ANTIBODY FORMATION IN VITRO*

By M. FISHMAN, Ph.D.

(From the Division of Applied Immunology, The Public Health Research Institute of The City of New York, Inc., New York)

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The successful initiation of a primary antibody response in tissue culture has been reported by several workers (1–4). In a previous report from this laboratory (3) it has been suggested that the sequential action of at least two different types of cells was responsible for the formation of antibody in tissue culture. One type was a mononucleated cell with phagocytic activity, referred to as a macrophage, and the other was a non-phagocytic cell belonging to the lymphocytic series. The macrophage, presumably a non-antibody—producing cell, was thought to interact with the antigen yielding a product which in some manner stimulated the production of specific antibody in the lymphocytic cells. The role of the macrophage, and the nature of the active substance(s) that result from the interaction of antigen and macrophages, were further investigated. The results of these experiments in which tissue culture and chick embryo techniques were used are presented in this paper.

Materials and Methods

Lymph Node Cells.—Young Wistar strain rats (60 gm) obtained from Blue Spruce Farms, Altamount, New York, were used as the source of lymph node cells. The animals were sacrificed by a blow on the head and the two lumbar nodes, the renal node, and an intestinal node (5) were excised. The nodes from 10 rats were teased manually in 6 ml of a Special Buffer Solution (SBS) of the following composition: NaCl 0.8 per cent, KCl 0.04 per cent, Na_2HPO_4 0.0568 per cent, KH_2PO_4 0.0095 per cent, $MgSO_4$ 0.0060 per cent, phenol red 0.002 per cent, and $CaCl_2 \cdot 2H_2O$ 0.014 per cent. The suspension of teased cells was then filtered through sterile gauze to remove tissue fragments as well as clumps of cells. After centrifuging the cells at $200 \times g$ for 10 minutes in the cold, the cells were washed once with SBS and then counted. Approximately 20×10^6 cells were obtained per rat; not more than 5 per cent of these cells were macrophages.

Rabbit lymph node cells were obtained by teasing the mesenteric and popliteal nodes in a similar manner. A much higher yield of lymph node cells (480×10^6) per rabbit was obtained; approximately 15 per cent of the cells were macrophages.

Macrophages.—Rat macrophages were obtained by injecting 5 ml of a beef heart infusion broth fortified with 10 per cent proteose-peptone No. 3 (6) intraperitoneally into 200 to 250 gm Wistar rats obtained from Blue Spruce Farms. The rats were sacrificed by a blow on

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the head 48 hours later and their peritoneal cavities were flushed with SBS. The macrophages were harvested by centrifugation in the cold at $200 \times g$ for 10 minutes and washed twice with 10 ml of saline buffered at pH 7.6. The average number of macrophages per rat was 40×10^6 cells. Exudates with noticeable red blood cell contamination were discarded. For a typical experiment the pooled exudates from 15 rats were used.

Rabbit macrophages were obtained by tapping the peritoneal exudate of animals injected intraperitoneally 4 to 5 days previously with 50 ml of sterile bayol F (light mineral oil). The exudates were pooled and placed in a separatory funnel to remove most of the oil. The cells were centrifuged at $200 \times g$ for 10 minutes in the cold and washed twice with buffered saline. Exudates obtained in this manner contained up to 400×10^6 macrophages per rabbit.

Macrophage Treatment of the Bacteriophage.—Macrophages and bacteriophage were mixed and incubated under conditions favorable for surface phagocytosis. Specifically, approximately 100×10^6 macrophages were centrifuged at $200 \times g$ for 10 minutes in the cold, and to the packed cells were added 3 to 5 ml of a bacteriophage T2 suspension containing approximately 5×10^5 plaque-forming units (pfu). The macrophages were dispersed in the bacteriophage suspension and the mixture allowed to incubate, without stirring, at 37°C for 30 minutes. After incubation, the macrophages were sedimented by centrifugation at $140 \times g$ in the cold for 8 minutes and the supernatant withdrawn. The cells were washed twice with 10 ml amounts of buffered saline and then broken up by means of a TenBroeck tissue grinder. The homogenized preparation was brought to its original volume and then was rendered cell-free and sterile by passage through a bacterial filter. Seitz (sterilizing), Selas No. 3, or Millipore filters (HA) could be used interchangeably. The entire filtrate was mixed with sufficient tissue culture medium to bring the volume to 50 ml which were then distributed in equal amounts among 10 tissue culture plates.

Bacteriophage counts were made on the phage suspension that was added to the macrophages, on the supernatant after incubation and centrifugation, and on the homogenized macrophage preparations. This last fraction, which is of prime interest, contained generally about 10 per cent of the bacteriophage input.

Tissue Culture.—In the course of the present work tissue culture media described by Eagle (7) and by Puck (8), and also Parker's 199 medium (9) and Trowell's medium (10) were employed. Since the last mentioned proved to be the most satisfactory it was used in all the experiments described here. Crystalline chloromycetin used in this medium was obtained through the courtesy of Parke, Davis & Co. The insulin was purchased from Delta Chemical Works, Inc., New York. In some experiments a zinc-free amorphous preparation of insulin kindly donated by Eli Lilly and Co. was used. Results obtained with media containing either of these two insulin preparations were similar.

