

ANTIBODY FORMATION INITIATED IN VITRO*

II. ANTIBODY SYNTHESIS IN X-IRRADIATED RECIPIENTS OF DIFFUSION CHAMBERS CONTAINING NUCLEIC ACID DERIVED FROM MACROPHAGES INCUBATED WITH ANTIGEN

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Previous reports (1, 2) have described a system in which a culture of lymph node cells from non-immunized rats was stimulated, *in vitro*, to produce specific antibody. This stimulus was provided by a cell-free homogenate of peritoneal exudate cells, consisting mainly of macrophages, which had been incubated with the antigen in question. The observations that both streptomycin and ribonuclease rendered the system ineffective directed attention to the possibility that nucleic acid was an essential factor. The experiments to be presented show that the specific stimulatory activity resides in a purified ribonucleic acid fraction of the cell-free homogenate.

Tests for activity of the purified material were conducted with the aid of diffusion chambers. Such chambers, charged with non-immune rat lymph node cells and the material to be tested, were inserted intraperitoneally into x-irradiated rats. In some experiments the material alone was placed into the chambers which were then inserted into non-irradiated recipients. Activity was measured by titrating the sera of the recipient rats for specific antibody.

Material and Methods

Animals.—200 to 250 gm Wistar rats were used as recipients for the diffusion chambers, and as the source of exudate cells, hereafter referred to as macrophages. 60 to 80 gm rats served as lymph node donors. The procedures for obtaining both macrophages and lymph node cells have been described in a previous paper (2).

X-Irradiation Factors.—Rats were exposed to 500 r total body irradiation through the facilities of the Brookhaven National Laboratories. The x-ray factors were 250 KVP, 15 ma, 0.5 mm Cu + 1.0 mm Al; distance from target to skin, 40 inches; roentgens in air per minute, 28.0.

Macrophage-Antigen Interaction.—The procedure for obtaining sterile cell-free homogenates of macrophage-bacteriophage T2 mixtures has been described (2). In brief, the macrophages

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were incubated with the antigen for 30 minutes at 37°C. Then they were washed and homogenized, and the homogenate was passed through a bacterial filter. A ratio of macrophages to T2 of approximately 200 to 300:1 was used in all experiments. For the preparation of RNA a mixture of 10^9 macrophages and 5×10^6 pfu of T2 was employed.

RNA extraction.—The method described by Gierer and Schramm (3) was applied to the extraction of RNA from peritoneal exudate cells. To a filtrate prepared from 2 gm (wet weight) of cells (approximately 10^9 cells) suspended in 10 ml of a 0.02 M phosphate buffer pH 7.3 were added 10 ml of water-saturated phenol. The presence or absence of versene in a final concentration of 0.02 per cent in the phosphate buffer appeared to have no detectable effect. The filtrate-phenol mixture was stirred for 10 minutes by means of a magnetic stirrer. The aqueous top layer, obtained by centrifuging the mixture at 1400 g for 10 minutes, was re-extracted with phenol for 2 minutes. 2 and at most 3 extractions were usually sufficient to remove most of the protein. After the final phenol extraction, the aqueous layer (1 volume) was subjected to 7 successive extractions with doubly distilled ether (1 volume each time) to remove the phenol. This was followed by flushing of the aqueous layer with N_2 gas for 30 minutes to remove the ether. All procedures were conducted at 5°C. The ultraviolet absorption of each preparation at 260 and 280 μ m was then determined as well as the hyperchromic effect after treatment with RNAase which had first been heated at 80°C for 10 minutes to inactivate contaminating enzymes.

Sucrose Gradients.—A 5 to 20 per cent sucrose gradient in 0.02 M phosphate buffer, pH 7.3 with or without added Mg^{++} was prepared by the method described by Britten and Roberts (4). 0.2 ml of the RNA material was placed over 4.4 ml of the sucrose gradient. After centrifuging the gradients for 3 hours at 35,000 rpm in a SW-39 swinging bucket rotor, drops were collected through a puncture in the bottom of the centrifuge tubes into a series of tubes each of which received 20 drops (0.2 ml).

Diffusion Chambers.—Millipore¹ or polypore² filter membranes with a rated pore size of 0.1 μ were glued to the top and bottom of lucite cylinders which were 0.5 cm high and had an outer diameter of 2.5 cm and an inner diameter of 2.1 cm (5). The assembled chambers were sterilized by exposure to ultraviolet light. They were filled with 0.5 ml of the test reagents with the aid of a syringe and needle through a hole in the lucite ring which was then sealed with a plastic pin³ (6) and Millipore¹ cement. The filled chambers were implanted, within a few minutes after filling, into the peritoneal cavities of rats. The operation was carried out under ether anesthesia, and the incision was closed with one set of wound clips.

