1 mg./ml. of BGG. The other ½ of the cells was incubated in a similar manner with 1 mg./ml. of casein. Casein was chosen as the control antigen since it showed the least serologic cross-reaction in the titration system.

The cell control consisted of trypsinized kidney cells taken from animals in the secondary response experiments only. Kidneys from 4 of the 5 animals were used. The kidneys were chosen in preference to other organs due to relative ease of culturing the cells, and since one would not expect antibody synthesis in kidney cells. It was also reasonable to select tissues from the secondary series of animals since these animals might be expected to produce more antibody than animals from either of the other systems.

All cells were planted in medium 199 75 per cent: NRS 25 per cent in roller tubes. The medium was changed every 2nd. day at which time antibody determinations were carried out on the pooled culture fluids. The cells were transplanted when cell counts revealed cells in excess of 1.5×10^6/ml.

The results of the 16 experiments are given in Tables V, VI, and VII. All titrations were done with BGG coated erythrocytes. It may be noted that in any given system, the antibody titers did not vary significantly over the course of the experiment until the precipitous fall in titers when the cells appeared no longer capable of synthesizing antibody.

The data for the series of experiments are summarized in Table VIII. Geometric mean titers (GMT) were calculated from the individual titers of each experiment including all titers greater than 8. The over-all mean hemaggluti-

TABLE V

HA Titers of Culture Fluids of Peritoneal Exudate Cells Taken from Primary Rabbits

Cell type and antigen	Exp. No.	No. subpasses	Days in culture											
			2	4	6	8	10	12	14	16	18	20	22	24
PEC* with BGG	175	2	64 (2)	32 (2)	32 (<2)	ND‡	ND	2 (<2)						
	220	2	64 (2)	8 (<2)	8 (2)	16 (2)	32 (2)	ND	16 (<2)	8 (2)	16 (<2)	4 (2)	2 (<2)	2 (2)
	225	3	32 (<2)	16 (2)	16 (2)	16 (2)	16 (<2)	ND	32 (<2)	16 (<2)	32 (2)	ND	32 (<2)	8 (2)
	230	1	64 (2)	32 (2)	32 (2)	ND	ND	64 (<2)	128 (2)	64 (<2)	64 (<2)	ND	16 (2)	2 (2)
	254	2	16 (<2)	32 (<2)	32 (2)	32 (2)	32 (2)	32 (<2)	32 (<2)	16 (2)	ND	2 (2)		
PEC with casein	220	2	2	<2	4	4	4	ND	2	<2	2	4	<2	2
	225	3	2	<2	2	4	<2	ND	2	4	4	ND	4	2
	230	1	<2	4	2	ND	ND	<2	<2	<2	4	ND	<2	2
	254	2	2	<2	2	4	<2	2	2	4	ND	<2		

Figures in parentheses indicate hemagglutination inhibition titers using BGG in the diluent.

* PEC, peritoneal exudate cells.

‡ ND not done.

nating titers in tissue cultures of cells from rabbits which received either primary or secondary injections of BGG were essentially the same, *i.e.* 30 and 32, respectively, and the antibody persisted in these two systems for essentially the same time; *i.e.*, 21 and 23 days, respectively.

The mean titer for the cells exposed to BGG *in vitro* was 28, and the antibody persisted for an average of 13 days. Hence, the only difference between the completely *in vitro* system and the other two seemed to be the duration of the response. The number of subpasses which the cells could undergo was not related to the source of the cells. The cells were passed only as long as antibody was being formed.

It was felt probable that cells from hyperimmunized rabbits would produce

more antibody than cells in any of the systems described above. It was also desirable to test an antigen other than BGG.

TABLE VI

HA Titers of Culture Fluids of Peritoneal Exudate Cells and Trypsinized Kidney Cells Taken from Secondary Rabbits

Cell type and antigen	Exp. No.	No. subpasses	Days in culture												
			2	4	6	8	10	12	14	16	18	20	22	24	26
PEC* with BGG	215	2	128 (2)	128 (2)	4 (2)	32 (<2)	ND‡	ND	64 (<2)	16 (2)	24 (<2)	8 (2)	2 (2)		
	241	1	ND	64 (<2)	32 (2)	64 (2)	32 (<2)	16§ (2)							
	245	1	16 (<2)	32 (2)	32 (2)	16‖ (<2)									
	267	1	16 (2)	64 (<2)	64 (2)	32 (<2)	32 (2)	ND	32 (<2)	16 (<2)	32 (2)	ND	32 (<2)	2 (2)	
	273	3	32 (<2)	32 (2)	16 (2)	64 (2)	32 (<2)	32 (2)	ND	ND	32 (2)	16 (2)	32 (2)	2 (2)	
PEC* with casein	215	2	2	<2	2	4									
	241	1	ND	2	<2	2	4	4	<2						
	245	1	2	4	<2	2									
	267	1	2	<2	2	4	2	ND	2	<2	2	ND	4	<2	
Kidney with BGG	215	1	2	4	4	4	ND	ND	8	<2	4	4	<2		
	241	1	ND	4	8	8	4	4							
	245	1	2	<2	2	<2	2								
	267	1	2	4	<2	4	4	ND	8	4	4	ND	<2	2	

Figures in parentheses indicate hemagglutination titers using BGG in the diluent.
* PEC, peritoneal exudate cells.
‡ ND, not done.
§ Lost to contamination.
‖ Cells died.

Accordingly, rabbits 3-46, 8, 8-5, and 8-6 each were given 10 intravenous injections of 25 Lf's each of alum-precipitated diphtheria toxoid every 2 to 3 days until a total of 250 Lf's had been injected. Monocytes were grown out as usual and the culture fluids assayed for antibody to diphtheria toxoid. For such titrations the cells were coated with 0.125 mg. of toxoid per ml. of cells, according to the method of Stavitsky (6).

The titration results appear in Table IX. Although the titers were 3 to 5 times higher than in the other systems, the antibody persisted for only 1 week. These results were felt to be possibly due to some difference in the response of rabbits to diphtheria toxoid compared to BGG.

Accordingly, rabbits 3-50 and 7-4 each were given a total of 110 mg. of AP-BGG in 11 intravenous injections over a 3 week period. Monocytes were grown out as usual and the culture fluids assayed for antibody with formalinized erythrocytes coated with BGG.

The results appear in Table X. The similarities in both the magnitude and the duration of the antibody response to BGG compared to the response to diphtheria toxoid are striking. This picture of higher titers and shorter duration of response appeared to be related to the hyperimmune state of the animals rather than to the antigen itself.

TABLE VII

HA Titers of Culture Fluids of Peritoneal Exudate Cells Incubated with BGG in Vitro

Cell type and antigen	Exp. No.	No. sub-passes	Days in culture									
			2	4	6	8	10	12	14	16	18	20
PEC* with BGG	180	1	ND‡	32 (<2)	ND	32 (<2)	16 (2)	2 (2)				
	253	2	128 (<2)	32 (2)	32 (<2)	16 (<2)	32 (2)	16 (2)	ND	32 (2)	32 (<2)	2 (<2)
	265§	0	16 (<2)	64 (<2)	32 (2)	32 (2)	16 (<2)	32 (<2)	2 (2)			
	270	1	ND	64 (2)	32 (<2)	32 (2)	8 (2)	2 (2)				
	272	0	8 (2)	16 (<2)	16 (2)	16 (2)	2 (<2)					
	275	2	32 (<2)	32 (2)	32 (2)	16 (2)	ND	16 (<2)	32 (2)	2 (2)		
PEC with casein	180	1	ND	<2	ND	2	4	2				
	253	2	2	2	4	<2	4	4	ND	<2	ND	2
	270	1	ND	2	2	ND	4	<2	ND			
	272	0	2	<2	ND	<2	2					
	275	2	<2	4	ND	4	ND	2	ND	2		

Figures in parentheses indicate hemagglutination titers using BGG in the diluent.

* PEC, peritoneal exudate cells.

‡ ND, not done.

§ Mineral oil remained in this animal for 6 days.

Effect of Endotoxin on Antibody Responses of Monocytes.—The next two series of experiments were designed to determine the effect of endotoxin on the antibody-forming ability of monocytes.