Rat lymph node cells were placed in tissue culture plates so that each plate containing 5 ml of medium received 6×10^6 cells. The plates were incubated in an atmosphere of 6 per cent CO_2 in air. Within the first 24 hours of incubation lymphocytic cells attached themselves firmly to the glass from which they could be detached with trypsin (0.5 per cent) but not by versene (0.02 per cent disodium versenate). The viability of the cultured cells was examined by vital staining techniques and it was found that a considerable portion of the apparently intact cells remained viable for about 12 days. After this time the cells deteriorated rapidly.

Neutralizing Activity of Tissue Culture Fluids.—After varying periods of incubation the fluids from the rat lymph node cell cultures were collected and appropriately pooled. Usually the fluids from 10 replicate plates made up one pool. After centrifugation at 10,000 RPM in the cold for the removal of cells and debris the supernates were treated with saturated ammonium sulfate solution (one-half or one-third saturation), usually in the presence of added serum which served as a carrier. The resulting precipitates were suspended and dissolved in that volume of buffered saline which would result in a tenfold concentration of the globulin

fraction in the final solution obtained after overnight dialysis in the cold against buffered saline.

In determining the anti-T2 activity of the concentrated tissue culture fluids, 0.2 ml amounts of a phage suspension containing approximately 500 pfu were added to a series of tubes into which had been placed 0.2 ml amounts of serial twofold dilutions of the fluid concentrates. The mixtures were incubated at 37°C for 30 minutes after which time 0.1 ml aliquots were removed and added to 2 ml of semisolid nutrient agar at 45°C. This semisolid agar together with 0.1 ml of an *E. coli* suspension was then immediately poured over the surface of solidified nutrient agar in Petri plates. Duplicate plates were made for each tissue culture fluid dilution. The average plaque count in control plates prepared under these conditions was 100. Since differences in the plaque counts between replicates did not exceed 10 per cent of the mean, and making allowance for possible other sources of variation, a decrease in plaque count exceeding 20 per cent of the control was considered to indicate a significant degree of neutralization. The average degree of neutralization encountered in the reported experiments was between 25 and 45 per cent when tissue culture fluids were concentrated without added carrier, and somewhat higher when concentration was performed after the addition of serum as carrier.

Chick Embryos.—In some of the experiments to be reported chick embryos were substituted for tissue culture. White Leghorn embryos, obtained from Shamrock Farms, New Brunswick, New Jersey, were injected intravenously when 18 days old with 0.2 ml of a mixture consisting of equal parts of a suspension of rat lymph node cells and a filtered homogenate of rat macrophages which had been incubated with T2 bacteriophage. This amount of filtrate represented the yield from an incubation mixture composed of 5×10^6 rat macrophages and 2×10^4 T2 bacteriophage particles. The number of lymph node cells injected into each embryo was approximately 3×10^6. The absolute amounts of the various cellular elements injected into each embryo were thus approximately one-half of those introduced into each tissue culture plate. The eggs were incubated in a commercial model incubator, and the hatched chicks were bled periodically starting on the 5th day after hatching.

RESULTS

1. The Production of Antibody in Cultures of Lymph Node cells.—Attempts to initiate the formation of antibody against bacteriophage T2 *in vitro* led to the conclusion that interaction between the antigen and macrophages was an essential prerequisite. Table I shows the results of a representative experiment in which three groups of 10 plates each were employed.

Each plate contained 5 ml of Trowell's medium seeded with approximately 6×10^6 lymph node cells obtained from "normal" rats. To the plates of group P were added 12,000 plaque-forming units (pfu) of T2 in 2.8 ml of buffered saline. The plates in group MP received 2.8 ml of a Seitz filtrate obtained from the homogenate of rat macrophages which had been incubated with T2 for 30 minutes at 37°C. Plates in the third group (M) received 2.8 ml of a similar filtrate from homogenized rat macrophages which, however, had not been exposed to the bacteriophage. Culture fluids were pooled in each group after 8 days of incubation. The globulins were precipitated by half-saturated ammonium sulfate, then were dissolved in and dialyzed against buffered saline in the cold overnight. The final volume was adjusted so as to obtain a tenfold concentration of the globulins.

It may be seen that significant neutralization activity (43 per cent) arose only in the plates of group MP. In other similar experiments antibody was not

always produced. Failure to find antibody production by the cells of group MP in any given experiment could not always clearly be attributed to technical causes. Such failures occurred on the average in 4 of 5 attempts. In all experiments in which antibody was found, it was present in fluids from group MP and in no instance in fluids from groups P or M. It has been previously reported (3) that under these experimental conditions the earliest detectable antibody appeared in plates of group MP on the 5th day of incubation and that the level rose or remained the same, on continued incubation, until the 8th to 12th day. This observation has been confirmed in the work presented here.