Antibody Assays.—Sera of the rats which had received chambers were assayed for the presence of neutralizing antibody against bacteriophage T2 in the usual way (2). All rats to be used as recipients were first tested for the presence of neutralizing activity in their sera. Only those whose sera, diluted 1:4, neutralized no more than 15 per cent of the test dose of T2 were used. About half of the animals in our stock qualified as recipients by this criterion.

RESULTS

Preliminary experiments showed that the diffusion chamber technic resulted in better antibody production than the tissue culture method previously described (2). In Table I are representative results of such experiments.

Chambers placed into animals of groups 1, 2 and 3 were charged with a Seitz-filtered homogenate derived from macrophages which had been incubated with T2 bacteriophage; those

¹ Obtained from Millipore Filter Corporation, Watertown, Massachusetts.

² Obtained from Gelman Instrument Company, Chelsea, Michigan.

³ Obtained from Williams Gold Refining Company, Buffalo, New York

placed into animals of group 4 received a similarly prepared filtrate from macrophages which had not been exposed to bacteriophage. In addition, chambers placed into group 1 rats received active lymph node cells from normal rats, and chambers placed into group 2 rats received heated lymph node cells.

It will be seen that in x-irradiated rats antibody was produced only in the first group of animals, namely the recipients of chambers containing both normal active lymph node cells and filtrate from specifically stimulated macrophages. The presence or absence of active lymph node cells in chambers placed into non-irradiated rats was without effect on antibody production against T2; among such animals antibody was produced in recipients of chambers contain-

TABLE I

Serum Antibody in Recipients of Chambers Containing Cell-Free Homogenates of Macrophages

Group	Material in chamber	X-irradiated rats (500 r)	Non-irradiated rats
1	M-T2 + LN	6/11*	9/10
2	M-T2 + LNΔ	0/6	8/10
3	M-T2	0/8	4/5
4	M	0/7	0/5

M-T2, Seitz-filtered homogenate of macrophages which had been incubated with T2 bacteriophage. 0.5 ml per chamber, equivalent to 3×10^7 macrophages.

LN, preparation of teased lymph node cells from normal rats, active or inactivated (Δ) by heating at 80°C/15 minutes; 10^6 to 10^7 cells per chamber.

M, Seitz-filtered homogenate of macrophages. Concentration and amount same as in M-T2.

* Number of rats with significant antibody titers/number of rats in group. Significant serum titers: Neutralization of at least 30 per cent of test dose of phage by serum diluted 1:4 or greater. Results are based on serum titers 6 days after implantation of chambers.

ing filtrate from macrophages which had been incubated with T2. Only recipients of chambers charged with the filtrate from non-stimulated macrophages consistently failed to produce antibody.

It is noteworthy that non-irradiated rats which showed a rather uniformly good response to the administration of filtrate from stimulated macrophages in chambers failed to make antibody when the same material was injected intravenously or intraperitoneally. Antibody titers in the fluids of chambers charged with lymph node cells were not systematically examined because the amounts of fluid obtained were small and the titers were in no case greater than those found in the recipients' sera. The serum titers of the recipients of chambers were considerably greater than the levels of antibody found in tissue culture fluids (2), probably between 10- and 50-fold higher.

The results just presented encouraged attempts to characterize the active

principle in the homogenate of macrophages which had been allowed to phagocytose bacteriophage T2.

A suspension of peritoneal exudate cells containing 1.5×10^6 cells was incubated with 5×10^6 pfu of T2 for 30 minutes at 37°C. The macrophages were harvested by centrifugation, washed to remove free T2, and were then homogenized in 0.02 M phosphate buffer of pH 7.3. The homogenate was extracted with phenol in the manner described above. The resulting RNA solution was placed into chambers after appropriate dilution so that each chamber received an estimated 100 μ g of RNA. The concentration of RNA was estimated by assuming that an optical density of 1.0 at 260 m μ corresponds to a concentration of 30 μ g RNA/ml.