Rabbits 7-5, 8-4, 3-5, and 3-6 were each given 40 mg. of AP-BGG together with 10 μg. of endotoxin intravenously. The day following the antigen and endotoxin injection, each animal received 100 ml. of sterile mineral oil intraperitoneally. The rabbits were sacrificed 3 days after the oil injection, and monocytes grown out as usual.

The titration results, given in Table XI, show a remarkable similarity to the results obtained with the hyperimmunized rabbits shown in Tables IX and X.

To determine the effect of endotoxin when the antigen was added *in vitro*, rabbits 3-7, 1, 2, 9-4, and 9-5 each received 10 μg. of endotoxin intravenously. Twenty-four hours later each animal was injected intraperitoneally with 100 ml. of sterile mineral oil. The animals were sacrificed 3 days after the oil injection, and the monocytes incubated at

TABLE VIII

GMT of Culture Fluids of Peritoneal Exudate Cells

	Experiment	Cell type and antigen	No. subpasses	Duration of experiment, *days*	Day antibody absent	GMT	No. titrations
Primary	175	PEC* with BGG	2	15	12	32(<2)	3
	220		2	26	24	16(2)	11
	225		3	26	26	18(2)	9
	230		1	26	24	45(2)	9
	254		2	20	20	24(<2)	9
		PEC with casein average of 4 experiments	2	23	—	2	32
Secondary	215	PEC with BGG	2	22	22	34(2)	9
	241		1‡	13‡	13	32(2)	5
	245		1	8§	8	14(<2)	5
	267		2	25	24	28(<2)	9
	273		3	24	24	28(<2)	10
		PEC with casein average of 4 experiments	1	24	—	2	29
		Kidney with BGG average of 4 experiments	1	24	—	2	29
Completely *in vitro*	180	PEC with BGG	1	11	11	20(<2)	3
	253		2	19	18	16(<2)	8
	265		0	16	13	26(<2)	6
	270		1	20	12	24(<2)	4
	272		0	12	10	16(<2)	3
	275		2	15	15	28(<2)	6
		PEC with casein average of 5 experiments	1	15	—	1	24

Figures in parentheses represent hemagglutination inhibition titers.

* PEC, peritoneal exudate cells.

‡ Experiment lost to contamination on 13th day.

§ Cells died.

37°C. for 1 hour in medium 199 containing 1 mg./ml. of BGG. The cells were washed 5 times in medium 199 and were planted at 5×10^5/ml. in medium 199 75 per cent:NRS 25 per cent.

The titration results are given in Table XII. Once again the response appeared to be 3 to 5 times higher than in the *in vitro* system without endotoxin (Table VII). It would appear that the endotoxin caused the monocytes to respond to antigenic stimulation in a manner essentially similar to that of

monocytes taken from hyperimmunized animals. However, the duration of the response was somewhat less when the BGG was added *in vitro* than that found in the primary animals given endotoxin. This agrees with the earlier findings without endotoxin (Table VII).

TABLE IX

HA Titers of Culture Fluids of Peritoneal Exudate Cells Taken from Rabbits Hyperimmunized with Diphtheria Toxoid

Experiment No.	Rabbit No.	Days in culture							
		2	4	6	8	10	12	25	30
276	3-46	256 (4)	32 (2)	2 (<2)	2 (<2)	2 (2)	2 (2)	2 (<2)	2 (2)
289	8	128 (2)	256 (2)	ND*	128 (<2)	2 (2)	ND	ND	ND
327	8-5	128 (8)	ND	256 (<2)	2 (<2)	2 (2)	ND	ND	ND
	8-6	256 (8)	ND	256 (4)	2 (<2)	2 (2)	ND	ND	ND

Figures in parentheses indicate hemagglutination inhibition titers using 2×10^{-4} mg./ml. of diphtheria toxoid in the diluent.

* ND, not done.

TABLE X

HA Titers of Culture Fluids of Peritoneal Exudate Cells Taken from Rabbits Hyperimmunized with BGG

Experiment No.	Rabbit No.	Days in culture					
		2	4	6	8	10	12
278	3-50	128 (4)	64 (2)	64 (2)	2 (2)	2 (2)	2 (2)
316	74	256 (2)	ND*	256 (4)	256 (2)	2 (2)	2 (2)

Figures in parentheses indicate hemagglutination inhibition titers using BGG in the diluent.

* ND, not done.

A condensation of the data accumulated for the various systems studied is given in Table XIII. It may be seen that cells from hyperimmune rabbits produced about 5 times as much antibody, but for about ½ to ⅓ as long as did cells from primary or secondary animals. Endotoxin, under the conditions studied, appeared to make cells, either from primary animals or exposed to BGG *in vitro*, behave as if they had been taken from hyperimmunized rabbits.

TABLE XI

HA Titers of Culture Fluids of Peritoneal Exudate Cells Taken from Rabbits Given 40 mg. of AP-BGG Together with 10 μg. of Endotoxin

Experiment No.	Rabbit No.	Days in culture					
		2	4	6	8	10	12
311	7-5	512 (4)	512 (2)	128 (2)	2 (<2)	ND*	ND
323	8-4	512 (16)	512 (8)	256 (4)	2	ND	4 (4)
329	3-5	128 (4)	128 (4)	256 (4)	4 (<2)	ND	ND
	3-6	128 (8)	128 (4)	128 (8)	4 (2)	ND	ND

Figures in parentheses indicate hemagglutination inhibition titers using BGG in the diluent.
* ND, not done.

TABLE XII

HA Titers of Culture Fluids of Peritoneal Exudate Cells Taken from Rabbits Given 10 μg. of Endotoxin, and Incubated in Vitro with BGG

Experiment No.	Rabbit No.	Days in culture				
		2	4	6	8	10
332	3-7	ND*	512 (4)	32 (8)	2 (<2)	
341	1	256 (8)	4 (2)	2 (<2)	ND	
	2	256 (8)	4 (2)	2 (2)	ND	
344	9-4	128 (4)	128 (8)	8 (4)	2 (<2)	2 (2)
	9-5	256 (8)	256 (4)	8 (2)	4 (2)	2 (2)

Figures in parentheses indicate hemagglutination inhibition titers using BGG in the diluent.
* ND, not done.

Time-Titer Curves.—McKenna and Stevens (1) removed rabbit spleens 2 hours after a secondary injection of BGG and planted the tissue. Antibody titers were detectable as early as 4 hours after planting the spleens and maximal titers in the culture fluids were reached after 15 to 24 hours. Two experiments were carried out to determine whether a similar time-titer relationship existed in cultures of monocytes.

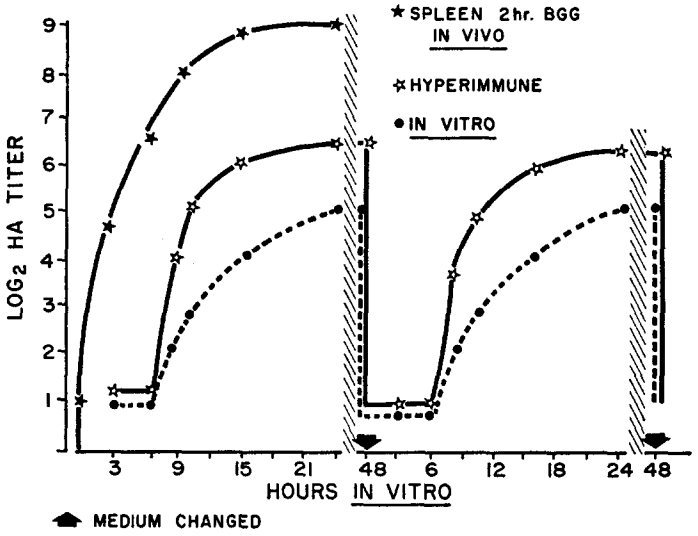
Rabbit 7-4 was hyperimmunized with BGG as described in the previous section, and monocytes from rabbit 8-2 (normal) were exposed *in vitro* to 1 mg./ml. of BGG as previously described. Titrations were carried out on pooled culture fluids at each medium change at the

TABLE XIII

Comparison of Geometric Mean Hemagglutinating Titers of Culture Fluids of Peritoneal Exudate Cells

Antigenic experience	No. Experiment	No. Rabbits	GMT	Day Antibody Absent	No. of Titrations
Primary	5	5	30	21	41
Secondary	5	5	32	23	28
In vitro	6	6	28	13	30
Hyperimmune	4	5	147	9	24
Primary and ET* *in vivo*	3	4	223	8	12
ET *in vivo* and antigen *in vitro*	3	5	97	6	12

* ET, endotoxin.