The rather low yield of antibody (usually just enough to cause 25 to 35 per

TABLE I

Neutralization of Bacteriophage T2 by Fluids from Rat Lymph Node Cells Maintained in Tissue Cultures for 8 Days

Group	Material added to tissue culture	No. of plaques/0.2 ml after incubation		Per cent neutralization
		0* min.	30 min.	
MP	Macrophage-T2 phage extract	44	25	43
P	Phage alone	58	54	7
M	Macrophage extract alone	56	55	2

* 0 time results represent the number of phage added to the tissue culture fluids as found in an aliquot removed immediately after mixing. Similar counts were also obtained when phage was added to a dilution of fluids showing no neutralizing activity.

cent neutralization) was, to a minor extent, a result of incomplete recovery of the precipitated globulin during the ammonium sulfate fractionation. Shown in Table II are the results of an improved fractionation-concentration procedure in which normal rat serum was added to the fluids as carrier. It may be seen that in the presence of normal rat serum in final concentration of 1 per cent significantly more antibody activity was recovered than in the absence of such carrier. It was also evident that an increase in carrier concentration to 5 per cent final concentration led to no further increase in antibody recovery. In this and subsequent experiments no normal rat serum with phage-neutralizing activity was used.

In an effort to determine whether the antibody found in culture fluids on the 8th day of incubation was the sum of antibody produced in the culture during the entire incubation period the following control experiment was carried out:

Ten rats were injected intraperitoneally with 1 ml of a phage suspension containing 1×10^8 pfu every 3rd day for 2 weeks. After 1 week's rest, they were bled from the heart and their sera assayed for T2 neutralizing activity. The results showed that a 50 per cent reduction of

an original phage input of 1000 pfu occurred with a 1:400 dilution of the pooled serum after 30 minutes' incubation at 37°C. This neutralization of phage displayed the kinetics of a first order reaction with a calculated K value of 4. The serum was diluted so that its neutralizing activity matched that of tissue culture fluids, and aliquots were then introduced into plates which contained the usual amount of Trowell's medium seeded with lymph node cells from normal rats. Incubation was carried out under the standard conditions, and at the times indicated in Fig. 1 fluids were harvested and processed in the usual manner.

It was found that neutralizing activity suffered no decrease during the first 5 days of incubation. After this period there was a rapid loss of antibody activity; none could be detected by the 8th day. It cannot be said whether this loss of activity was the result of catabolism, inactivation, or adsorption to cells, cellular debris, or glass. Unpublished experiments suggest that on prolonged incubation cultured lymph node cells may remove some of the gamma globulin from tissue culture medium. In these experiments fluorescein-labeled anti-rabbit gamma globulin detected rabbit gamma globulin on or in rabbit lymph node cells maintained in tissue culture in the presence of 20 per cent normal rabbit serum after 6 to 8 days of incubation but not before this time. Thus it seems possible that only part of the antibody produced by the cells was detected in the tissue culture fluids harvested after 8 days of incubation.

TABLE II

Recovery of T2 Neutralizing Activity from Culture Fluids of Rat Lymph Node Cells in the Presence of Added Carrier

Dilution of fluids	Per cent neutralization		
	A	B	C
1:2	46	72	76
1:4	29	49	52
1:8	15	30	25
1:16	4	0	0

A, no carrier used in precipitation of globulins. B and C, normal rat serum added as carrier in 1 per cent or 5 per cent final concentration, respectively.

2. The Specificity and Other Properties of Antibody Formed in Tissue Culture.—Tissue culture fluids in which antibody against T2 had appeared after proper stimulation of the cells were tested for neutralizing activity against the serologically unrelated phages T1 and T5 and none was found.

The precipitability of the neutralizing activity of tissue culture fluids by half, or by third saturation with ammonium sulfate strongly suggested that the activity was associated with globulins but for more precise identification of the neutralizing substance the serum from rabbits immunized against rat serum gamma globulin was employed. Immunoelectrophoretic examination of

the serum showed its antibody to be directed mainly against gamma globulin but in addition it contained traces of antibody against a rat beta globulin. The results shown in Table III demonstrate that preincubation of the tissue culture globulins with rabbit antiserum diluted 1:5 led to the loss of T2 neutralizing activity, presumably because of aggregation of the rat anti-T2 gamma globulin. The addition of the rabbit immune serum after T2 and the tissue

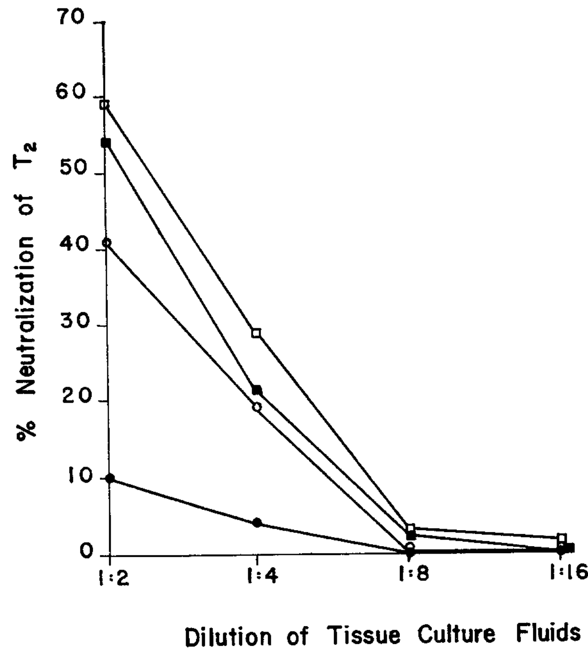

FIG. 1. Recovery of neutralizing serum antibody added to rat lymph node cell tissue culture in trace amounts. ■, 0 days post-incubation, □, 2 days post-incubation, ○, 5 days post-incubation, ●, 8 days post-incubation.

culture globulins had been allowed to react had no effect on the degree of phage neutralization observed. Other experiments showed that higher dilutions (1:1000) of the rabbit anti-rat globulin serum gave similar results. These observations are consistent with the concept that neutralization of T2 was mediated by antibody gamma globulin produced by the lymph node cells in response to the proper stimulus.