The data presented in Table II show the responses of rats which received chambers containing the above preparation or control preparations. Again, as

TABLE II
Serum Antibody in Recipients of Chambers Containing RNA from Macrophages

Group	Material in chamber	X-irradiated rats (500 r)	Non-irradiated rats
1	RNA(M-T2) + LN	8/12*	10/12
2	RNA(M-T2)	0/12	7/9
3	RNA(M-T2) + LN + RNAase	0/11	0/11
4	RNA(M) + LN + T2	0/11	0/11

RNA(M-T2) + LN, approximately 100 μ g RNA extracted from macrophages incubated with T2 bacteriophage + 10^6 to 10^7 lymph node cells from normal rats/chamber.

RNA(M-T2), lymph node cells omitted.

RNA(M-T2) + LN + RNAase, RNAase added to the mixture to give approximately 1.5 μ g of RNAase to 100 μ g RNA/chamber. RNAase heated at 80°C/15 minutes prior to use.

* See Table I. Results are based on titers observed 4 days after implantation of chambers.

in the previous experiment, both x-irradiated and non-irradiated rats were used. Among the irradiated rats antibody was produced in recipients of chambers containing normal lymph node cells plus RNA from macrophages that had been incubated with T2 (group 1). In such irradiated animals no antibody was formed if the lymph node cells were omitted (group 2). In non-irradiated rats the presence of lymph node cells in the chambers was unnecessary for antibody production. Antibody to T2 was not formed if the RNA from T2-stimulated macrophages was treated with RNAase (20 μ g of RNAase was used for 1.5 mg RNA) as shown by the results in group 3. When a mixture of 100 μ g of RNA from rat non-stimulated macrophages, 10^8 pfu of T2, and lymph node cells was placed into diffusion chambers no antibody formation ensued (group 4).

The next experiment was an initial attempt to characterize the size of the active principle in the RNA preparation from specifically stimulated macrophages by subjecting it to centrifugation in a sucrose gradient (5 to 20 per cent sucrose). After optical densities at 260 and 280

$m\mu$ had been measured for all the fractions, pools of these fractions were made which represented the bottom, middle, and top thirds of the gradient. The OD ratios at 260/280 $m\mu$ were 1.5, 1.9, and 2.1, respectively. Each of these three pools was then tested for its ability to initiate antibody production, employing diffusion chambers charged with the fraction and active lymph node cells, and x-irradiated rats.

TABLE III
Serum Antibody in Recipients of Chambers Containing Sucrose Gradient Fractions of RNA

Group	Material in chamber	Estimated $\mu\text{g RNA}^*$	X-irradiated rats (500 r)
1	RNA(M-T2) + LN	150	8/13‡
2	RNA(M-T2) + LN F-3	190	4/8
3	RNA(M-T2) + LN F-2	360	1/8
		180	0/9
4	RNA(M-T2) + LN F-1	75	0/8
5	RNA(M-T2)	150	0/12
6	RNA(M-T2) + LN + RNAase	150	0/14
7	RNA(M) + LN	200	0/9

F-3, top third layer of gradient.

F-2, middle layer of gradient.

F-1, bottom third layer of gradient.

* Assumed conversion factor of 30 $\mu\text{g RNA/ml} = \text{OD}_{260m\mu}$ of 1.0.

‡ See Table I. Results are based on serum titers 4 days after implantation of chambers.

TABLE IV
The Neutralizing Activity against T2 of Sera from Non-Immune X-Irradiated Rats (500 r)

Rat No.	Per cent neutralization by rat serum diluted 1:4			
	Time after x-irradiation, days			
	0	4	6	10
1	57	16	10	23
2	41	11	0	33
3	40	15	25	34
4	47	6	23	28
5	37	17	0	10
6	29	10	0	18

The results, shown in Table III, indicate that activity was present in the top fraction (group 2). The middle layer, when used in the larger of the two amounts employed, may have contained some activity (group 3) but none was found in the bottom fraction (group 4). Groups 1 and 6 were controls showing the activity of the starting preparation of RNA and its sensitivity to RNAase treatment. The results from group 5 demonstrate again the requirement for lymph

node cells in x-irradiated recipients of diffusion chambers. The results from group 7 show that effective RNA could not be obtained from macrophages which had not been incubated with the specific antigen.