TEXT-FIG. 3. Comparison of rates of antibody formation *in vitro*.

beginning of each time curve, and on 3 individual cultures at each time interval. In addition, cell counts were carried out at the beginning and the end of each time curve on 2 tube cultures different from those used for titrations. An adequate number of tubes was planted for these experiments so that no tube was titrated more than once.

The titration results for two 48 hour periods are presented graphically in Text-fig. 3. Each point on the graph represents a mean of 3 titrations. To compare these results with those found in the spleen system, the curve obtained in

cultures of 5 diced rabbit spleens taken from animals 2 hours after a secondary injection of BGG (1) is also plotted in Text-fig. 3. It is evident that the shape of the 3 curves and the times of maximal antibody titers are somewhat similar. The curves resemble those found by Stevens *et al.* (7) for the incorporation of S^{35}-methionine into antibody and into serum albumin. The average time for maximal incorporation was 36 hours for antibody and 6 hours for serum albumin.

The data obtained for the cells from rabbits 7-4 and 8-2, plotted in part in Text-fig. 3, were also used to calculate the number of antibody molecules synthesized per cell. The calculations were based on the combining tests carried out by Stevens and McKenna (2) and are shown in Table XIV. Further tests with 14 tissue culture fluids of PEC confirmed the results with spleen; *i.e.*, 0.01 μg. of BGG neutralized an HA titer of 32. If the amount of antibody formed was

TABLE XIV

Comparison of Cell Count with Antibody Molecules formed/Cell

Antigen treatment	Day	Cell count $\times 10^4$	HA	Molecules antibody/cell $\times 10^5$
Hyperimmune rabbit 7-4	2	7	256	300
	3	13	316	200
	7	62	256	33
	8	73	256	30
In vitro rabbit 8-2	2	8	32	32
	3	7	39	42
	7	50	16	3
	8	60	16	2

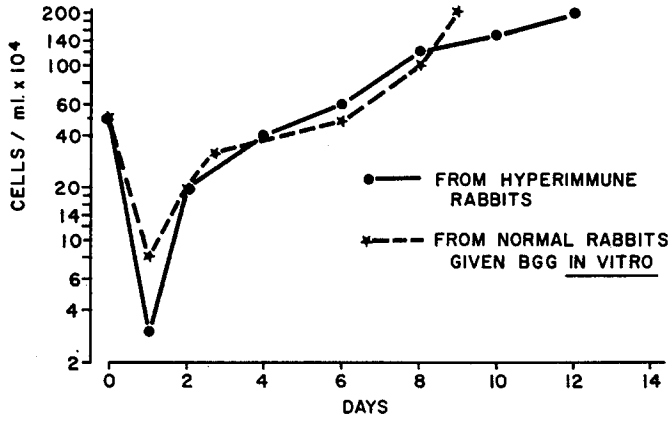
simply a function of the total number of cells, then the HA column should increase with an increase in cell count and the molecules of antibody formed per cell would be constant. However, the HA remained constant in each system, and as the cell count increased, the average number of molecules of antibody formed per cell decreased. In Text-fig. 4, the mean cell counts at various times have been plotted. It may be seen that there was no difference in the growth potentials in either system. Therefore, it appears that the titer attained in any given system is not a direct function of the total number of cells, but rather of their particular ability to react to an antigen with the formation of antibody.

Antigen Dose-Antibody Response Experiments.—To determine the effect of antigen concentration on the antibody response of monocytes exposed to BGG *in vitro*, rabbit 7-9 was given 100 ml. of sterile mineral oil intraperitoneally.

The animal was sacrificed 3 days later and the monocytes were recovered as usual. The cell yield of 4.4×10^7 cells was divided into 6 equal parts so that each of the 6 suspensions contained approximately 7×10^6 cells. Each aliquot of cell suspension was centrifuged at

700 R.P.M. for 3 minutes and the cells taken up in medium 199 containing various amounts of BGG. These mixtures were incubated at 37°C. for 1 hour and the cells washed 5 times with medium 199. The cells were planted and titrations were carried out in the usual manner.

The results, given in Table XV, show that no antibody was formed at concentration of BGG less than 0.1 mg./ml. Concentrations of BGG of 0.1 mg./ml.



TEXT-FIG. 4. Growth of monocytes *in vitro*. The points represent geometric mean counts for 3 hyperimmune and 5 normal rabbits.

TABLE XV

Antigen Dose-Antibody Response of Peritoneal Exudate Cells Incubated in Vitro with BGG

Mg. BGG/ml.	Mean HA titers
0	2(3)
0.001	2(3)
0.01	2(3)
0.1	32(3)
1.0	29(3)
10.0	16(3)

Figures in parentheses indicate hemagglutination inhibition titers using BGG in the diluent.
Means calculated over an 8 day period.

and 1.0 mg./ml. produced antibody in similar amounts. However, the drop in response at a concentration of 10.0 mg./ml. may be significant since this same pattern was found when diced spleens were incubated with 50.0 mg./ml. of BGG (2). The data suggest that, once a threshold concentration of antigen obtains, the cells respond with a maximal effort and are in substantial agreement with the earlier studies from this laboratory (2) in which diced rabbit spleens were exposed to BGG *in vitro*. Two differences between the two systems were noted, however. The peritoneal exudate cells appeared to be able to form antibody when exposed to 0.1 mg./ml. of BGG, while 0.5 mg./ml. was required to elicit a

response using diced rabbit spleens (2, 8). The other difference was that peritoneal exudate cells responded to antigen *in vitro* without endotoxin, while diced spleens did not form antibody unless the animals had been pretreated with endotoxin (2).

Stevens and McKenna (2) also found that sodium prednisolone phosphate at a concentration of 100 μg./ml. (2×10^{-4} M) completely inhibited antibody formation by diced rabbit spleens exposed *in vitro* to BGG together with the sol-

TABLE XVI

Effect of Prednisolone in Vitro on the Antibody Response of Peritoneal Exudate Cells Initiated in Vitro

Treatment of cells	HA on day					
	3			5		
	SD*	SD and BGG	SD and Casein	SD	SD and BGG	SD and Casein
BGG	128	8	256	64	2	32
BGG and 100 μg/ml. PP‡	16	4	8	2	2	<2

* SD, standard diluent.

‡ PP, sodium prednisolone phosphate.

uble corticosteroid. The effect of prednisolone on the antibody-forming ability of peritoneal exudate cells was studied as follows.

Rabbit 9-4 was given mineral oil as usual. Two days later, the animal received 10 μg. of endotoxin[2] intravenously. The animal was sacrificed 24 hours after the endotoxin injection and the peritoneal exudate cells were incubated for 1 hour at 37°C. with 1 mg./ml. of BGG alone and 1 mg./ml. of BGG plus 100 μg./ml. of prednisolone. After washing, the cells were planted and titrations done as before.

The results, presented in Table XVI, show that prednisolone suppressed antibody synthesis, essentially in the same manner as in the diced spleen system. The antibody formed when BGG was used alone was inhibited by the specific antigen (BGG) but not by the same concentration of casein in the standard diluent.

Reactivity of Monocytes to More Than One Antigen.—In efforts to determine if a given cell population could form antibodies to more than one antigen, a search was made for a soluble antigen other than BGG which would incite peritoneal exudate cells to form antibody when exposed to the antigen *in vitro*. Another requisite of this antigen was that it should not cross-react in the titration scheme. Early work using purified diphtheria toxoid and an antigenic derivative

[2] Endotoxin was used to have this system comparable to the diced spleen system referred to above.

of *Shigella paradysenteriae* made in this laboratory according to the method of Harris *et al.* (9), were unsuccessful. However, preliminary experiments showed that crystalline egg albumin (EA) was satisfactory for the purpose. For these and subsequent titrations of antibody to EA, formalinized erythrocytes were coated with 5 mg. of EA/ml. of cells as recommended by Boyden (10).