The kinetics of T2 phage neutralization by the antibody produced by lymph node cells *in vitro* were investigated. The results presented in Fig. 2 show that the antibody produced *in vitro* may not adhere to the first order kinetics commonly found in the neutralization of phage by antisera (11). They were similar to those reported by Jerne and Avegno (12) for antibody produced by

horses during the early course of immunization and to those found in early bleedings from immunized rats (unpublished data).

In a typical experiment (Fig. 2) 2 ml of a globulin solution from tissue culture fluids of properly stimulated rat lymph node cells were mixed with 2 ml of a

TABLE III

Inhibition of T2 Neutralizing Activity of Tissue Culture Fluids by Rabbit Anti-Rat Globulin

Dilution of tissue culture fluids	Per cent neutralization		
	Immune rabbit serum (anti-rat globulin) added		Normal rabbit serum (control)
	Before T2	After T2	
1:2	6	43	53
1:4	0	21	22
1:8	0	6	11
1:16	0	0	0

0.1 ml of rabbit anti-rat globulin serum diluted fivefold was added to 0.5 ml of tissue culture fluids. Mixture incubated for 30 minutes at 37°C. 0.5 ml of a phage suspension (460 pfu) was used.

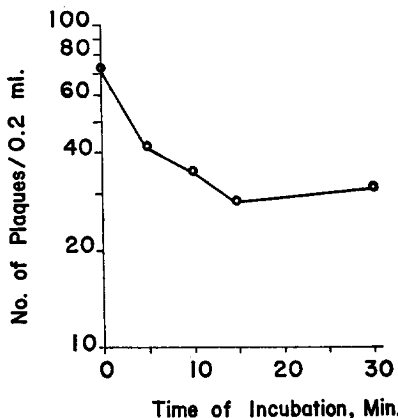

FIG. 2. The rate of bacteriophage T2 neutralization by tissue culture fluids.

suspension of T2 containing 3.7×10^4 pfu/ml. Samples were removed at varying times during incubation of the mixture at 37°C and after appropriate dilutions were assayed for residual phage activity. The time course of inactivation is best described by a rapid decline of infectivity during the first 5 minutes ($K = 0.226$), followed by a continued inactivation at a lower rate ($K = 0.074$) during the next 10 minutes of incubation, after which time no further neutralization occurred. The mixture was found to contain some residual neu-

tralizing activity after this period as shown by its neutralizing action against newly added phage.

3. Substitution of the Chick Embryo for Tissue Culture.—Since there was no assurance that maintainance of rat lymph node cells in Trowell's medium under the conditions described provided optimal conditions for antibody synthesis, intact chick embryos were used to nurture the rat lymph node cells.

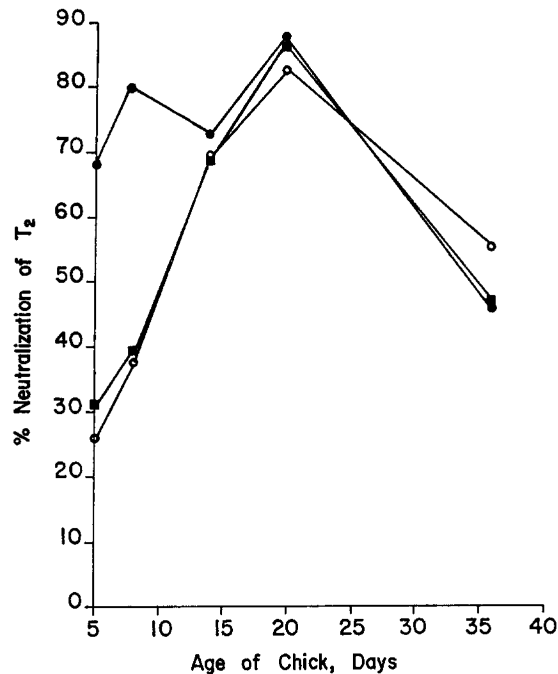

FIG. 3. Bacteriophage T2 neutralization by sera (chick serum diluted 1:2 with buffered saline) from chicks that were injected *in ovo* with T2, rat lymph node cells plus T2, or rat lymph node cells plus macrophage-treated T2. ●, MPL, ○, P, ■, PL.