The recipient rats used in all the experiments cited had been carefully selected for low neutralizing titers prior to insertion of the chambers (see Material and Methods), and the possibility of the occurrence of spontaneous fluctuations in

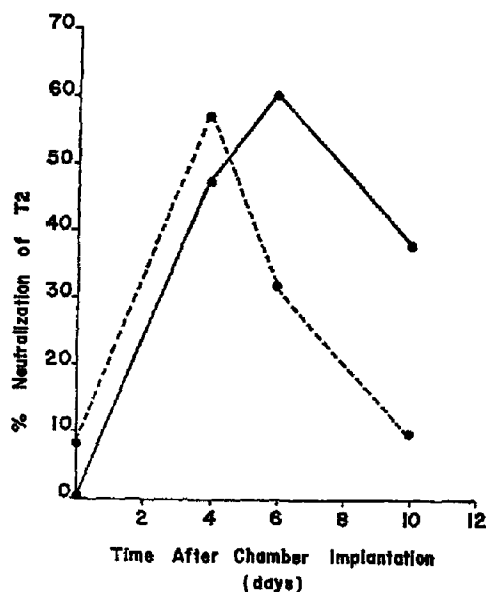


FIG. 1. Antibody formation in rats receiving chambers containing material from antigen-stimulated macrophages. ●—●, homogenates from antigen-stimulated macrophages; ●—●, RNA from antigen-stimulated macrophages.

titer was largely ruled out by numerous "negative" controls (see Tables I to III) and the absence of significant fluctuations in untreated animals. More cogent evidence came from experiments (Table IV) showing that x-irradiation caused a decline in "natural" neutralizing titers, which reached minimal levels on about the 6th day after irradiation. The rise of T2 antibody following implantation of chambers on the day after x-irradiation occurred during this same period when "normal" antibody was declining; *i.e.*, during the first 4 to 6 days after insertion of the chambers (Fig. 1). Also illustrated in Fig. 1 is a difference in the time required for the attainment of peak titers in recipients of chambers containing filtrate (6 days) or RNA (4 days). It is not known at this time whether this difference is attributable to qualitative or to quantitative factors.

DISCUSSION

It has been previously reported (2) that antibody formation against T2 bacteriophage could be initiated *in vitro* through the sequential actions of two cell types. The essential first step was phagocytosis of the antigen by peritoneal exudate cells (macrophages) obtained from normal rats. Homogenization of such cells then yielded an RNAase-sensitive, cell-free material which initiated the formation of specific antibodies in cultures of lymph node cells from normal rats. The present data confirm the critical role of macrophage RNA by showing that the capacity to initiate antibody formation resides in a purified RNA preparation from specifically stimulated macrophages. As indicated by the results of gradient centrifugation, the RNA in question appears to be of low molecular weight. Preliminary data have shown it to be non-dialyzable, and it appears to be phosphorylated. Studies of such RNA prepared from macrophages which had been labeled with tritiated cytidine have shown it to be readily incorporated by lymph node cells maintained in tissue culture (7). Treatment of the RNA with RNAase produced not only the anticipated hyperchromic effect but, more importantly, destroyed its biological activity.

The data presented here do not exclude the possibility that the RNA contained antigenic fragments derived from the bacteriophage complexed with ribonucleic acid from the macrophages, especially in light of the reported adjuvant effect of diffusion chambers (8). Indeed, complexes of antigen with nucleoprotein, or with ribonucleic acid, have been implicated in antibody formation by others (9, 10). This earlier work, in contrast to the present data, dealt with materials extracted from the tissues of actively immunized animals. Preliminary attempts to detect antibody-binding material in the RNA preparations described here have failed. Appropriately labeled bacteriophage will be used in future experiments. However, whether label from the antigen will be found in the lymph node cells or not, the destruction of biological activity upon RNAase treatment clearly points to a critical role of RNA.

"Transformation" of mammalian cells by RNA has been reported by several workers (11-13). In the recent work of Mannick (13) the transfer of specific immunological activity has been described. In this author's experiments the RNA was obtained from lymph node cells of immunized animals, whereas in the present work macrophages from normal animals, stimulated *in vitro* with antigen were the source.

Experiments in progress seek to reveal more information on the physico-chemical nature of the active RNA preparation.

SUMMARY

The diffusion chamber technique permitted the demonstration of specific antibody formation in x-irradiated recipients of such chambers filled with

normal lymph node cells and a cell-free homogenate of macrophages which had been incubated *in vitro* with T2 bacteriophage.

The activity of the cell-free homogenate was retained in its RNA fraction isolated by means of the phenol method.

No antibody formation occurred if such RNA was treated with RNAase.

On sucrose gradients (5 to 20 per cent), the active RNA was found to be present in the top third layer.

The question of the possible presence of antigen complexed to the RNA is discussed.

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