A dose-response experiment was carried out to determine the amount of EA required to initiate antibody synthesis *in vitro*. The results are shown in Table XVII. As with BGG (Table XV) 0.1 mg./ml. of EA was an adequate concentration to elicit antibody synthesis. However, in contrast to BGG, as the concentration of EA was increased, the antibody titers increased. This suggests that the response of peritoneal exudate cells exposed *in vitro* to EA did not follow the all-or-none pattern seen in the response to BGG.

TABLE XVII

Antigen Dose-Antibody Response of Peritoneal Exudate Cells Incubated in Vitro with EA

Mg. EA/ml.	HA
0	2(3)
0.001	2(3)
0.01	2(3)
0.1	16(3)
1.0	39(3)
10.0	52(3)

Figures in parentheses indicate hemagglutination inhibition titers using EA in the diluent.

Rabbits 9-8, 1-01, 1-02, 1-03, and 1-04 each were given mineral oil and sacrificed as usual. Cells were recovered from each animal and divided into 3 equal parts. One part was taken up in medium 199 containing 1 mg./ml. of BGG. The second part was suspended in medium 199 containing 4 mg./ml. of EA, and the 3rd part was suspended in medium 199 without antigen. All suspensions were incubated at 37°C. for 1 hour, and the cells were washed and planted as usual. Titrations were carried out using formalinized erythrocytes coated with the homologous antigen for the cells incubated with antigen and with erythrocytes coated separately with each antigen for peritoneal cells incubated without antigen.

The titration results, given in Table XVIII, show that a given population of monocytes is equally reactive to serologically unrelated antigens when exposed to these antigens separately.

On the other hand, when monocytes taken from 2 animals hyperimmunized with diphtheria toxoid were exposed to BGG *in vitro*, no antibody to BGG was formed. This suggests that these monocytes were all oriented to form antibody to diphtheria toxoid and were not reactive to BGG. These cells produced HA titers of 256 to diphtheria toxoid.

Phagocytosis Studies.—Several attempts were made to determine whether peritoneal exudate cells had *in vitro* phagocytic ability both on primary isolation and after various lengths of time in tissue culture. The three lines of approach

used were with India ink, and immune and non-immune phagocytosis of *Salmonella typhosa*, *Escherichia coli*, and *Shigella paradysenteriae* (Flexner).

A suspension of India ink was centrifuged at 30,000 R.P.M. for 2 hours and the sedimented particles resuspended in distilled water. This was done to remove the suspending shellac which might possibly be toxic to the monocytes.

TABLE XVIII

HA Titers of Culture Fluids of Peritoneal Exudate Cells Exposed to Either BGG or EA in Vitro

Experiment No.	Rabbit No.	Antigen treatment of cells	Homologous antibody titers on day				
			2	4	6	8	10
343		BGG	32	32*			
	9-8	EA	16	16			
		None	<2	<2			
345		BGG	32	ND‡	32	64	2
	1-01	EA	16	ND	16	<2	2
		None	2	ND	<2	<2	<2
		BGG	64	ND	64	32	4
	1-02	EA	8	ND	16	<2	4
		None	2	ND	<2	<2	2
349		BGG	64	32*			
	1-03	EA	32	32			
		None	2	<2			
		BGG	32	32*			
	1-04	EA	64	16			
		None	2	<2			

* Lost to contamination. See text for details of titration method.

‡ ND, not done.

Antisera to *S. typhosa*, *E. coli*, and *Sh. paradysenteriae* were prepared in rabbits as follows.

Several slants of nutrient agar were inoculated with each of the bacteria and incubated at 37°C. for 18 hours. The growth was harvested in 0.9 per cent saline and the organisms killed with a final concentration of 0.5 per cent formaldehyde at 4°C. for 24 hours. The cells were washed 4 times and resuspended in sterile saline to contain 1×10^9 cells/ml. Two rabbits were immunized with 3 intravenous and 2 intraperitoneal injections of each bacterial suspension and were bled 1 week after the last injection. The agglutinating titers of the sera against 0.2 ml. of a 1 per cent suspension of bacteria were 1:2048 for *S. typhosa* and 1:1024 for both *E. coli* and *Sh. paradysenteriae*.

To study the phagocytosis of India ink (carbon) particles, fresh cells or cells removed from the culture tubes with 0.25 per cent trypsin after various lengths of time *in vitro*, were washed and suspended in 0.9 per cent saline so as to contain approximately 1×10^6 cells per ml. To the cell suspensions, various amounts of India ink from 0.1 ml. through 1.0 ml. were added. The tubes were placed on a roller drum at 15 R.P.H.

at 37°C. for a minimum of 30 and a maximum of 120 minutes. After incubation, smears were made of the cells, some of which were air-dried while others were examined wet. Microscopic examination of the cells showed no evidence of phagocytosis in 3 experiments using freshly harvested cells and in 4 experiments using cells which had been in culture not in excess of 1 month.

Tests for immune and non-immune phagocytosis were carried out as follows.

Again both freshly harvested and cultured cells were used at approximately 1×10^6 cells/1.0 ml. of saline. To the cell suspensions were added in sequence 0.1 ml. of either normal rabbit serum or antiserum specific for the bacteria being used, and 0.1 ml. of bacterial suspension containing 1×10^8 cells. These mixtures were placed on a roller drum at 15 R.P.H. at 37°C. for a minimum of 30 and a maximum of 120 minutes. After incubation, smears were made and the cells fixed in 10 per cent formalin. The cells were stained by the method of Lucké *et al.* (11).

No evidence of phagocytosis was seen in 2 experiments using fresh cells or in 3 experiments using cells which had been in culture not in excess of 1 month.

To determine if the bacteria used were amenable to phagocytosis,

0.1 ml. of fresh citrated rabbit blood was mixed with 0.1 ml. of either normal rabbit serum or rabbit antiserum to the bacteria used. Stained smears showed many intracellular bacteria in polymorphonuclear leucocytes. All three genera of bacteria were phagocytized by polymorphonuclear leucocytes.

Morphologic Studies.—

Monocytes from rabbits hyperimmunized with BGG and monocytes from normal animals were grown in the usual manner. At intervals, the cells were removed from each of 2 tube cultures with 0.25 per cent trypsin, and counted in a hemocytometer. Two hyperimmune rabbits and 4 normal rabbits were studied.

The results were almost identical with those shown in Text-fig. 4, *i.e.*, after an initial drop to about 10 per cent of the original count, the cells proliferated with a doubling time of 55 to 60 hours. No differences were noted in the growth rates nor in total counts in the two systems.

For studies on morphologic variation, Kalter's quadruple stain (12) was used. This staining technique involves safranin, crystal violet, fast green, and orange 2. According to Kalter, cell nuclei stain red, nucleoli purple, and cellular cytoplasm pink to red with the exception of Henle's loop which stains light green. Fibroblasts, in contrast to other animal cells, stain bright green with purple nuclei.

Monocytes in culture took on the angular, elongate appearance of fibroblasts rather than the amorphous shape of macrophages. Thus, Kalter's technique was thought to be most useful for studying monocytes in culture to determine if the cells became fibroblasts. Maximow (13, 14) and Bloom (15) felt that there was an orderly and irreversible progression to fibroblasts irrespective of the source of the original cell, whether monocytes or lymphocytes.

For these studies, monocytes and kidney cells from secondary animals immunized with BGG were grown on 11 x 22 mm. coverslips in Leighton tubes. The cells were stained *in situ* on the coverslips and mounted on microscopic slides for study.

The results of a typical series of stained slides are given in Figs. 1 to 5. On first isolation, many of the peritoneal exudate cells were seen to have brick-red cytoplasm with eccentric horseshoe-shaped nuclei. These appeared to be typical monocytes; some contained oil droplets. Some lymphocytes and polymorphonuclear leucocytes also were seen (Fig. 1). After 3 days in culture, some cells were seen elongating, throwing out long spikes of cytoplasm. Nuclear changes involved rounding of the nucleus with the nucleoli becoming more prominent (Fig. 2). After 1 week (Fig. 3), little further change was noted, except that the cells were beginning to stain a dirty green. On the 24th day (Fig. 4), the cells appeared mostly a dirty green. However, when these cells were compared with the kidney cells from whole kidney after 24 days in culture (Fig. 5), two chief differences were observed. In the first place, the kidney cells stained a bright green, as expected for fibroblasts, in contrast to the dirty green of the monocytes. Secondly, the monocytes were larger than the kidney cells.