Eighteen day old embryos were injected intravenously with T2 phage alone (group P), T2 phage and rat lymph node cells (PL), or rat lymph node cells and Seitz filtrate from homogenized rat macrophages which had been preincubated with T2 phage (MPL). The amounts of these reagents injected were approximately half the amounts introduced into tissue culture plates containing 5 ml medium; hence, assuming a blood volume of 2 ml for the embryos, the final concentrations would be comparable. Blood samples were taken after hatching and tested for T2 neutralizing capacity.

Representative data from such experiments are shown in Fig. 3. It will be noted that the MPL group of chicks showed a rapid rise of serum antibody which attained a maximum on the 5th to 8th day after hatching whereas chicks

in the other two groups showed a much slower increase in antibody which did not reach a maximum level until the 20th day after hatching. It will also be noted that no perceptible difference was found between chicks in groups P and PL, an observation which suggested that the antibody produced was chicken antibody and that the rat lymph node cells injected into group PL birds had made no significant contribution to the antibody formed.

TABLE IV

Detection of Rat Globulin in the Serum of Chicks Injected in Ovo with Rat Lymph Node Cells plus Macrophage-Treated Bacteriophage T2

Inhibiting antigen added to rabbit anti-rat globulin serum		Dilution of inhibiting antigen	Inhibition of HA*
Rat gamma globulin (255 μg N)		1:1600	+
		1:3200	+
		1:6400	+
		1:12,800	+
		1:25,600	±
		1:51,200	−
Normal chick serum		1:2	−
		1:4	−
		1:8	−
		1:16	−
Chick serum from group MPL	6 to 8 day old chicks	1:2	+
		1:4	+
		1:8	±
		1:16	−
	16 day old chicks	1:2	−
		1:4	−
		1:8	−
		1:16	−

* Inhibition of the agglutination of tanned erythrocytes coated with rat gamma globulin by a standard amount of rabbit anti-rat gamma globulin serum.

It remained to be proven that the antibody detected in the 5 to 8 day old chicks of group MPL was rat antibody produced by the rat lymph node cells in response to the stimulus of the rat macrophage–T2 phage digestion mixture. As a first approach to this problem an attempt was made to demonstrate rat gamma globulin in the sera of these chicks. For this purpose the rabbit immune serum against rat gamma globulin used in a previous experiment (see above) was employed in a hemagglutination inhibition test based on the method of Boyden (13). This serum agglutinated tanned red cells, coated with electrophoretically purified rat gamma globulin, to a titer of 1:100,000. When this

serum was used in a 1:40,000 dilution its agglutination activity could be completely inhibited by 0.009 μg of rat gamma globulin nitrogen. Data presented in Table IV show that similar inhibition could be demonstrated with serum from chicks in group MPL obtained on the 6 to 8th day after hatching; normal chick serum was devoid of inhibiting activity as were the sera from chicks in groups P and PL. Serum from chicks of group MPL obtained 16 days after hatching no longer appeared to contain rat gamma globulin.

Though the results just presented indicated that rat gamma globulin had been produced in chicks of group MPL during the period of particular interest, more direct evidence in support of the notion that at least part of this gamma globulin was antibody against T2 appeared desirable. To obtain additional

TABLE V

Neutralization of Bacteriophage T2 by Serum from Chicks Injected as 18 Day Embryos with Heated or Viable Rat Lymph Node Cells plus Macrophage-Treated T2

Dilution of chick* serum	Per cent neutralization of T2	
	Δ Lymph node cells + macrophage-treated T2	Lymph node cells + macrophage-treated T2
1:2	10	70
1:4	12	48
1:8	2	10
1:16	0	0

* Chicks 6 to 8 days old.

evidence two groups of 18 day old embryos were injected with Seitz filtrate of homogenized rat macrophages that had been incubated with T2 phage. The first group of embryos received, in addition, viable rat lymph node cells, while the control group was injected with rat lymph node cells that had been inactivated by heat. Results of this experiment, shown in Table V, demonstrated that the high levels of neutralizing activity characteristically attained by chicks in group MPL on the 5th to 8th day appeared only in the experimental group but not in the control group of chicks.

The levels of antibody against T2 attained in chicks of group MPL were on the average higher than those obtained with the tissue culture technique.

4. Studies on the Reaction between Macrophages and Bacteriophage T2.—Under the experimental conditions described in the preceding sections antibody production by lymph node cells from normal rats was induced only by bacteriophage which had been preincubated with rat macrophages. Experiments now to be described were designed to elucidate the nature of this apparently essential step.

(*a*) *The effect of variation in antigen dose.*—

Aliquots of a suspension of rat macrophages were incubated with varying amounts of T2 phage under the standard conditions employed in previous experiments. The sedimented and washed macrophages of each mixture were then homogenized and the filtrates of these homogenates were injected into 18 day old chick embryos together with rat lymph node cells.

The results presented in Table VI show that in chicks of the experimental group (MPL) optimal results were obtained with the filtrates from mixtures of macrophages and T2 phages which had received approximately 4×10^5 pfu, a ratio of approximately 250 macrophages per bacteriophage. When greater amounts of phage were used the filtrates were less active. Similarly, when the amount of phage added to the macrophages was decreased to approximately 4×10^4 pfu the filtrates were less active. Also shown in the table are the results of control experiments which, consistent with earlier findings, showed that 8 day old chicks injected *in ovo* with varying amounts of phage, with (PL) or without (P) lymph node cells, had little or no neutralizing activity in their sera.

TABLE VI

Effect of Antigen (T2) Concentration on the Neutralizing Antibody Titers Attained by 8 Day Old Chicks Injected in Ovo

Group	Number of phage added to 10^8 macrophages	Per cent neutralization by chick serum diluted 1:2
MPL	3.6×10^7	0
	3.6×10^6	35
	3.6×10^5	85
	3.6×10^4	51
P	3.6×10^7	0
	3.6×10^6	20
	3.6×10^5	10
PL	3.6×10^7	0
	3.6×10^6	2
	3.6×10^5	0

In the *in vitro* system similar results were obtained: Increases in the phage-macrophage ratios to values greater than 1:20 yielded preparations which induced little or no antibody in cultures of lymph node cells.

In view of the results just presented it appeared of interest to determine just how much of the phage was adsorbed to or ingested by the macrophages. It was found that in each of these mixtures in the table approximately 90 per cent of the phage remained in the supernatant.

Relevant to the problem under consideration is also the question whether different preparations of macrophages could be expected to have similar activity and also whether one could anticipate that all the cells in a given macrophage

preparation would be equally active. Some preliminary studies with an antigen that could be directly visualized on or in macrophages (*E. coli*) showed that only 10 to 20 per cent of the macrophages in a given preparation would interact with the bacterial cells. This finding suggested that macrophage preparations may be heterogeneous with respect to their reactivity with a given antigen, or alternatively, that only a fraction of the cells in any given preparation may be in the proper physiological state to interact with antigens in general. These considerations suggest a number of explanations for the apparent need for very large numbers of macrophages in the procedure presented here.

(*b*) *Variation in the time of incubation of macrophage-phage mixture.*—

TABLE VII

Effect of Length of Incubation of Macrophages with T2 on the Ability of the Macrophage-T2 Extract to Stimulate Antibody Formation in a Rat Lymph Node Cell Tissue Culture

Length of incubation of macrophages with T2 phage suspension	Additional incubation after removal of free phage	Per cent neutralization of T2
min.	*min.*	
10	0	8
30	0	63
10	20	55

A series of tubes, each containing 10^8 macrophages and 5×10^5 T2 phages, were incubated for varying periods of time at 37°C. The cells were sedimented, washed, homogenized, and the filtrates of the homogenates were then added to cultures of rat lymph node cells.

The results, shown in Table VII, indicated that 30 minutes of incubation, but not 10 minutes sufficed to provide an active filtrate.

A change in experimental procedure yielded results which suggested that the production of an active filtrate depended on a reaction, or a chain of reactions, more complex than simple adsorption or ingestion of the antigen. It was found that if one removed free T2 bacteriophage after 10 minutes of incubation, then suspended the macrophages in buffered saline and continued incubation for an additional 20 minutes, such macrophages yielded an active filtrate (Table VII).

(*c*)*Substitution of macrophages from other species for those from rats.*—One possible reason for the apparent need for preliminary macrophage action on T2 phage appears to be that the resulting "solubilization" would lead to an increase in immunogenicity. If this were the correct explanation, then macrophages from other animal species should be capable of replacing those from the rat.

Tests were carried out in which filtered extracts from incubation mixtures of T2 phage with macrophages from the rat or the rabbit, or from mixtures of HeLa cells with phage, were injected into 18 day old chick embryos together with normal rat lymph node cells. The chicks were bled on the 5th day after hatching and their sera were assayed for neutralizing activity against T2 phage.

It may be seen from Table VIII that the rat lymph node cells were stimulated into antibody production by the filtrate from the T2 phage–rat macrophage mixture only, and not by the filtrates from the other mixtures (lines 2 and 3).

Also shown in Table VIII are the results of parallel experiments in which

TABLE VIII

Effect of Substitution of Heterologous Cells for Rat Macrophages in the Initiation of Antibody from Rat Lymph Node Cells

Lymph node cell source	Macrophage source	Per cent neutralization*
1 Rat	Rat	70
2 Rat	Rabbit	11
3 Rat	HeLa	0
4 Rat	—	3
5 Rabbit	Rabbit	42
6 Rabbit	Rat	20
7 Rabbit	—	19

* A 1:2 dilution of serum from 7 day old chicks injected as 18 day embryos.

lymph node cells from normal rabbits were used instead of those from rats. A low level of neutralizing activity was found in the sera of chicks which had received T2 phage and rabbit lymph node cells (line 7). Significantly higher levels were present in the sera of chicks injected with rabbit lymph node cells plus filtrates from incubation mixtures of T2 phage and the homologous (rabbit) macrophages. The heterologous macrophages (in this case from the rat) again were inactive. It has previously been pointed out (Materials and Methods) that lymph node cell preparations from rabbits always contained significantly more macrophages than did similar preparations from rats, an observation which may explain the low but measurable response in phage–rabbit lymph node cell preparations recorded here.

In vitro experiments yielded results in complete agreement with those just described. These observations lend support to the concept that the essential reaction product of the T2 phage–macrophage interaction is not necessarily solubilized phage antigen but rather a substance containing components of macrophage origin with or without phage antigens.