The cells used for the illustrations were all taken from experiment 267, and both monocytes and kidney cells underwent one subculture. It is of interest to note that the antibody titer declined between the 22nd and the 24th day in this experiment (Table VI), and the cells became predominantly a dirty green on the 24th day. It is felt, however, that the cells remained as monocytes throughout this time since the monocytes were somewhat larger than the kidney cells (fibroblasts) and formed syncytia rather than sheets. The dirty green stain taken on by the monocytes in late tissue culture may reflect a tendency toward the common primitive mesenchymal ancestor of monocytes and fibroblasts.

DISCUSSION

Although lymphocytes and neutrophils were present in the initial cell suspension of peritoneal exudate cells (PEC), there was no evidence that these cells attached to the glass after planting. Hence they would be removed with the first medium change. Even when a mixed population of this type is attached to glass with plasma or tissue juice, the lymphocytes and granulocytes quickly disappear (16, 17). Therefore, we believe that the ability to produce antibody has been demonstrated by peritoneal exudate cells which originally resembled monocytes. At no time were cells resembling either plasma cells or eosinophils observed either in the original cell suspension or in culture.

The ability of monocytes to produce antibody has been shown after primary, secondary, and hyperimmunizing injections of bovine-γ-globulin (BGG) as well as by cells taken from normal rabbits and exposed to BGG *in vitro*. The monocytes produced antibody to three different antigens: BGG, egg albumin (EA),

and diphtheria toxoid. However, positive results with diphtheria toxoid were only obtained with hyperimmune animals.

In an earlier paper from this laboratory, Stevens and McKenna (2) demonstrated antibody formation to BGG, initiated *in vitro*, using diced spleens from rabbits previously treated with endotoxin. When spleens were used, it was necessary to pretreat the animals with endotoxin, else no antibody was formed when the spleens were incubated with antigen. However, it was not necessary to pretreat the animals with endotoxin in order to elicit antibody formation by monocytic cells after exposure of these cells to either BGG or egg albumin (EA) *in vitro*. It must be remembered, however, that mineral oil was used to evoke the monocytes. As is well known, mineral oil has adjuvant properties and may have acted in a manner similar to the endotoxin in that it made the cells responsive to *in vitro* stimulation by antigen. It is not believed that mineral oil and endotoxin react in the same fashion pharmacologically, but the end result of antibody formation might well be the same.

One of the most striking features of these experiments was that there was no difference either in the magnitude or the duration of the antibody responses between cells taken from primary or secondary rabbits (Table XIII). This shows both differences from and similarities to the spleen system. When the antigen was given *in vivo* to rabbits, as was done for the primary and secondary studies reported here, the secondary response in the spleen system was much greater than the primary response (1). However, when the primary injection of antigen was given *in vivo* and the secondary contact was *in vitro*, the spleen showed no differences between primary and secondary *in vitro* contact (2). The strictly analogous experiment has not been made with PEC.

The magnitude of the response of normal monocytes exposed to BGG *in vitro* was essentially the same as that of cells from either primary or secondary rabbits. This differs from the results with spleen when both primary and secondary rabbits gave higher responses than did the completely *in vitro* system (1, 2). In contrast, the primary and secondary monocyte series did differ from the *in vitro* series in the duration of antibody formation in tissue culture; *i.e.*, 3 weeks versus 2 weeks, respectively (Table XIII).

The cells from hyperimmune rabbits gave considerably higher titers but they persisted only about 1 week. Pretreatment of the animals with endotoxin produced responses most closely resembling those seen in the hyperimmune series; *i.e.*, high titers persisting for only 1 week. The shorter span of antibody formation in these two groups was unexpected and we can offer no explanation.

Ehrich *et al.* (18) were unable to demonstrate antibody in extracts of peritoneal exudate cells during maximal antibody formation and this was interpreted to mean that the antibody was formed by cells other than those in the peritioneal exudates. An extract scheme, however, is static, while a tissue culture system is dynamic. Even in the dying spleen system McKenna and Stevens (1) found

about 10-fold less antibody in splenic extracts than in culture fluids of the same spleens at maximal titers.

Roberts (17) reported that he was unable to elicit antibody formation to *S. typhosa* in tissue cultures of either immune or normal peritoneal exudate cells, despite the fact that these cells were reported to contain intracellular bacteria. The PEC were maintained in plasma clot cultures in Hanks' solution and rabbit serum. It may be recalled that Hanks' solution supplemented with 30 per cent normal rabbit serum would not support the replication of monocytes for more than 1 week (Table II). Harris *et al.* (19) also reported the failure of macrophage rich peritoneal exudates to produce antibody after transfer into recipient rabbits. These findings contrast with the results reported here and those of Dixon *et al.* (5) as well as the successful transfer of delayed hypersensitivity with peritoneal exudates rich in macrophages (20). The antigens chosen may have a bearing on the different results since we were unable to obtain completely *in vitro* antibody synthesis to diphtheria toxoid or to Harris' fraction derived from *Sh. paradysenteriae.*

Roberts, as well as Dixon *et al.* (5), called the cells macrophages. We were unable to demonstrate phagocytosis by the PEC or after subsequent culture. However, even such strongly phagocytic cells as Kupffer cells have been shown to be non-phagocytic in culture although such cells on injection into an animal regained their phagocytic ability (21). The cells in our cultures did not exhibit the undulating membranes said to be characteristic of macrophages but rather were sharply angular and either elongate or stellate. Since the cells when originally isolated resembled monocytes more than macrophages (Fig. 1), the former name seemed more appropriate.

An abstract by Houghton in 1948 (22) stated that reticulo-endothelial cells produced antibody to "selected antigens" in tissue culture when taken either from immunized animals or from normal animals and exposed to antigen *in vitro*. The details of the study have not been published so critical appraisal is impossible.

It was of interest that autologous serum had no apparent effect on either the functional ability or the morphology of the monocytes, since we had previously found that 40 per cent autologous serum increased the antibody titers about 9-fold over those attained with homologous serum in cultures of diced rabbit spleens (2).

If we assume that all the cells in culture are actively producing antibody and that each cell is producing a like amount, then we may calculate the amount of antibody synthesized per cell by using the combining ratio referred to earlier. If an HA titer of 32 represents 0.07 μg. of antibody/0.5 ml. then 1 ml. will contain 0.14 μg. of antibody. Since 2.5×10^6 cells/ml. counted on the 3rd day, produced a titer of 32, then 1 cell produced 6×10^{-7} μg. of antibody. Dixon *et al.* (5) transferred an average of 4.8×10^8 cells which produced an average of 16

μg. antibody N/ml. of serum in 3 kg. recipient rabbits having an extracellular fluid volume of about 480 ml. The amount of antibody produced per cell calculated from these data would be 1×10^{-4} μg. These calculations show, as might be expected, that the amount of antibody formed in the hyperimmune system *in vivo* was much greater, *i.e.* about 170-fold, than that formed *in vitro*. Dixon's group felt that some of the transferred cells differentiated into plasma cells *in vivo* (23). As mentioned earlier, no transformation to plasma cells was noted in any of our monocytic tissue cultures.

Jerne (24), Talmage (25), and Burnet (26) have postulated a clonal hypothesis as a possible explanation for antibody formation. The essence of such an hypothesis is, to quote Talmage, "The process of natural selection requires the selective multiplication of a few species out of a diverse population." According to this theory, the only role of the antigen would lie in inducing proliferation of the particular cell(s) that were already producing γ-globulin with a high affinity for the particular antigen.

The usual system for measuring antibody formation involves serum antibody titrations. In such a system, antibody is rarely detected before the 3rd day after immunization, and maximal titers are not attained in less than 1 week. Since there is time for cellular replication under these conditions, the clonal theory is compatible with many of the facts known concerning antibody synthesis. However, results from this laboratory (2) indicate much earlier formation of antibody and, therefore, raise doubt concerning the ability of the clonal theory to provide a comprehensive picture of antibody formation.