(d)Some properties of the effective filtrate from macrophage-phage interaction.— Set forth are several of the properties of the active filtrates which shed some light on the physicochemical nature of the active substance(s).

Stability at low temperatures: Freshly prepared filtrates which successfully induced antibody formation in tissue culture were stored at either 4°C or −80°C. On reusing these filtrates after varying periods of storage, it was observed that those stored at 4°C no longer exerted an "induction" effect after the 2nd day of storage. The filtrate stored at −80°C showed little loss of activity when tested after 9 days. Upon longer storage at −80°C the biological activity of these filtrates appeared to be diminished.

TABLE IX

The Heat Stability of the Rat Macrophage–T2 Phage Extract Used to Initiate Antibody Formation in Tissue Culture

Extracts heated at temperatures	Per cent neutralization of T2 by fluids from tissue cultures after incubation	
	6 days	10 days
°C/30 min.		
37	45	75
56	40	ND*
80	30	ND
100	19	12

* Not done.

Thermostability: Shown in Table IX are results of a representative experiment in which aliquots of filtrate obtained from homogenized rat macrophages which had been incubated with T2 phage were heated at various temperatures for 30 minutes. Each aliquot was then tested for its ability to stimulate antibody production against T2 phage in cultures of rat lymph node cells. It will be noted that the biological activity of the extracts was rather heat-stable. Partial or complete loss of activity occurred upon exposure, for 30 minutes, to temperatures in the 80–100°C range.

Effect of treatment with ribonuclease: Studies in progress with various enzymes have revealed one finding of particular interest: Antibody production by rat lymph node cells against T2 phage was completely inhibited by the action of ribonuclease.[1] This is demonstrated by the results shown in Table X which are based on an experiment in which 2 micrograms of ribonuclease had been added to each milliliter of Trowell's medium.

Further experiments shown in Table XI indicated that the inhibition of

[1] Three times recrystallized, salt-free, and free of protease activity, obtained from Worthington Biochemical Corporation, New Jersey.

antibody formation was not caused by a deleterious effect of ribonuclease on the lymph node cells. It will be noted that incubation of lymph node cells with as much as 50 micrograms of ribonuclease at 37°C for 30 minutes, followed by washing of the cells, failed to impair the ability of the cells to produce antibody in response to the proper stimulus. These findings, together with similar observations of Dineen (14), suggested strongly that the enzyme exerted its inhibiting effect not by action on the lymph node cells but rather by acting on

TABLE X

Effect of RNAse on Antibody Synthesis in Rat Lymph Node Cell Tissue Culture

Dilution of fluids*	Per cent neutralization of T2	
	Without RNAse	With RNAse
1:2	62	0
1:4	28	0
1:8	18	0
1:16	0	0

* Fluids harvested after 7 days' incubation.

TABLE XI

The Ability of RNAse-Treated Rat Lymph Node Cells to Form Antibody in Vitro

Lymph node cells (LN)	No. of plaques/0.2 ml after incubation		Per cent neutralization* of T2
	0 min.	30 min.	
RNAse-treated LN	61	35	43
Non-treated LN	62	32	48

* Tissue culture fluids diluted 1:2.

an RNAse-sensitive component in the filtrate from the macrophage-T2 phage reaction mixture. Since the bacteriophage antigen concerned in neutralization of phage by antibody is believed to be a protein, it may be assumed that the vulnerable component in the mixture was ribonucleic acid from the rat macrophages.

Inhibition of antibody formation in the presence of streptomycin: In the course of preliminary experiments it was found that Trowell's medium supported antibody production by rat lymph node cells in response to the stimulus of an active filtrate if the antibiotic used was penicillin (1000 units/ml) or chloromycetin[2] (0.03 microgram/ml), but consistently failed when the medium

[2] Parke, Davis and Company.

contained streptomycin in a concentration of 0.02 microgram/ml. Typical results are shown in Fig. 4. Since it has been reported (15) that streptomycin has no adverse effect on the rate and amount of antibody formation by lymph node cells from immunized animals, and in view of (*a*) the known interaction between streptomycin and nucleic acid (16) and (*b*) the deleterious effect of ribonuclease on the system described above, these results lend additional support to the notion that ribonucleic acid from the macrophage might play an important role in the initiation of antibody formation by normal lymph node cells.

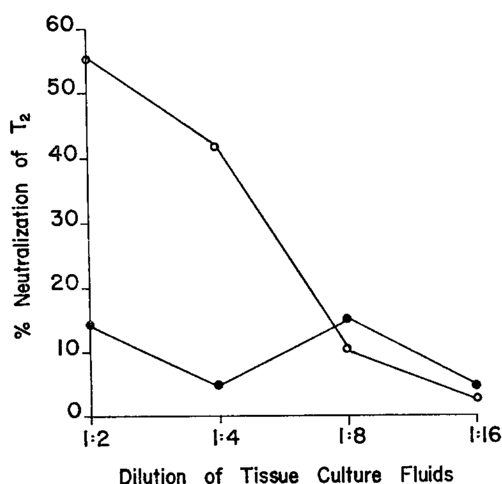

FIG. 4. Effect of antibiotics on antibody formation in tissue culture. ○, penicillin (1000 units/ml), ●, streptomycin (0.02 μg/ml).

DISCUSSION

The role of the macrophage in antibody production has been described by Burnet (17) as an entirely passive one; by removing excess antigen the macrophages merely protect antibody-producing cells from the repeated exposure to antigen which might exert a deleterious effect on these cells. The opposite view is held by a number of other workers who consider the macrophage as an active producer of antibody (4, 18). Still others believe that the macrophage solubilizes antigens and thereby merely enhances the stimulus to antibody-producing cells (19). McMaster (20) has recently reviewed the evidence against antibody formation by macrophages.

In our hands all attempts to demonstrate the formation of antibody against T2 phage or bovine serum albumin by macrophages in tissue culture have failed. Likewise we have not been able to observe antibody production against these two antigens in cultures of mononucleated cells from rabbit peritoneal exudate as described by McKenna and Stevens (4).

The recent work by Halpern (21) has lent new impetus to the notion that the phagocytic activity of the reticulo-endothelial system plays a significant role in antibody production. The results presented in the current paper lend further support to this concept. Moreover, evidence presented here indicates that the solubilization of the antigen alone is not the correct explanation for the role of the macrophage in antibody formation, since it has been shown that macrophages and lymph node cells must be obtained from animals of the same species in order to secure an active system. In addition, the observed inhibition of the formation of neutralizing antibody against T2 phage (presumably antibody against phage tail protein) by streptomycin and by RNAse suggests that the active product of the macrophage-antigen interaction might consist, entirely or in part, of RNA. That complexes between RNA and antigenic fragments might play an important role in antibody formation has been indicated by the findings of Garvey and Campbell (22). It seems possible that the RNAse–sensitive material transferred from the macrophages to the lymph node cells in tissue culture is the template for antibody production by the latter cells. This concept is currently under further investigation.

Thiery (23) and Sharp and Burwell (24) have recently described structural units in the lymph nodes of immunized animals which consist of phagocytic cells that are surrounded by lymphocytic cells. Similar "islands" have been observed in this laboratory in tissue cultures of teased lymph node cells from immunized rabbits. If these units could be clearly related to antibody production they would suggest the physical means by which RNAse-sensitive material is transferred from macrophages to antibody-producing cells.

The disturbing factor in this presentation is the low yield of antibody and the low frequency of successful experiments. Both of these factors would necessarily go hand in hand. Many reasons can be given to explain these factors some of which were mentioned in appropriate prior parts of this paper. One of the more important reasons is the fact that the tissue culture cells were non-proliferating. Although it has been shown by Sterzl (25) and Holub and Říha (26) that non-dividing cells from immune, as well as those transferred from non-immune animals, are still capable of synthesizing antibody, the amount of antibody would be very low. A system which would allow for the reproduction of competent cells such as chick spleen injected into an homologous embryo (27) facilitates attainment of higher antibody titers.

Recently experiments with diffusion chambers have confirmed the notion that the low antibody titer in tissue culture was due to suboptimal nutritional environment for lymph node cells. Rats that received 500 r total body x-irradiation, served as recipients of 0.1 μ Millipore filter chambers which contained rat lymph node cells with T2 and/or macrophage-treated T2 bacteriophage. The titers of the T2 neutralizing antibody in sera of rats with chambers containing lymph node cells plus macrophage-treated T2 were considerably higher than any reported in the present paper.

The lymph node cells in tissue culture do show signs of becoming modified. At the end of 5 to 7 days of incubation, certain cells possess a basophilic cytoplasm which differentiates them from the original lymphocytes. However, no typical plasma cells were detectable in any of the plates examined.

SUMMARY

Neutralizing activity against T2 bacteriophage appeared in cultures of lymph node cells from normal rats in response to their *in vitro* stimulation with a cell-free filtrate derived from homogenized rat macrophages which had been incubated with T2 bacteriophage.

This activity was specifically directed against T2 bacteriophage. It resided in a fraction of the culture fluid which had the salting-out properties of serum globulin. Phage neutralization was inhibited by antibody specific for rat serum gamma globulin.

Antibody production against T2 bacteriophage in cultures of lymph node cells from normal animals failed to occur if (*a*) T2 bacteriophage alone was added, (*b*) if the incubation period of macrophages and T2 phage was unduly shortened, (*c*) if the cell-free filtrate was heated at 80–100°C for 15 minutes, (*d*) if more than an optimal amount of T2 bacteriophage was added to the macrophages. Additional factors which prevented the formation of antibody were the heat inactivation of the lymph node cells or the addition to the culture medium of either streptomycin or ribonuclease. Finally, it was found that macrophages and lymph node cells had to be obtained from animals of one and the same species.

All essential findings on the production of antibody to T2 bacteriophage *in vitro* could be confirmed by substitution of the chick embryo for the tissue culture medium.

The results are discussed in terms of a possible mechanism of antibody production in which an RNAse-sensitive substance resulting from the interaction of macrophages and antigen is capable of stimulating antibody synthesis in lymphocytic cells.

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