Data from the previous paper (2) and the present one are presented in Table XIX to demonstrate the short period required for antibody formation to occur after initiation *in vitro*. In the upper part of the table it may be seen that antibody appeared in the short space of 1 hour, and only when the spleen was exposed to BGG. It is probably safe to conclude that no cell division took place in an hour; hence the resultant antibody could not be a consequence of cellular multiplication of the cell(s) already forming antibody to BGG. In the lower section of the table, the results obtained with monocytes exposed to two antigens are presented. Once again, antibody was synthesized only under the aegis of antigen and maximal titers were attained in 24 hours. Since the doubling time of both immune and normal rabbit monocytes was shown to be about 55 hours (Text-fig. 4), less than a 50 per cent increase in cell numbers would be expected in 24 hours. Such an increase could hardly account for the observed responses to both BGG and EA. Both of these systems were studied *in vitro*. However, earlier studies (1) showed antibody in spleens removed 4 hours after a primary intravenous injection of BGG.

These comments have all referred to early primary responses. In contrast, the minimal time required to sensitize an animal for a true anamnestic response is about 3 weeks and the magnitude of the secondary response increases with time.

Such an extended period suggests that multiplication of cells is required rather than multiplication of subcellular units. Previous work indicated that the replication of subcellular units involved in antibody formation occurred in a few hours (Table VI in reference 1).

We suggest that the observed results could be interpreted as follows. The monocyte system contains no lymphoid cells and shows no secondary response. Although by hyperimmunization increased titers can be obtained, hyperimmunization differs markedly from the secondary response *per se*. The former involves repeated introduction of antigen usually in large amounts. The essence of the secondary response is the altered reactivity of the animal to reintroduction of the same antigen when the original antigen and antibody may be low or

TABLE XIX

Summary of Antibody Titers Showing Speed of Antibody Formation

Antigen	Cells coated with	HA	
		0 time extract	1 hr. extract
Spleen			
BGG	BGG	<2	128
None	"	<2	2
Monocytes			
		24 hr. fluids	
BGG	BGG	26	
EA	EA	24	
None	EA	2	
None	BGG	2	

The data for the spleen system were taken from Table X of reference 2.

undetectable. The spleen system, of course, contains both monocytic and lymphoid cells. While monocytes retain most of their functional abilities *in vitro*, lymphoid cells are much less hardy and rapidly lose these abilities *in vitro*. Hence when the spleen system is initiated *in vivo*, both the monocytic and lymphoid response is possible and was observed when *in vivo* secondary spleens were placed in culture (1). However, when the antigen is introduced *in vitro* to the primary or secondary spleen (*i.e.*, primary *in vivo*), the lymphoid cells are unable to react with the antigen and only the monocytic response is obtained (2).

The true primary response is considered to be a monocytic response, occurring over the first few hours to days. Lymphoid cells then become involved in an undetermined fashion and the majority of the antibody formed even after primary contact with antigen may be due to lymphoid cells. These lymphoid cells divide,

producing daughter cells capable of responding anamnestically to reintroduced antigen. This forms the basis of the secondary response. While such mitoses could lead to a clonal-like picture, they would not represent genetically determined clones in the sense envisioned in the clonal theory. A good primary antigen such as BGG would stimulate the monocytic series strongly with relatively poor carry-over to the lymphoid series. In contrast, a poor primary antigen such as diphtheria toxoid would stimulate the monocyte series poorly but carry its effect to the lymphoid series very strongly, thereby preparing the animal for an excellent secondary response.

In contrast to the lymphoid system, the monocyte-macrophage system of defense appears to have no secondary response; *i.e.*, no "memory" system. It is of interest to consider this from an evolutionary point of view. Among the invertebrates, the number of offspring produced is often staggering, while among most of the vertebrate classes emphasis is placed on fewer offspring with higher survivals. It would seem that the immune mechanisms of invertebrates could be less efficient than those of vertebrates and yet ensure species survival because of the large number of offspring. Thus, a "memory" system may have evolved, in which an initial contact with an infectious agent would enable the survivors to respond with greater immunity to subsequent contacts with the same or antigenically related agents. It is suggested, therefore, that antibody formation by monocytes and/or macrophages may be a primitive, relatively inefficient defense mechanism while more efficient antibody formation by lymphoid cells may be an evolutionary development of vertebrates. In comparing the speed and low level of antibody formed by diced spleens *in vitro* (2) and by monocytes in this paper, it is tempting to consider it analogous to the rapid, low-level immunity which is alleged to develop in insects (27, 28).

SUMMARY

Cells from peritoneal exudates of rabbits sacrificed 3 days after an intraperitoneal injection of sterile mineral oil were grown in tissue cultures in medium 199 (75 per cent); normal rabbit serum (25 per cent). Antibody produced by the cells was assayed by an hemagglutination technique in which the antigens used were adsorbed to formalinized tanned sheep erythrocytes. These sensitized cells agglutinate in the presence of antibody specific to the adsorbed antigen. It has been demonstrated that:

Peritoneal exudate cells produced hemagglutinating antibody to bovine gamma globulin (BGG) in a replicating tissue culture system for approximately 3 weeks when taken from animals given either primary or secondary injections of BGG. The mean hemagglutinating titer was 30 for the primary and 32 for the secondary systems. Since the other cell types did not persist, it is felt that monocytes were responsible for these results.

Monocytes taken from normal rabbits and exposed to either BGG or egg albumen (EA) *in vitro* produced titers of 28 for about 2 weeks.

Monocytes taken from rabbits given hyperimmunizing injections of BGG produced titers of 147 for about 1 week.

Endotoxin from *Salmonella typhosa* caused the monocytes to form antibody as if they had been taken from hyperimmunized rabbits. This was true both when the antigen was given *in vivo* together with the endotoxin as well as when the cells were exposed to antigen *in vitro*. The titers were 223 and 97, respectively.

Neither freshly harvested nor cultured monocytes were phagocytic for carbon particles or bacteria *in vitro*. Monocytes in tissue culture appeared to assume the morphology of fibroblasts, but did not stain with the characteristics of fibroblasts. The morphologic changes and staining characteristics of monocytes in tissue culture have been described.

The implications of these findings have been discussed and an attempt made to integrate them into general biological theory.

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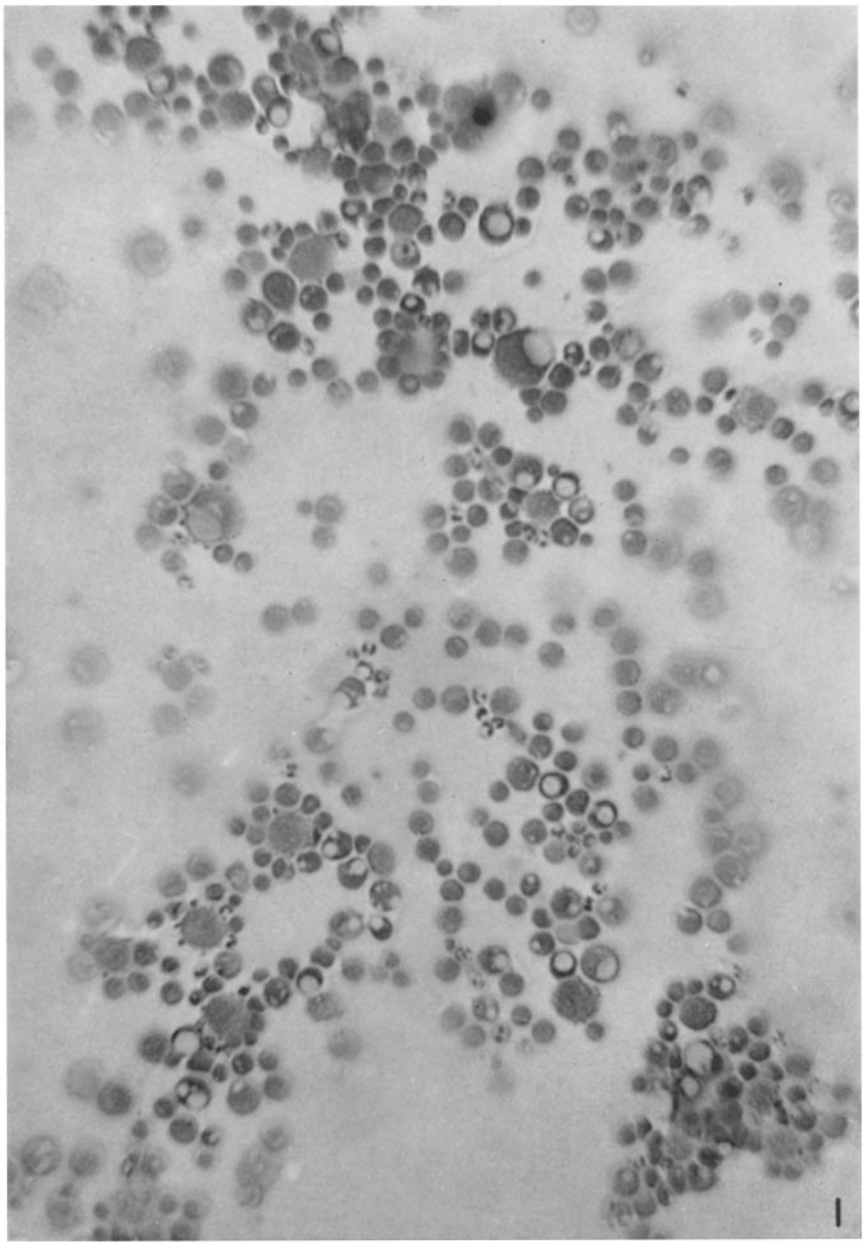
EXPLANATION OF PLATES

All cells were stained with Kalter's quadruple stain and were photographed at ×450.

PLATE 48

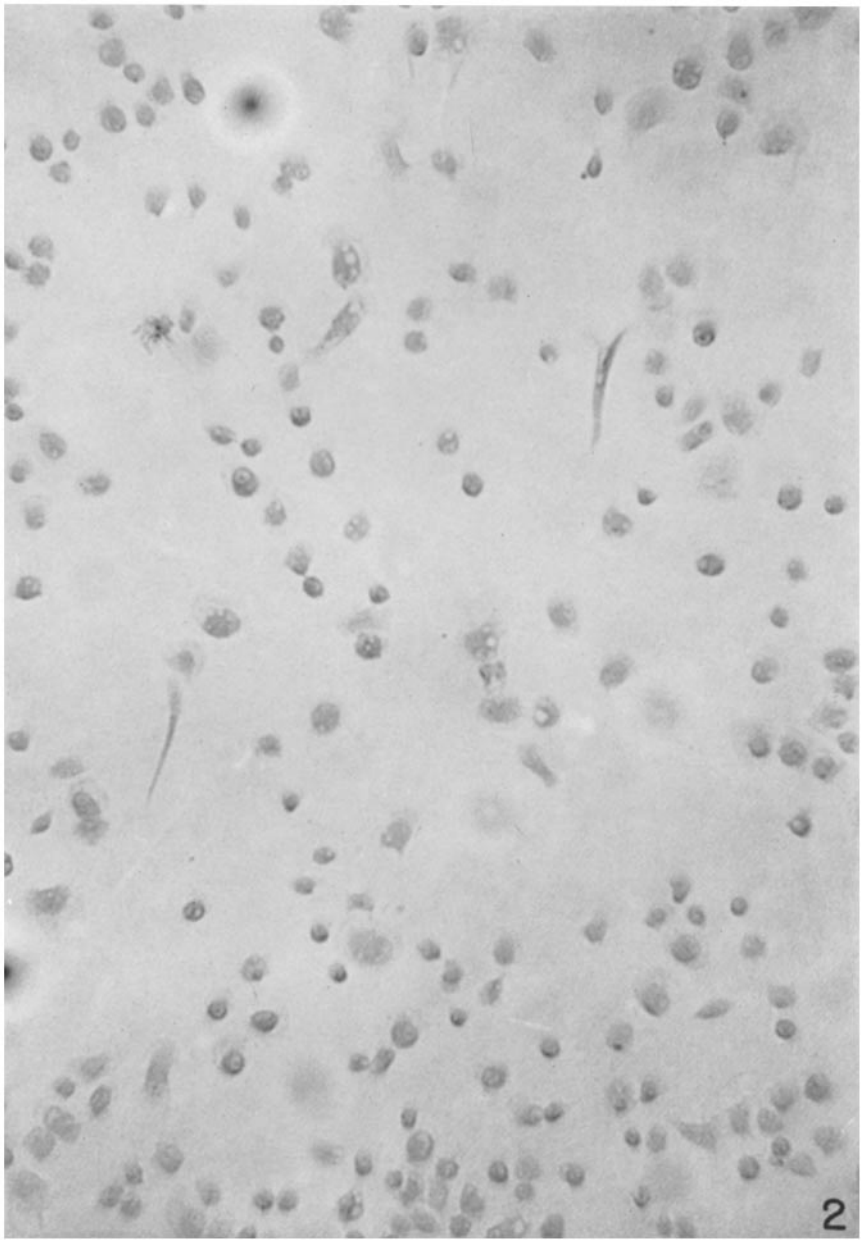
FIG. 1. Freshly harvested peritoneal exudate cells taken 3 days after intraperitoneal injection of 100 ml. of sterile heavy mineral oil.



(McKenna and Stevens: Antibody formation by peritoneal exudate cells)

PLATE 49

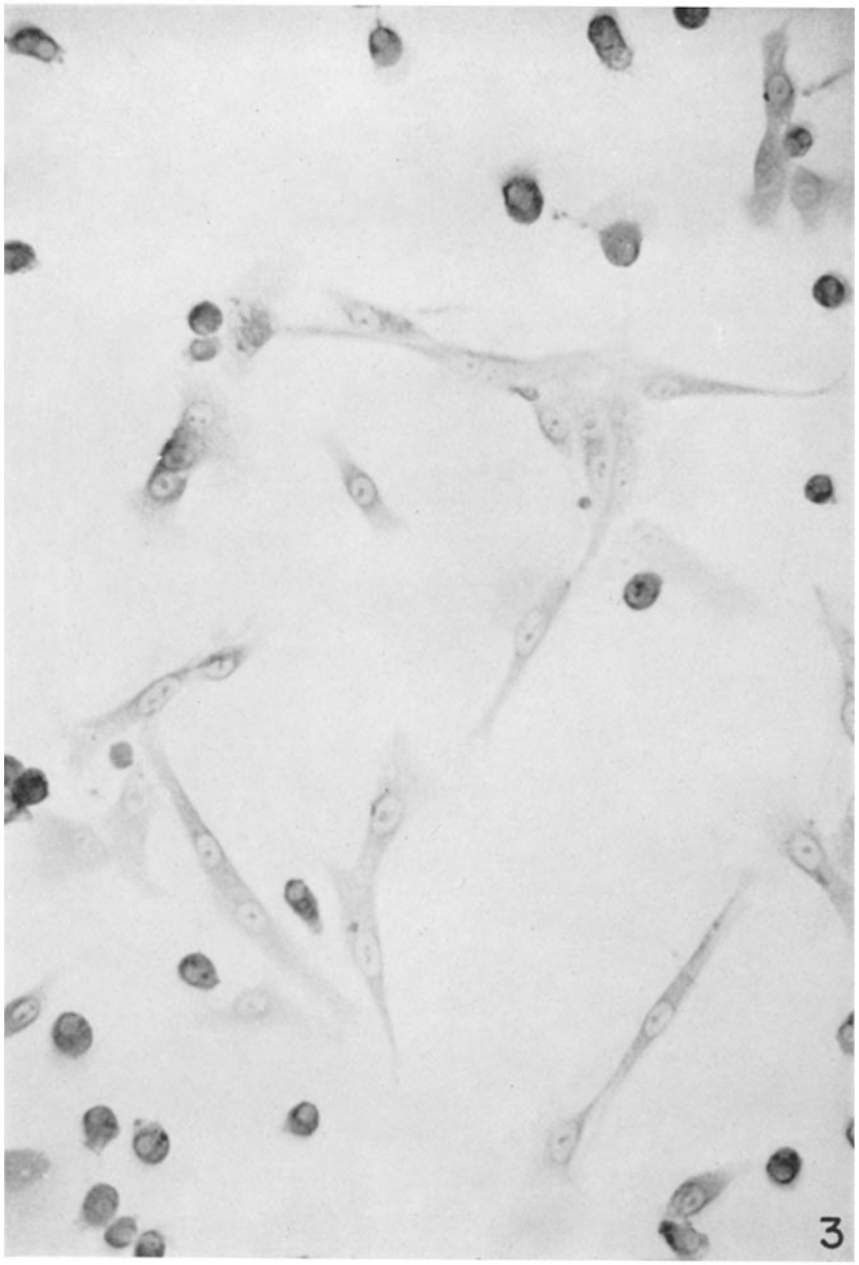
FIG. 2. Peritoneal exudate cells after 3 days in culture.



(McKenna and Stevens: Antibody formation by peritoneal exudate cells)

PLATE 50

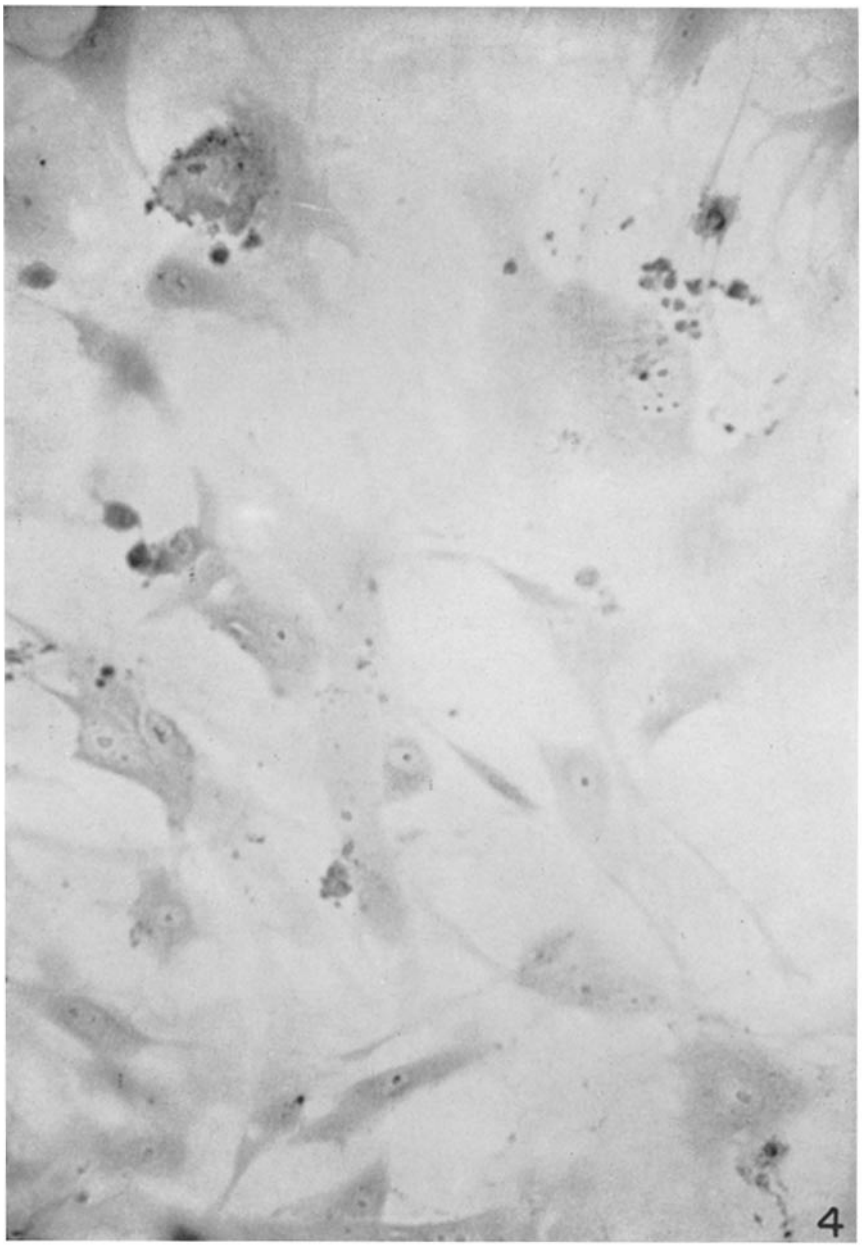
FIG. 3. Peritoneal exudate cells after 1 week in culture.



(McKenna and Stevens: Antibody formation by peritoneal exudate cells)

PLATE 51

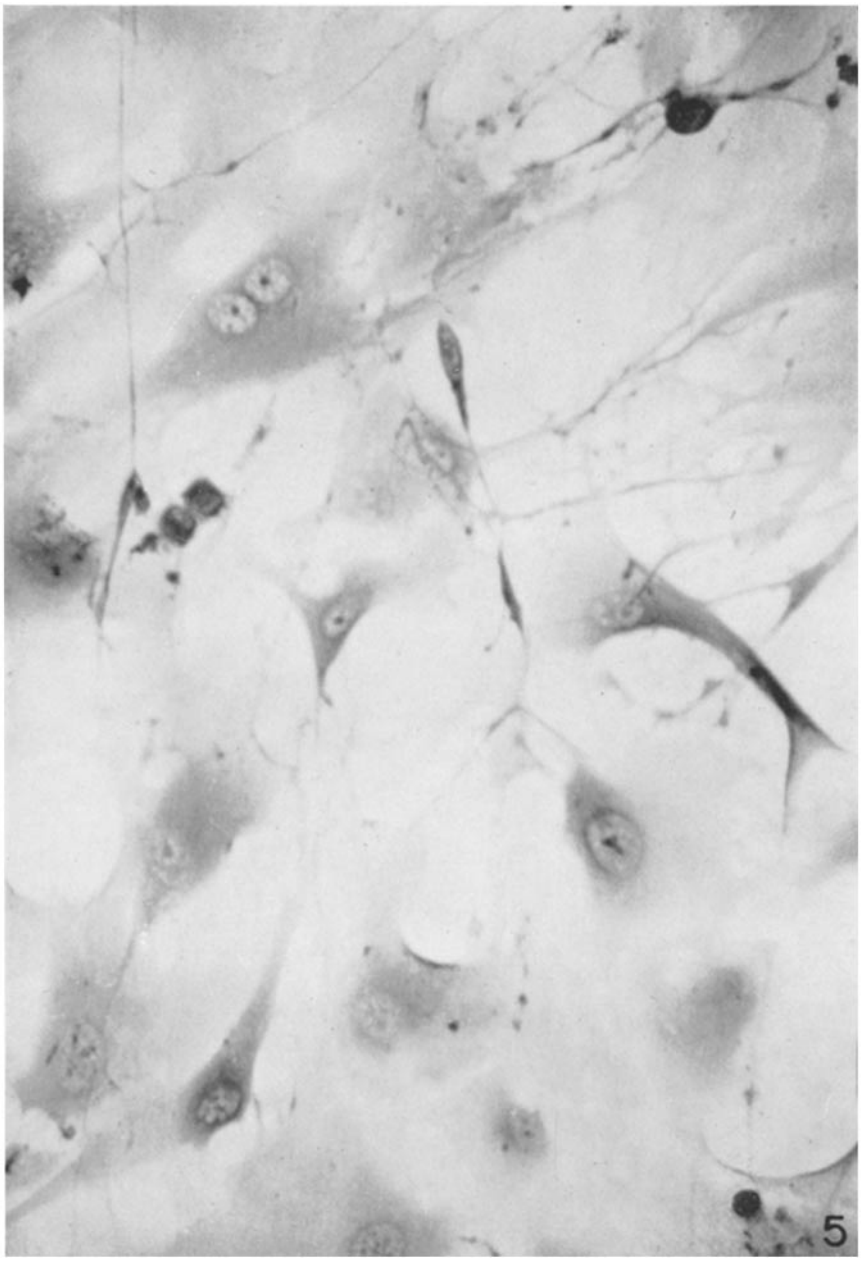
FIG. 4. Primary peritoneal exudate cells 24 days in tissue culture.



(McKenna and Stevens: Antibody formation by peritoneal exudate cells)

PLATE 52

FIG. 5. Primary trypsinized rabbit kidney cells after 24 days in culture.



(McKenna and Stevens: Antibody formation by peritoneal exudate cells)